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Compositional and Structural Heterogeneity of Avidin Glycopeptides†

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ABSTRACT: Avidin, purified to apparent homogeneity by cation-exchange chromatography, was resolved into three incompletely separated fractions on carboxymethylcellulose columns at pH 9. These fractions were indistinguishable by specific biotin-binding activity, N-terminal analysis, carbohydrate composition, and polyacrylamide gel electrophoresis at acid pH and in sodium dodecyl sulfate. Although some heterogeneity was detected by discontinuous gel electrophoresis at basic pH, no definitive source for the chromatographic heterogeneity of purified avidin was identified. Commonly encountered sources of charge heterogeneity such as variable phosphorylation and sialylation were shown by specific analyses to be not responsible for the apparent charge heterogeneity of avidin. Purified avidin (780 mg) was reduced, carboxymethylated, and exhaustively digested with Pronase. The resulting neutral glycopeptides were fractionated by Dowex 50 chromatography into five major components containing asparagine as the only amino acid. After purification of the glycopeptides by gel filtration, compositional analysis by gas-liquid chromatography showed that the glycopeptide fractions ranged from 1.2 to 2.0 in the ratio of mannose to *N*-acetylglucosamine. Examination of the glycopeptides by 250-MHz proton nuclear magnetic resonance (NMR) spectroscopy showed that all of these fractions were heterogeneous. Oligosaccharides were prepared by cleavage of the glycopeptides with endoglycosidase H, followed by high-resolution gel filtration, and major oligosaccharide fractions were characterized by NMR spectroscopy. Some samples were also examined by two-dimensional *J*-resolved proton NMR. Comparison of the spectra of avidin samples with those of ovalbumin glycopeptides and oligosaccharides prepared by the same methods showed that the avidin carbohydrate contains at least three distinct oligosaccharide structural types of similar composition and size. In addition to oligomannosidic and bisected hybrid components like those of ovalbumin, the avidin carbohydrate also contains nonbisected hybrid structures similar to those of bovine rhodopsin.

Avidin, a glycoprotein synthesized in the oviduct and deposited in the albumen fraction of eggs, binds the water-soluble vitamin biotin very tightly and specifically with a dissociation constant of $\sim 10^{-15}$ M (Green, 1975). The protein has been extensively characterized due to its remarkable ligand-binding activity and unusual solution stability [see Green (1975) for review]. Avidin is a tetrameric protein, composed of subunits of identical amino acid composition and sequence (DeLange & Huang, 1971). Of the ten asparagine residues of each subunit, only Asn_{17} is glycosylated. It occurs in the general tripeptide sequence -Asn(CHO)-X-Ser(Thr)-, where in the case of avidin, X is methionine, followed by threonine (DeLange, 1970). The remaining nine asparagine residues in each subunit neither occur in the general tripeptide sequence nor are glycosylated (DeLange & Huang, 1971), consistent with the conclusion that the general tripeptide sequence is a prerequisite for glycosylation (Hart et al., 1979). Carbohydrate accounts for about 10% of the avidin molecular weight of 68 000 (Green, 1975). Previous analyses have shown that the carbohydrate units of avidin are composed of an average of four to five mannose and three *N*-acetylglucosamine residues

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per subunit (DeLange, 1970; Green & Toms, 1970); however, no structural characterization of the avidin carbohydrate moiety has been reported.

Chemical modification of avidin by a variety of reagents (Green, 1975) has shown that biotin-binding activity is dependent on factors associated with the protein portion of the molecule (Green, 1963). For this reason, the importance of the carbohydrate as a participant in biotin-binding activity is probably minimal or nonexistent. Although periodate-oxidized avidin is slightly less active than the native protein, the reagent simultaneously modified both tryptophan and carbohydrate residues (Green, 1963). The existence of the nonglycosylated biotin-binding protein streptavidin (Chaiet & Wolf, 1964) is also consistent with the conclusion that the avidin carbohydrate has no functional role in ligand-binding activity. Experimental assessment of the functional role of the carbohydrate moiety in other properties of the protein has not been reported.

Although 85–90% of egg white proteins are glycosylated, primary structures have been determined only for ovalbumin, ovotransferrin, and ovomucoid glycans (Montreuil, 1980). The ovalbumin glycan has been the most intensively studied and has been shown to be a heterogeneous mixture of at least nine oligomannosidic and bisected hybrid (Yamashita et al., 1978; Carver & Grey, 1981) structures. Additional structures, present in low abundance and not previously characterized, have also been recently detected by 360-MHz proton NMR[1] spectroscopy (Atkinson et al., 1981). In this paper, the heterogeneity of the avidin carbohydrate is shown by fractionation of the Pronase-derived glycopeptides. Compositional and spectroscopic evidence is presented that indicate that the heterogeneity of the avidin glycan arises from the presence of three structural types, including oligomannosidic, bisected hybrid, and nonbisected hybrid components. The latter compounds have not previously been reported among egg white glycoproteins. A preliminary account of this work has been presented (Bruch & White, 1982).

Experimental Procedures

Avidin Purification. Avidin was purified from the egg whites of 65-dozen chicken eggs by repetitive cation-exchange chromatography (Green & Toms, 1970). Microgranular carboxymethylcellulose cation-exchange resin (Whatman CM-52) was substituted for the fibrous support material described in the original method (Melamed & Green, 1963). All resin was washed with EDTA prior to use (Melamed & Green, 1963), and double-distilled water was used throughout the purification to prevent metal ion mediated photoxidation of avidin (Fraenkel-Conrat et al., 1952; Green, 1963). Following isolation of avidin from homogenized egg white (homogenate volumes ranged from 9 to 22 L) by batch adsorption (Green & Toms, 1970), partially purified avidin was rechromatographed on two additional carboxymethylcellulose columns in the manner previously described (DeLange, 1970). Columns were washed with two bed volumes of 0.05 M ammonium acetate, pH 9.0, prior to elution with a stepwise gradient of ammonium carbonate, pH 9.0 [0.5–1.2% (w/v) in 0.1% increments, equal volumes of each buffer], in a total volume equal to twice the column bed volume. Column effluents were monitored continuously at 280 nm for protein. Preliminary screening of column fractions for biotin-binding activity was done by the spectrophotometric dye-binding method (Green, 1970). Specific activity was determined by the radioligand method (White & Hughes, 1981). Fractions containing purified avidin were combined, dialyzed against water, and lyophilized.

Analytical Methods. Avidin was dansylated (Gray, 1967) and hydrolyzed in sealed tubes for 18 h in 6 M HCl at 110 °C. The dry hydrolysates was dissolved in ethanol and spotted on micropolyamide thin-layer chromatography plates (Schleicher and Schuell, F1700), which were developed by two-dimensional ascending chromatography with 1.5% (v/v) formic acid in water in the first direction and toluene/acetic acid (9:1) in the second direction (Schulze & Neuhoff, 1976). Polyacrylamide gel electrophoresis in gels containing sodium dodecyl sulfate was performed as described previously (Weber & Osborn, 1969). Calibration curves for determination of molecular weights were constructed with a mixture of standard proteins (Bio-Rad Catalog No. 161-0304). Discontinuous polyacrylamide gel electrophoresis at pH 2.3 and 8.9 was performed as described previously (Davis, 1964).

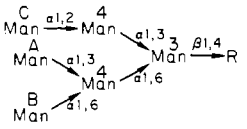
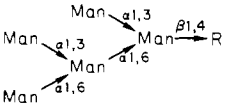
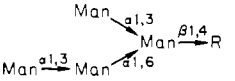
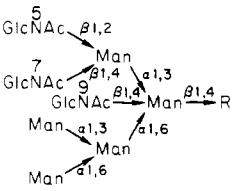
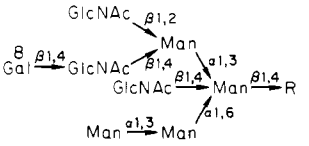
Neutral sugars were estimated by the orcinol method (Francois et al., 1962); mannose was used as the standard. Hexosamines were liberated by hydrolysis in 4 M HCl at 100 °C for 4 h under nitrogen (Marshall & Neuberger, 1972), and the neutralized hydrolysates was analyzed by the Morgan–Elson method (Rondle & Morgan, 1955) with glucosamine hydrochloride as the standard. Sialic acid was determined by the thiobarbituric acid method (Warren, 1959), with *N*-acetylneuraminic acid as the standard, following hydrolysis in 0.05 M H_2SO_4 at 80 °C for 1 h under nitrogen (Sprio, 1966). Carbohydrate analysis of glycopeptides was also performed by gas–liquid chromatography of the alditol acetates (Miller et al., 1982). Reducing sugars were determined by the ferricyanide method (Park & Johnson, 1949). Phosphate was determined colorimetrically (Chen et al., 1956) in acid-washed glassware with KH_2PO_4 as the standard, following ashing of samples in 10% $Mg(NO_3)_2$ (Ames, 1966).

Isolation and Fractionation of Avidin Glycopeptides. Avidin (780 mg) was dissolved in 6 M guanidine hydrochloride, pH 8.0, containing 2 mM EDTA (Hirs, 1967). Following reduction and carboxymethylation of the protein (Huang & DeLange, 1971), the alkylated product was dialyzed against water and lyophilized. The residue was suspended in 0.15 M Tris–acetate, pH 7.4, containing 1.5 mM $CaCl_2$ (40 mL) and equilibrated at 40 ± 1 °C. Pronase [1680 proteolytic units/mg at pH 7.5 with casein as the substrate, according to Narahashi (1970)] was initially added at 1% of the substrate weight and again at 12, 24, and 36 h of incubation at the indicated temperature. After 48 h, the reaction mixture was chromatographed on a Sephadex G-25 column (2.5 × 85 cm) that was eluted with 0.1 M acetic acid (Huang et al., 1970). Fractions were analyzed by the orcinol and ninhydrin (Moore, 1968) methods, and those containing the glycopeptides were combined and dried by rotary evaporation. Glycopeptides isolated by this procedure were subjected to three additional treatments with Pronase (1% of substrate weight). Following isolation of the glycopeptides with the Sephadex G-25 column, acetic acid was removed by rotary evaporation, and the glycopeptides were dissolved in a small volume of water and lyophilized.

Avidin glycopeptides obtained by exhaustive Pronase digestion were fractionated by chromatography on Dowex 50 (Huang et al., 1970). The column was eluted with seven bed volumes of the initial buffer, followed by one bed volume of 0.3 M sodium acetate, pH 5.0. Fractions were analyzed for

[1] Abbreviations: endo H, endo-β-*N*-acetylglucosaminidase H; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GP, glycopeptide; H-1, anomeric proton; Man, mannose; OL, oligosaccharide; 2DJ, two-dimensional *J*-resolved proton NMR spectroscopy; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid. All sugars are of the D configuration.

Table I: Structures of Reference Ovalbumin Glycopeptides

glycopeptide	structure[a]	reference
$Man_6GlcNAc_2$		Tai et al., 1975
$Man_5GlcNAc_2$		Tai et al., 1975
$Man_4GlcNAc_2$		Tai et al., 1977
$Man_5GlcNAc_5$		Yamashita et al., 1978
$GalMan_4GlcNAc_5$		Yamashita et al., 1978

[a] R = GlcNAc(2) β1,4 GlcNAc(1) β1 Asn.

peptide material by the fluorescamine method (Udenfriend et al., 1972) with asparagine as the standard. Fluorescence measurements were obtained with a Turner Model 110 filter fluorometer at 360 nm excitation and 460 nm emission wavelengths. The glycopeptide fractions from the Dowex 50 column were dried, redissolved in water, and applied to a column (2.5 × 75 cm) of Bio-Gel P-4 (50–150 mesh). The column was eluted with water at ambient temperature (Conchie & Strachan, 1978), and fractions were assayed by the fluorescamine and orcinol methods. Fractions containing the desalted purified glycopeptides were combined and lyophilized.

Isolation of Avidin Oligosaccharides. Endo-β-*N*-acetylglucosaminidase H (endo H) from *Streptomyces plicatus* (Tarentino et al., 1978) was obtained from Health Research Inc., Albany, NY. The specific activity of the enzyme was >33.5 units/mg with dansyl-Asn-$GlcNAc_2Man_5$ as the substrate (Tarentino et al., 1978). Glycopeptides were incubated with endo H (1–5 milliunits/μmol of glycopeptide) at 37 °C for 24 h in 0.05 M sodium acetate, pH 5.5. The reaction mixtures were deionized, reduced with $NaBH_4$, again deionized, and lyophilized (Tai et al., 1975). Borate was removed as methyl borate by repeated evaporation of methanol under nitrogen at 50 °C. The oligosaccharide alditols were dissolved in water, and 0.1 mL of a marker solution containing 10 mg/mL each of blue dextran and glucitol was added. The mixtures were chromatographed on a column (1.6 × 170 cm) of Bio-Gel P-4 (−400 mesh), which was eluted with water at ambient temperature. Fractions were assayed spectrophotometrically, following periodate oxidation (Avigad, 1969). Oligosaccharide molecular weights were estimated from the retention volumes of the marker compounds and the oligosaccharides, with purified ovalbumin oligosaccharides as standards for calibration of the column (Natowicz & Baenziger, 1980; Hubbard & Robbins, 1980).

Reference Glycopeptides and Oligosaccharides. 2-Acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose was obtained from Sigma and used without further purification. Ovalbumin glycopeptides were prepared by exhaustive Pronase digestion and fractionated by Dowex 50 chromatography (Huang et al., 1970). In this manner, the reference ovalbumin glycopeptides $Man_6GlcNAc_2Asn$, $Man_5GlcNAc_2Asn$, $Man_4GlcNAc_2Asn$, $GalMan_4GlcNAc_5Asn$, and $Man_5GlcNAc_5Asn$ were obtained. The compositions and structures of the ovalbumin glycopeptides were determined by gas–liquid chromatography and conventional proton NMR spectroscopy. Some samples were also characterized by 2DJ proton NMR (Bruch & Bruch, 1982). The structures of the ovalbumin reference glycopeptides were found to correspond to those previously described by other investigators, as shown in Table I. Oligosaccharides were prepared from the ovalbumin glycopeptides by endo H treatment (Tai et al., 1975). Examination of the ovalbumin reference compounds demonstrated the utility of the 250-MHz spectrometer to glycopeptide structural analysis as described elsewhere (Bruch, 1982; Bruch & Bruch, 1982).

Proton NMR Spectroscopy. Conventional and 2DJ proton spectra were recorded with a Bruker WM-250 spectrometer equipped with an Aspect 2000 computer (Bruch & Bruch, 1982). Spectra were usually obtained at a probe temperature of 40 °C, although some experiments were conducted at other temperatures. Chemical shifts, measured relative to internal sodium 3-(trimethylsilyl)propanesulfonate, and coupling constants measured from the 2DJ spectra were assigned to specific monosaccharide residues of complex carbohydrates by comparison to proton spectra recorded at 180, 250, 360, and 500 MHz (Bruch & Bruch, 1982, and references cited therein).

Results and Discussion

Avidin Purification and Characterization. Purified avidin was obtained from egg white in three column steps by repetitive cation-exchange chromatography on carboxymethylcellulose

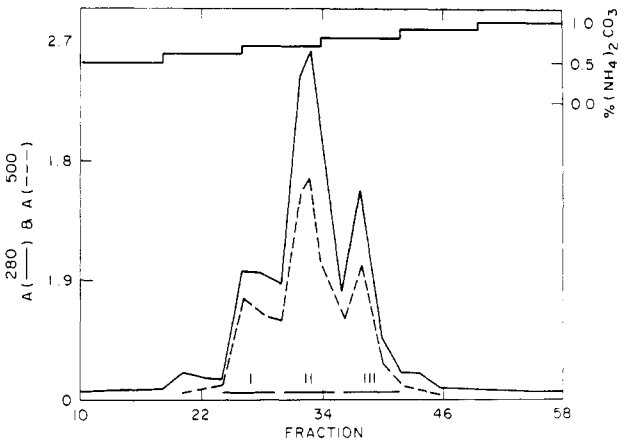


FIGURE 1: Elution profile of avidin on the third carboxymethylcellulose column. The column (2.5 × 45 cm) was eluted with an ammonium carbonate gradient. Fractions of 15 mL were collected at a flow rate of 1 mL/min. Protein (—) was detected A_{280} measurements, and biotin-binding activity (– –) was determined by the spectrophotometric dye-binding assay. Fractions were combined as indicated by the numbered bars.

at pH 9.0. Avidin was eluted as a single broad peak from the first column but was subsequently eluted as three incompletely separated components when rechromatographed on the second and third columns. Figure 1 shows a typical elution profile obtained for avidin eluted from the third column. The same elution profile was obtained when the fractions were assayed by the radiometric method, indicating that the observed elution profile was not due to nonspecificity of the dye-binding assay. The separation of the individual avidin fractions (Figure 1) was not improved by rechromatography on a longer column (1 × 85 cm) or with a linear ammonium carbonate gradient. The specific activity determined by the radiometric assay was constant at 14 units/mg for all three fractions, which is the expected maximum specific activity (Green, 1975). Purified avidin was also examined by incubation of avidin and [^{14}C]-biotin assay mixtures at 80 °C for 30 min. Under these conditions, free and bound biotin equilibrates, and all binding sites would become labeled (White & Hughes, 1981). When biotin was tested over a range of avidin concentrations, no dilution of the radioactive label was observed in these experiments, indicating that the avidin chromatographic heterogeneity was not due to differences in endogenous biotin content.

Additional analyses were performed to evaluate the homogeneity of the purified avidin fractions. N-Terminal analysis of the fractions gave only dansylalanine, the expected N-terminal amino acid of avidin (DeLange, 1970). Polyacrylamide gel electrophoresis of the avidin fractions under denaturing conditions in gels containing sodium dodecyl sulfate gave single bands of about the same relative mobility. The average subunit molecular weight determined by gel electrophoresis was 16 700 (±700), in good agreement with previous estimates (DeLange, 1970). When examined by discontinuous gel electrophoresis at pH 2.3, single sharp (~1 mm) bands of similar relative mobility (0.71 ± 0.02) were obtained for each avidin fraction and for a mixture containing about equal amounts of each fraction. By contrast, electrophoresis under the same conditions at pH 8.9 gave single broad (3–4 mm) bands for each sample, which had more divergent mobilities (0.48 ± 0.05).

On the basis of the specific activity measurements, N-terminal analysis, and the electrophoretic results, the avidin preparation was considered homogeneous. Compositional analyses were therefore conducted in an attempt to define the source of the chromatographic heterogeneity of purified avidin. Phosphate analysis of the individual fractions failed to detect phosphate (0.1 mol/mol of avidin). This result indicated that the heterogeneity of avidin did not arise from variable phosphorylation of the protein and also showed that the preparation was free of the avidin–nucleotide complexes reported previously (Fraenkel-Conrat et al., 1952). The carbohydrate compositions of the three fractions were virtually identical, as determined by colorimetric methods (Table II). The 5:3 mannose:*N*-acetylglucosamine stoichiometry is in good agreement with previous analyses (DeLange, 1970; Green & Toms, 1970). Following mild acid hydrolysis, no sialic acid was detected (<0.1 mol of *N*-acetylneuraminic acid/mol of avidin), also consistent with previous results. Since the carbohydrate composition of the avidin fractions was similar in all cases, all fractions containing biotin-binding activity were combined in preparations used for glycopeptide isolation. Avidin from four preparations, representing 65-dozen eggs, was combined for glycopeptide isolation.

Table II: Carbohydrate Composition of Avidin

	residues/subunit[a]	
fraction[b]	hexose[c]	hexosamine[d]
I	5.18 (5)	3.71 (4)
II	5.52 (6)	3.17 (3)
III	5.05 (5)	3.25 (3)
$\bar{x} \pm s$[e]	5.25 ± 0.24	3.38 ± 0.29

[a] Assumes avidin subunit molecular weight is 16 700. Values in parentheses are the nearest integer number of residues. [b] Avidin fractions as defined in Figure 1. [c] Determined by the orcinol method and calculated as mannose. [d] Determined by the Morgan-Elson method and calculated as *N*-acetylglucosamine. [e] Mean ± standard deviation, $N = 3$.

Isolation and Fractionation of Avidin Glycopeptides. Following sulfhydryl reduction and carboxymethylation, avidin was readily attacked by Pronase. The glycopeptides were isolated from the reaction mixture by gel filtration and were eluted as a single component near the column void volume with almost complete separation from the nonglycosylated degradation products. The recovery of carbohydrate applied to the column was about 95% on the basis of the neutral sugar content. Analysis of the isolated glycopeptides showed that the ratio of neutral sugar to hexosamine was the same as that for the intact glycoprotein. The glycopeptide fraction also lacked reducing sugar activity. These results indicate that the Pronase preparation lacked exoglycosidase (Levvy et al., 1966), endoglycosidase (Conchie & Strachan, 1978), and 4′-L-aspartylglycosylamine amidase (Yamashina, 1972) activities.

The glycopeptides were fractionated by Dowex 50 column chromatography, resulting in the elution profile shown in Figure 2. The glycopeptide mixture was resolved into at least five major components with the low ionic strength buffer, corresponding to glycopeptides that contained asparagine as the sole amino acid (Huang et al., 1970; Conchie & Strachan, 1978). No other amino acids could be detected in these samples by proton NMR spectroscopy (Wüthrich, 1976). This result demonstrates that the avidin carbohydrate exhibits extensive glycan microheterogeneity. The material eluted with the higher ionic strength buffer (fractions 6 and 7 in Figure 2) corresponds to glycopeptides containing more than one amino acid. These fractions were very heterogeneous and were not examined in detail. The seven glycopeptide fractions represented 79% of the fluorescamine-positive and 95% of the oricinol-positive material applied to the column. Glycopeptides containing only asparagine represented 77% of the total neutral

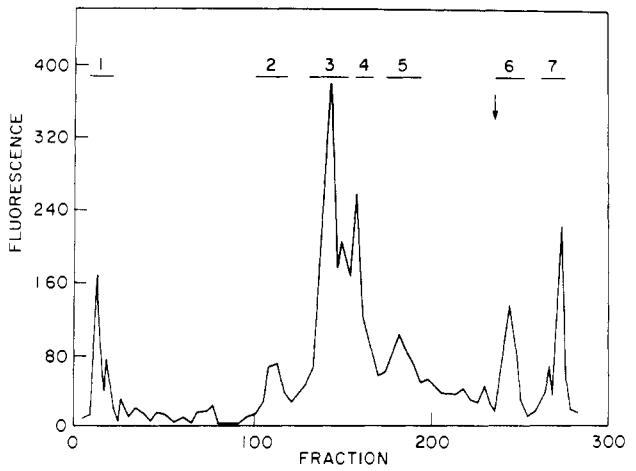


FIGURE 2: Dowex 50 column chromatography of avidin glycopeptides. The column (2.5 × 50 cm) was equilibrated and eluted with 1 mM sodium acetate, pH 2.6. Fractions of 8 mL were collected at a flow rate of 0.5 mL/min, which were assayed by the fluorescamine method. Fluorescence in relative units was measured with 360 nm excitation and 460 nm emission wavelengths. The arrow marks the start of elution with 0.3 M sodium acetate, pH 5.0. Fractions were combined as indicated by the numbered bars.

Table III: Carbohydrate Distribution and Composition of Avidin Glycopeptides

GP[a]	recovery (%) hexose[b]	recovery (%) Asn-(CHO)[c]	composition[d] Man	composition[d] Gal	composition[d] GlcNAc
1	14.7	18.9	4.97 (5)	0.16 (0)	3.90 (4)
2	11.4	14.8	5.26 (5)	0.17 (0)	4.36 (4)
3	22.4	28.9	6.03 (6)	0.12 (0)	3.39 (3)
4	12.5	16.1	5.38 (5)	*e*	3.50 (4)
5	16.3	21.3	3.89 (4)	*e*	3.24 (3)
6	12.7				
7	9.9				

[a] Glycopeptides numbered in order of elution from Dowex 50 (Figure 1). [b] Percent recovery of total hexose in all glycopeptides. [c] Percent recovery of total hexose in glycopeptides containing only asparagine. [d] Values are in residues per mole of glycopeptide determined by gas–liquid chromatography and normalized to asparagine = 1.00 residue/mol of glycopeptide. Values in parentheses are the nearest integer number of residues. [e] Not detected.

sugar recovered in the glycopeptides, and Table III shows the distribution of neutral sugar in the seven fractions.

Table III also shows the results of carbohydrate analysis of the glycopeptide fractions by gas–liquid chromatography of the alditol acetates. The ratio of mannose to *N*-acetylglucosamine ranged from 1.2 to 2.0, demonstrating the compositional heterogeneity of the avidin glycan. Small amounts of galactose were also detected in glycopeptides 1–3. Since each glycopeptide fraction was purified by gel filtration prior to the compositional analysis, free galactose would have been separated from the glycopeptides by this procedure. Further, the amount of galactose in glycopeptides 1–3 greatly exceeded the maximum possible galactose contribution from the Pronase preparation. For these reasons, the presence of galactose in avidin glycopeptides probably indicates galactose-containing glycopeptides in low abundance.

250-MHz Proton NMR Spectra of Avidin Glycopeptides and Oligosaccharides. Avidin GP 1 was not retained on the Dowex 50 column and consisted of at least two components. Nonintegral intensities throughout the H-1 spectrum of GP 1 shown in Figure 3A indicate that the sample is a mixture of several components (Cohen & Ballou, 1980; Atkinson et al., 1981). Line assignments indicated in Figure 3A were made by comparison to the spectra of reference oligomannosidic ovalbumin glycopeptides (Table I) obtained at 250 MHz and from literature data at other field strengths (Bruch & Bruch, 1982, and references cited therein). These assignments indicate the presence in this sample of oligomannosidic structures similar to those of ovalbumin. Following cleavage of GP 1 by endo H, about 50% of the carbohydrate was recovered as a single component from the gel filtration column. The H-1 spectrum of this oligosaccharide fraction, shown in Figure 3B, could be completely assigned from the reference data. The assignments indicate that the sample is a mixture of oligomannosidic structures, containing six and seven mannosyl residues. These structures are identical with those of the corresponding ovalbumin glycopeptides. The structure of the Man_6 component is shown in Table I. The Man_7 structure is identical with the Man_6 component, with the addition of a β1,2-linked mannosyl residue (Man D_3) to Man B.

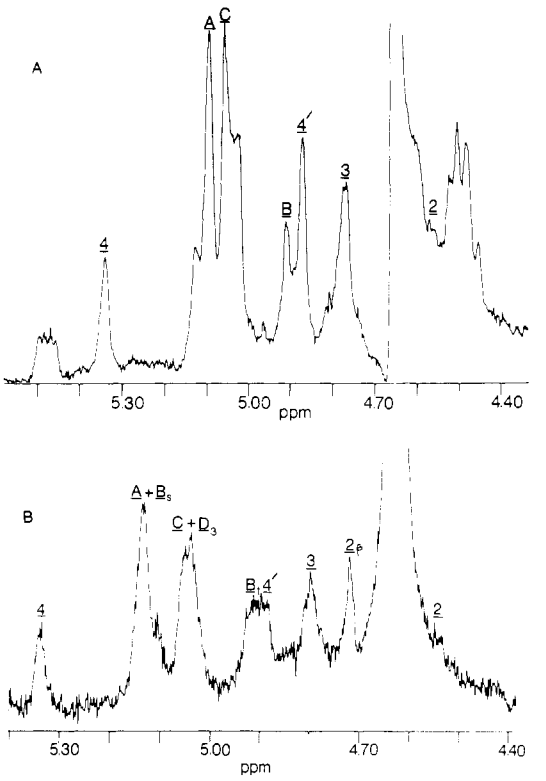


FIGURE 3: Anomeric proton NMR spectra of avidin glycopeptide 1 and oligosaccharides obtained from the glycopeptide: (A) H-1 spectrum of GP 1 (5 mg/mL neutral sugar) obtained with 260 scans; (B) H-1 spectrum of OL 1 obtained with 200 scans. Line assignments indicated by letters and numbers in the spectra correspond to monosaccharide residues as shown in Table I. B_s denotes Man B substituted with Man D_3, and B_t denotes terminal Man B.

The chemical shift positions of the series of unresolved doublets from 4.45 to 4.55 ppm in the H-1 spectrum of avidin GP 1 (Figure 3A) indicate the presence of structures containing nonreducing terminal *N*-acetylglucosamine and/or galactose residues, typical of hybrid and complex structures (Vliegenthart et al., 1981; Carver & Grey, 1981; Carver et al., 1981). Since endo H completely cleaved the glycopeptide, as determined by analysis of reducing sugars and proton NMR, the sample must contain hybrid structures (endo H susceptible) not endo H resistant complex structures (Tarentino et al., 1978). The multiplet at 3.25 ppm, characteristic of bisected structures (Carver & Grey, 1981), was absent in the spectrum GP 1. In combination, these observations indicate the presence of nonbisected hybrid structures in GP 1.

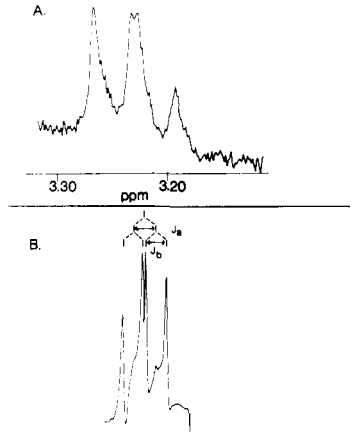


FIGURE 4: One-dimensional and two-dimensional *J*-resolved proton NMR spectra of 3.25-ppm multiplet: (A) One-dimensional proton spectrum of the region from 3.10 to 3.30 ppm of avidin oligosaccharide 3 at 40 °C plotted on an expanded scale; (B) 2DJ cross-section plot at 3.25 ppm. Coupling constants were measured from the plot as indicated in the figure.

Nonbisected hybrid structures of the type proposed for avidin are similar in composition and structure to the bisected hybrid structures of ovalbumin but lack the bisecting *N*-acetylglucosamine residue (GlcNAc 9 in Table I). These structures occurred at low abundance in GP 1 and could not be further characterized.

Because microheterogeneity complicates the *N*-acetyl methyl and H-1 regions of the proton spectra, assignment of lines in these regions to specific carbohydrate residues is often not possible. Similarities in chemical shifts and coupling constants for these signals in homogeneous preparations also often preclude accurate assignments except at high field (Vliegenthart et al., 1981). The 3.25-ppm multiplet is therefore a useful additional structural reporter group, since it can be observed readily in a region of the spectrum without interference from other resonances, regardless of field strength or sample purity. At 250 MHz, the signal appears as a multiplet at 3.25 ppm as shown in Figure 4A. Since no assignment has been made for this multiplet (Carver & Grey, 1981), this region was examined by 2DJ spectroscopy. A cross-section along the *J* axis at 3.25 ppm showed that the multiplet is a doublet of doublets of similar coupling constants (Figure 4B). The multiplet splitting and magnitude of the coupling constants (J_a = 9.2 and J_b = 8.8 Hz) were identical in the 2DJ spectra at 40 and 75 °C. The 2DJ spectrum thus provides an additional criterion (i.e., coupling constants) by which positive identification of the 3.25-ppm multiplet can be made.

Avidin GP 3 was the most abundant fraction from the Dowex 50 column and was also a mixture of at least two components. The H-1 spectrum of GP 3 is shown in Figure 5A. The chemical shift positions of the Man 3 residues show that this sample is a mixture of nonbisected (4.763 ppm) and bisected (4.736 ppm) structures. From the intensities of these lines, nonbisected structures account for about 30% of the sample and bisected components account for the remainder. The presence of the 3.25-ppm multiplet and the nonintegral intensities throughout the H-1 spectrum also support the Man 3 assignments. The similar intensities of the signals at 4.868, 5.087, and 5.340 ppm indicate that these correspond to the nonbisected components. Taken together, the assignments shown in Figure 5A for the nonbisected components are consistent with the presence of oligomannosidic structures in this sample. The remaining H-1 signals in the spectrum correspond to the bisected components. The resonances from

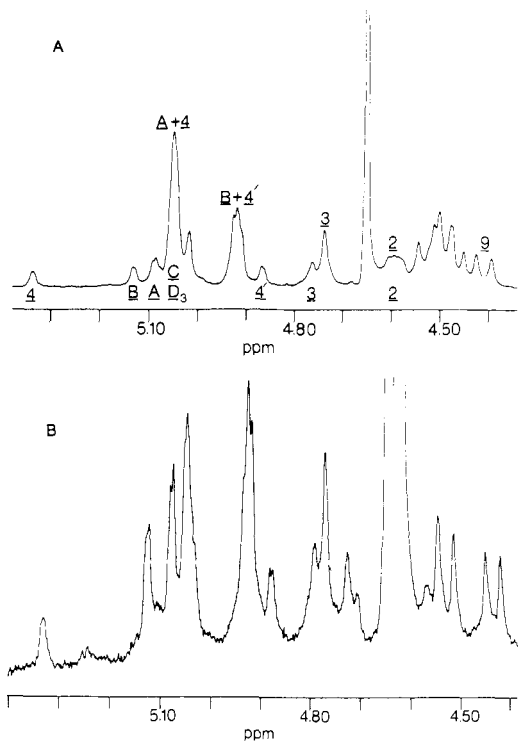
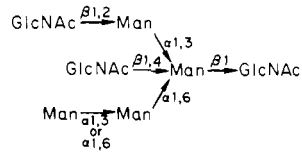


FIGURE 5: Anomeric proton NMR spectra of avidin glycopeptide 3 and oligosaccharides obtained from the glycopeptide: (A) H-1 spectrum of GP 3 (9 mg/mL neutral sugar) obtained with 120 scans; (B) H-1 spectrum of OL 3 obtained with 120 scans. Line assignments corresponding to the nonbisected components are indicated by designations under the appropriate signals. Resonances corresponding to the bisected components are indicated by designations above the appropriate signals.

4.40 to 4.60 ppm, together with the remaining assignments in Figure 5A, indicate the presence of bisected hybrid structures like those of ovalbumin.

Avidin GP 3 was completely cleaved by endo H, and virtually all of the carbohydrate was recovered as an oligosaccharide with a molecular weight of 10.3 glucose equiv. The H-1 spectrum of this oligosaccharide fraction (OL 3) is shown in Figure 5B. The region of the spectrum from 4.40 to 4.60 ppm contains two doublets at 4.431 ppm ($J_{1,2}$ = 7.9 Hz) and at 4.528 ppm ($J_{1,2}$ = 8.1 Hz). Since the 3.25-ppm multiplet was present, the doublet at 4.431 ppm corresponds to the bisecting GlcNAc 9 residue. The 4.528-ppm doublet corresponds to the remaining nonreducing terminal *N*-acetylglucosamine residue. On the basis of the chemical shift of this doublet, this signal corresponds to either GlcNAc 5 or 5′, β1,2 linked at Man 4 or 4′, respectively. The resonance at 5.017 ppm, for which the assignment was uncertain in the glycopeptide spectrum (Figure 5A), could be assigned to Man 4 or 4′ substituted at position 2 with *N*-acetylglucosamine (Vliegenthart et al., 1981; Carver & Grey, 1981). Since the H-1 signals of GlcNAc 5 and 5′ occur at virtually the same chemical shift, the two possible structures cannot be distinguished in this sample without additional purification. However, partial structures for the bisected components of composition $Man_4GlcNAc_3$ (10 glucose equiv) consistent with the spectral data can be proposed:



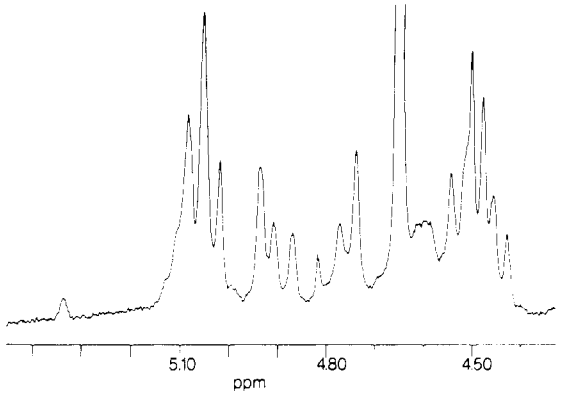


FIGURE 6: Anomeric proton NMR spectrum of avidin glycopeptide 5. The spectrum was obtained with 240 scans at 5 mg/mL neutral sugar concentration.

Avidins GP 4 and 5 were also complex mixtures of similar structures as shown by proton NMR spectroscopy. The H-1 spectrum of GP 5 shown in Figure 6 was similar to that of GP 4. Detailed line assignments were not attempted for either sample due to the complexity of the mixtures. Both glycopeptides were completely cleaved by endo H. In the case of GP 5, two oligosaccharide fractions were obtained by gel filtration. Oligosaccharide OL 5a accounted for 22% of the total neutral sugar recovered from the column, and OL 5b accounted for the remainder. The calculated molecular weights for OL 5a and 5b were 11.6 and 10.0 glucose equiv, respectively. The proton spectra of these fractions showed that both samples were very heterogeneous, precluding definitive line assignments. However, the spectrum of OL 5a lacked the 3.25-ppm multiplet, whereas this signal was present in the spectrum of OL 5b. Both spectra showed the presence of nonreducing terminal *N*-acetylglucosamine residues, indicating that OL 5a was a mixture containing nonbisected hybrid components, while OL 5b was a mixture of bisected hybrid components.

The results of these experiments demonstrate the compositional and structural heterogeneity of avidin glycopeptides. Each of the glycopeptide fractions was heterogeneous as were the corresponding oligosaccharides obtained by endo H cleavage. The complexity of these mixtures precluded complete structural analysis of the avidin carbohydrate. The analysis was further complicated by the similar composition and size of the oligosaccharides and by limited amounts of samples. However, the proton spectra show that the avidin carbohydrate consists of three distinct structural types. The presence in the avidin spectra of oligomannosidic and bisected hybrid structures of the type described for ovalbumin is indicated by resonances common to both sets of spectra. Some of these ovalbumin-like hybrid structures at low concentrations probably account for the presence of the previously unreported galactose-containing glycopeptides in avidin. Biantennary nonbisected hybrid structures of the type proposed for avidin have been reported from bovine rhodopsin (Liang et al., 1979) but have not been reported previously for egg white glycoproteins. These structures are similar to known bisected hybrid glycans, but lack the GlcNAc 9 residue.

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Characterization of Multiple Forms of Porcine Anterior Pituitary Proopiomelanocortin Amino-Terminal Glycopeptide†

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ABSTRACT: Chromatography on a molecular sieve column of a preparation of porcine proopiomelanocortin N-terminal glycopeptide purified from anterior pituitary resulted in the isolation of three forms of the peptide with respective apparent M_r 21 000, 17 500, and 13 500 on polyacrylamide/sodium dodecyl sulfate gel. Determination of the amino acid composition of each peptide revealed that the form with a molecular weight of 17 500 corresponds to the 80 amino acid residue porcine N-terminal glycopeptide (PNT 1–80) previously characterized [Larivière, N., Seidah, N. G., & Chrétien, M. (1981) *Int. J. Pept. Protein Res. 18*, 487–491]. The forms with molecular weight of 21 000 and 13 500 correspond respectively to longer and shorter forms of the N-terminal glycopeptide. The high molecular weight form contains 107 amino acid residues. Sequencing of the fragments obtained after cleavage of the molecule with cyanogen bromide and *Myxobacter* Lys-C protease indicated that an extension of 27 amino acid residues is linked to PNT 1–80 through a -Lys-Arg- sequence. The sequence of the extension is reported. The low molecular weight form corresponds to the first 61 residues of PNT 1–80. Pronase digestion of the peptide and dansylation of the digest revealed the presence of a residue of phenylalanine amide at position 61. A general model for the maturation of the N-terminal glycopeptide of proopiomelanocortin in porcine anterior pituitary is presented.

Adrenocorticotropic hormone (ACTH), β-lipotropic hormone (β-LPH), α- and β-melanotropic hormones (α- and β-MSH), and β-endorphin (β-END) are synthesized in the pituitary gland from a common glycoprotein precursor (Roberts & Herbert, 1977; Mains et al., 1977; Crine et al., 1978) called proopiomelanocortin (POMC) (Chrétien et al., 1979). These hormones are located on the central and C-terminal portions of the POMC structure leaving a large N-terminal fragment. Using recombinant DNA technology, Nakanishi et al. (1979) determined the nucleotide sequence of the mRNA coding for pre-POMC in bovine pars intermedia. This study revealed an amino acid sequence in the N-terminal portion of POMC homologous to the sequence of α- and β-MSH in ACTH and β-LPH, respectively. This new sequence was called γ-MSH (Nakanishi et al., 1979). More recently, investigations on the genomic DNA structure of human (Chang et al., 1980), bovine (Nakanishi et al., 1980), and rat (Drouin & Goodman 1980) POMC were published confirming the finding of Nakanishi et al. (1979). Pulse and pulse–chase experiments in rat pars intermedia (Crine et al., 1979, 1980a,b; Gossard et al., 1980) have shown that during maturation of POMC, the N-terminal portion of the molecule is released as two glycosylated peptides with apparent molecular weights (M_r) on polyacrylamide/sodium dodecyl sulfate ($NaDodSO_4$) gel of 17 000 and 19 000.

Recently, we purified the N-terminal fragment of POMC from the anterior lobe of porcine pituitary glands and from whole human pituitary glands and reported their complete amino acid sequence (Larivière et al., 1981; Seidah & Chrétien, 1981). The peptide from porcine anterior lobe was found to be 80 amino acids long while its human homologue was 76 amino acids long. However, during the purification procedure, a certain heterogeneity of the preparations could be detected by gel filtration and two-dimensional polyacrylamide gel electrophoresis.

In this paper we present the chemical characterization of two additional forms of the N-terminal fragment of porcine POMC differing in their length from the peptide of 80 amino acids. One has 107 amino acid residues and represents probably the N-terminal portion of POMC up to the putative -Lys-Arg- sequence preceding the ACTH sequence (Nakanishi et al., 1979, 1980; Chang et al., 1980; Drouin & Goodman 1980); the other has 61 amino acids, it contains the γ-MSH sequence, and its C-terminal phenylalanine is amidated. This latter form is possibly a maturation product of the 80 amino acid peptide.

Materials and Methods

Isolation of Porcine N-Terminal Peptides and Two-Dimensional Polyacrylamide Gel Electrophoresis. The methods for the isolation and purification of the peptides by high-performance liquid chromatography (HPLC) have been previously reported (Larivière et al., 1981). One subsequent step of gel filtration on a column (125 × 1.5 cm) packed with Sephadex G-75 superfine (Pharmacia Fine Chemicals) eluted

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