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Structural Studies on Rabbit Transferrin: Isolation and Characterization of the Glycopeptides†

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ABSTRACT: The structure of rabbit transferrin was investigated with regard to number, size, and composition of the heteropolysaccharide units and their relative location on the polypeptide chain. The composition and molecular weight of the Pronase glycopeptides revealed that rabbit transferrin contains two heteropolysaccharide units, each composed of 2 sialic acid residues, 2 galactose residues, 3 mannose residues, and 4 *N*-acetylglucosamine residues. The composition and molecular weight of the tryptic glycopeptides further substantiated the existence of two identical heteropolysaccharide units and revealed that both units have identical amino acid residues in the immediate vicinity of the carbohydrate attachment sites to the polypeptide chain, suggesting a sequence homology surrounding the two glycosylation sites. Characterization of the cyanogen bromide fragments from rabbit transferrin indicated that both heteropolysaccharide units are located within a single polypeptide fragment representing approximately one-third of the molecule.

The transferrins represent a group of iron binding proteins that play a vital role in iron metabolism (Aisen & Brown, 1977). This role includes binding, transport, and transfer of iron to a variety of tissues such as the erythrocyte precursor where this iron is utilized in hemoglobin biosynthesis. The mechanism of iron transfer from rabbit transferrin to the rabbit reticulocyte has been extensively studied (Aisen & Brown, 1977; Morgan, 1974). The initial event involves binding of transferrin to specific receptors located on the reticulocyte surface (Jandl et al., 1959; Jandl & Katz, 1963; Baker & Morgan, 1971). These receptors have recently been solubilized from the rabbit reticulocyte (Leibman & Aisen, 1977) and from the differentiated Friend erythroleukemic cell (Hu et al., 1977). The mechanism of subsequent release of iron from transferrin to the reticulocyte is at this time poorly understood.

The critical importance of species variability in assessing the mechanism of iron transfer from transferrin to the reticulocyte was recently demonstrated (Harris & Aisen, 1975a). In the homologous system of rabbit transferrin and rabbit reticulocytes (Harris & Aisen, 1975a) or human transferrin and human reticulocytes (Harris & Aisen, 1975b), the two iron binding sites of transferrin are functionally equivalent. However, in the heterologous system involving human transferrin and rabbit reticulocytes, the sites are functionally nonidentical, possibly indicating an artifact of the heterologous system (Harris & Aisen, 1975a).

Ultimately, the elucidation of the detailed mechanism of iron transfer, using the homologous system of rabbit transferrin and rabbit reticulocytes, will require a detailed knowledge of the chemical structure of rabbit transferrin. At present the amino acid and carbohydrate composition (Hudson et al., 1973), molecular weight and single chain nature (Hudson et al., 1973; Palmour & Sutton, 1971; Green & Feeney, 1968), iron binding properties (Hudson et al., 1973) and preliminary crystallographic data (Al-Hilal et al., 1976) have been reported for rabbit transferrin. The purpose of this investigation was to determine the number, composition and size of the heteropolysaccharide units of rabbit transferrin and to define their relative location on the polypeptide chain. The results of this study show that rabbit transferrin contains two heteropolysaccharide units that are identical with respect to size and monosaccharide composition. In addition, each carbohydrate unit contains identical amino acid residues in the immediate vicinity of the two glycosylation sites, and, finally, both units are located in a single polypeptide segment representing about one-third of the intact transferrin molecule. These results provide further support for the existence of sequence homology within the transferrin molecule.

Experimental Procedures

Materials

Urea was purchased from Aldrich and was recrystallized and deionized prior to use. Ethylenimine and cyanogen bro-

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mide were obtained from Pierce. Methanol was purchased from Burdick and Jackson Laboratories. Pyridine and acetic anhydride were purchased as acetylation kits from Applied Science Laboratories. The carbohydrate standards and column packing (3% SP-2340 on 100–120 Supelcoport) were obtained from Supelco. Tos-PheCH_2Cl[1]-treated trypsin was purchased from Worthington. All other chemicals were the best commercially available and used without further purification.

Methods

Purification of Rabbit Plasma Transferrin. Fresh rabbit plasma was purchased from Pel Freez Biologicals, Inc. Transferrin was purified according to the procedure described by Martinez-Medellin & Schulman (1972). Prior to ion-exchange chromatography on a SP-Sephadex G-50 column, apotransferrin was prepared by extensive dialysis against 0.1 M sodium acetate–0.1 M sodium citrate, pH 4.0. Individual fractions eluted from the ion-exchange column were checked for their ability to bind Fe(III) using the ferric nitrilotriacetate chelate (Harris & Aisen, 1975a). The amount of iron bound was determined by using ^{59}Fe-labeled iron nitrilotriacetate.

Pronase Digestion of Rabbit Transferrin. Apotransferrin was digested with Pronase according to the procedure described by Spiro & Bhoyroo (1974). The Pronase digest was desalted on a Bio-Gel P-6 column prior to redigestion with Pronase. Further purification of the Pronase glycopeptide was achieved using a DEAE-cellulose ion-exchange column (1.0 × 34 cm). The column was equilibrated with 1 mM ammonium bicarbonate, adjusted to pH 8.3, and eluted with a linear gradient of 200 mL of 1 mM ammonium bicarbonate and 200 mL of 500 mM ammonium bicarbonate.

Reduction and Alkylation of Rabbit Apotransferrin. Rabbit apotransferrin was reduced and alkylated using the procedure described by Brew & Hill (1970), and the extent of alkylation was determined by amino acid composition.

Tryptic Digestion of Reduced and Alkylated Rabbit Transferrin and Isolation of the Glycopeptides. Reduced and S-aminoethylated rabbit transferrin was dissolved in 0.05 M ammonium acetate (pH 4.0) at a concentration of 20 mg/mL. Trypsin (1% w/w) was added prior to adjusting the pH to 7.8 by the addition of 1.0 M ammonium bicarbonate. An additional 1% (w/w) of trypsin was added at 1 h and 3 h of digestion, and digestion was continued for 24 h at 37 °C under a toluene atmosphere. The digest was lyophilized and redissolved in 15.0 mL of 0.2 M pyridine-acetate (pH 3.55). A small amount of precipitate was removed by centrifugation. An aliquot from the supernatant solution was removed to quantitate the amount of hexose present, and the remainder of the digest was applied to a Dowex 50-X2 column. The glycopeptides were identified by analyzing each fraction for hexose by the phenol–sulfuric acid method (Dubois et al., 1956) scaled down to one-tenth the volume. The peptide material was identified using a fluorescamine assay (Nakai et al., 1974). Further purification of the glycopeptide was accomplished using a DEAE-cellulose ion-exchange column.

Cyanogen Bromide Cleavage of Reduced and Alkylated Rabbit Transferrin and Isolation of CBII. S-Aminoethylated rabbit transferrin was dissolved in 70% (v/v) formic acid at a concentration of 20 mg/mL. A 500-fold molar excess over methionine of crystalline cyanogen bromide was added and the reaction was carried out at room temperature for 24 h under nitrogen. The reactants and solvent were removed by evaporation under reduced pressure at 30 °C. The extent of cyanogen bromide cleavage was determined by the loss of methionine and quantitative appearance of homoserine. In a control experiment, the protein was hydrolyzed in 70% formic acid for 24 h at room temperature. The cyanogen bromide cleavage products were redissolved in 30 mL of 0.02 M sodium citrate, pH 3.60, and applied to a CM-cellulose column (1.6 × 20 cm) equilibrated in the same buffer. Selected fractions were analyzed by sodium dodecyl sulfate–urea gel electrophoresis by removing 25 μL from the fractions and adding 175 μL of 0.05 M sodium phosphate, pH 7.0, containing 2% sodium dodecyl sulfate and 8 M urea. The fractions containing CB II were pooled, dialyzed against 0.05 M ammonium acetate, pH 4.0, and then lyophilized. The partially purified CB II fraction was dissolved in 0.1 M sodium phosphate, pH 6.0, containing 6 M urea at a concentration of 15 mg/mL and a portion (4.0 mL) was applied to a Sephacryl S-200 column (2.0 × 90 cm) equilibrated in the same buffer. Selected fractions were analyzed by sodium dodecyl sulfate–urea polyacrylamide gel electrophoresis. Those fractions containing CB II were pooled, concentrated and rechromatographed on the same column. Purified CBII was dialyzed extensively against 0.05 M ammonium acetate, pH 4.0, and used as a stock solution for electrophoretic and compositional analyses.

Tryptic Digestion of CBII. Reduced and S-aminoethylated CBII (20 mg) was dissolved in 2.0 mL of 0.05 M ammonium acetate, pH 4.0. Trypsin (1% w/w) was added prior to adjusting the pH to 7.8 by the addition of 1.0 M ammonium bicarbonate. Subsequent additions of trypsin (1% w/w) were made at 1 h and 3 h of digestion. The digestion was carried out for 24 h at 37 °C under an atmosphere of toluene. Isolation of the tryptic glycopeptides followed the same procedure as described for those isolated from whole transferrin.

Analytical Procedures. Amino acids were analyzed on a Beckman 121 HP amino acid analyzer using a modified dual column method (Beckman manual). Hydrolysis was carried out under reduced pressure in sealed tubes at 110 °C for 24 h using glass-distilled, constant-boiling HCl. *S*-Aminoethylcysteine eluted between lysine and histidine on the basic column. The color value was found to be 92% of that obtained for lysine. The homoserine content was determined as described by Shechter et al. (1977). The hexosamines were determined on the basic column of the amino acid analyzer after hydrolysis of the protein with 4 N HCl at 100 °C for 6 h (Spiro, 1966). Sialic acid was determined by a micromodification of the thiobarbituric acid method described by Warren (1959) after hydrolysis of the protein with 0.1 N H_2SO_4 at 80 °C for 1 h. Neutral sugars were identified and quantitated as described by Kim et al. (1967) with the following modifications: (i) Dowex 1 resin was used to remove H_2SO_4; and (ii) Supelco SP-2340 column packing was used.

Molecular Weight Determination. The molecular weights of the Pronase and tryptic glycopeptides were estimated by thin-layer gel filtration in 6 M guanidine hydrochloride as previously described (Hung et al., 1977).

Electrophoresis. Disc gel electrophoresis was performed according to the procedures of Davis (1964). The concentration of acrylamide was varied between 7.5% and 12.5% with a constant ratio of bis(acrylamide) to acrylamide. Poly(acrylamide) gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described by Hudson & Spiro (1972). The acid–urea system that was used has been described by Panyim & Chalkley (1969). The system used for analysis of peptides was a modification of the method of Swank &

[1] Abbreviations used: Tos-PheCH_2Cl, tosylphenylalanyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl; DEAE, diethylaminoethyl; CB, cyanogen bromide; DNP, 2,4-dinitrophenyl; AERbTf, S-aminoethylated rabbit transferrin.

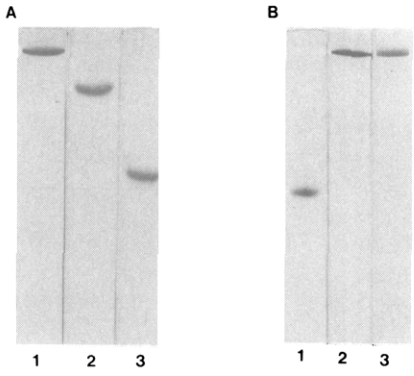

FIGURE 1: Polyacrylamide gel electrophoresis of purified rabbit transferrin. (A) Disc gel electrophoresis of 10 μg of rabbit transferrin at pH 9.5 on (1) 12.5%; (2) 10%; (3) 7.5% acrylamide. (B) Electrophoresis of 10 μg of reduced rabbit transferrin under a variety of denaturing conditions: (1) sodium dodecyl sulfate gel electrophoresis, 5% acrylamide; (2) acid–urea system, 10% acrylamide; (3) sodium dodecyl sulfate–urea gel electrophoresis, 12.5% acrylamide.

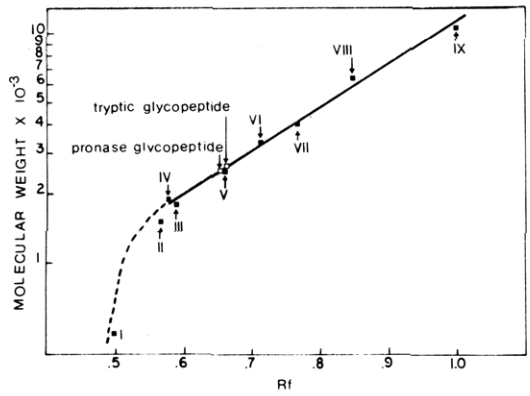

FIGURE 2: Molecular weight determination of the glycopeptides from rabbit transferrin. Molecular weight standards are: (I) glucose-galactose-hydroxylysine (481); (II) ovalbumin glycopeptide IV (1497); (III) tryptic peptide from avian pancreatic polypeptide (1759); (IV) ovalbumin glycopeptide III (1918); (V) human transferrin glycopeptide (2621); (VI) fetuin glycopeptide (3325); (VII) avian pancreatic polypeptide (4240); (VIII) CBI peptide from horse myoglobin (6220); (IX) CBI peptide from bovine α-lactalbumin (10 500).

Munkres (1971) as described by Downing et al. (1975). Gels were stained for protein using Coomassie brilliant blue, and for carbohydrate by the use of the periodate–Schiff stain (Segrest & Jackson, 1972). Paper electrophoresis was performed on Whatmann 3MM 20 × 20 cm sheets using a MRA electrophoretic chamber at 400 V and 25 mA. The mobility of the various peptides and glycopeptides was measured relative to DNP-alanine or lysine. The buffers used were: (a) pH 3.6, pyridine:glacial acetic acid:H_2O (1:10:89 v/v); (b) pH 6.5, pyridine:glacial acetic acid:H_2O (25:1:450 v/v).

Results

Purity of Rabbit Transferrin. The purity of each preparation of rabbit transferrin was examined by both disc gel electrophoresis (Figure 1A) and electrophoresis under a variety of denaturing conditions (Figure 1B). In all cases, a single component was observed and thus the heterogeneity observed with human transferrin (Roop & Putnam, 1967) was not seen. The apparent molecular weight obtained for rabbit transferrin from sodium dodecyl sulfate gel electrophoresis was 78 000. This value is in good agreement with the value of 76 700 obtained by sedimentation equilibrium (Hudson et al., 1973). In addition to the electrophoretic analysis, each preparation was examined for its iron binding ability, and, in all cases, purified rabbit transferrin was found to bind 2 mol of Fe(III) per mol of protein. Finally the ratio of absorbance at 460 nm and 405 nm of iron saturated transferrin was checked to ensure that heme-containing proteins were absent.

TABLE I: Amino Acid and Carbohydrate Composition of the Pronase Glycopeptide from Rabbit Transferrin.

components	residues/mol[a]
amino acid	
Asp	1.0
Ser	1.2
Gly	0.6
Glu	0.4
Ala	0.4
Lys	0.3
carbohydrate	
mannose	2.7
galactose	1.9
glucosamine	4.1
sialic acid	2.2

[a] Calculations based on a molecular weight of 2660 (Figure 2).

TABLE II: Calculation of the Number of Rabbit Transferrin Heteropolysaccharide Units.

mol wt of Pronase glycopeptide[a]	2660
carbohydrate content of glycopeptide[b]	85%
mol wt of heteropolysaccharide unit[c]	2261
carbohydrate content of rabbit transferrin[d]	5.5%
mol wt of rabbit transferrin[d]	76 700
mol wt of carbohydrate moiety of transferrin[e]	4210
no. of heteropolysaccharide units per polypeptide chain (4210/2261)	1.9

[a] Determined from experiment described in Figure 2. [b] Obtained from composition given in Table I. [c] Calculated from composition. [d] Hudson et al., 1973. [e] Calculated from composition of rabbit transferrin.

Pronase Digestion and Fractionation of Glycopeptides. In order to determine the composition, size, and number of carbohydrate units of rabbit transferrin, apotransferrin was digested extensively with Pronase and the resulting glycopeptides were fractionated by gel filtration and ion-exchange chromatography. The glycopeptides eluted as a single symmetrical peak on a Bio-Gel P-6 column. The glycopeptide fractions were pooled, redigested with Pronase, and reapplied to the P-6 column. The resultant glycopeptide fraction was then chromatographed over a DEAE-cellulose column and was found to elute as a single peak. The molecular weight of this glycopeptide fraction was found to be 2660 using thin-layer gel filtration in 6 M guanidine hydrochloride (Figure 2). Its amino acid and carbohydrate compositions are presented in Table I. Based on these data and the carbohydrate composition of the intact glycoprotein, rabbit transferrin contains two heteropolysaccharide units that are identical with respect to monosaccharide constituents and size. These calculations are shown in Table II.

Tryptic Digestion of Reduced and S-Aminoethylated Rabbit Transferrin. In an attempt to isolate the individual heteropolysaccharide units for future monosaccharide sequence studies and to locate these units on the polypeptide chain, the tryptic glycopeptides from reduced and S-aminoethylated rabbit transferrin were fractionated by ion-exchange chromatography. The glycopeptides were separated

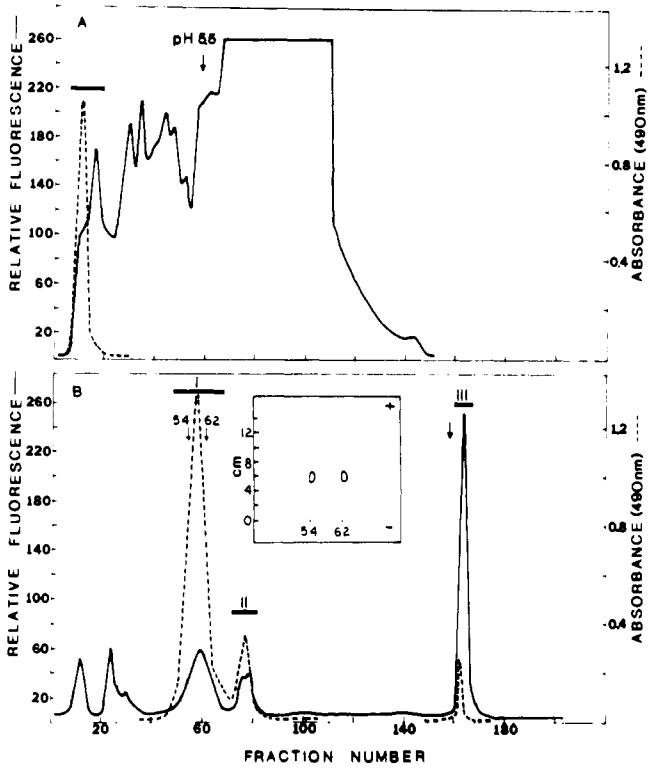

FIGURE 3: Purification of glycopeptides from a tryptic digest of aminoethylated rabbit transferrin. (A) A tryptic digest of 300 mg of reduced and alkylated rabbit transferrin was dissolved in 15.0 mL of 0.2 M pyridine-acetate (pH 3.55) and applied to a Dowex 50-X2 column (1.5 × 20 cm) equilibrated in the same buffer. Fractions of 3.8 mL were collected. Aliquots of 50 μL were removed from each fraction and assayed for hexose content by the phenol-sulfuric acid method, and for peptide content by the fluorescamine method. At the position indicated by the arrow, the column was eluted with 2.0 M pyridine-acetate, pH 5.5. The bar indicates those fractions pooled for further purification. (B) The glycopeptide fraction from the Dowex 50 column was applied to a DEAE-cellulose column (1.0 × 10 cm) equilibrated with 0.01 M ammonium bicarbonate, pH 7.8. Elution was carried out using a linear gradient consisting of 200 mL of 0.01 M ammonium bicarbonate and 200 mL of 0.10 M ammonium bicarbonate. At the completion of this gradient (arrow), the column was eluted with 0.50 M ammonium bicarbonate. Aliquots of 50 μL were removed from each fraction and assayed for hexose and peptide content. The three fractions designated I, II, and III were pooled for further study. (Inset) Paper electrophoresis at pH 6.4 of fractions 54 and 62. Peptides were detected with ninhydrin.

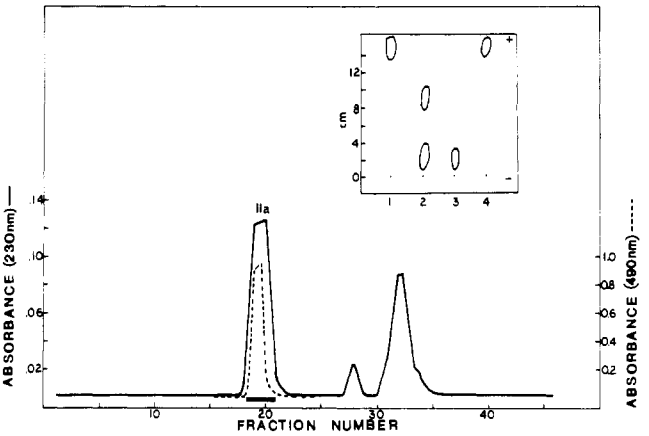

FIGURE 4: Further purification of fraction II. Fraction II (Figure 3) was chromatographed on a Bio-Gel P-4 column (1.0 × 84 cm) equilibrated with 0.10 M ammonium bicarbonate, (pH 7.8). Peptides were detected by measuring the absorbance at 230 nm. Aliquots of 200 μL were removed and assayed for hexose content. (Inset) Paper electrophoresis run at pH 6.4 of (1 and 4) DNP-alanine, (2) fraction II, and (3) fraction IIa. Peptides were detected with ninhydrin.

TABLE III: Amino Acid and Carbohydrate Composition of Fractions I and IIa and Tryptic Glycopeptide of CBII.

components	residues per mole[a] I	IIa	tryptic glycopeptide of CBII
amino acid			
Asp	1.02	1.06	1.04
Ser	1.78	1.88	1.87
Glu		1.08	
Leu	1.05	2.20	1.00
S-aminoethyl Cys	1.00	1.00	1.00
carbohydrate			
mannose	2.95	2.95	2.71
galactose	2.20	2.16	2.04
glucosamine	4.06	3.87	4.20
N-acetylneuraminic acid	2.05	1.70	2.00
mol wt[b]	2785	3027	2785
mol wt[c]	2700	ND[f]	2772
% yield[d]	80	7	78
R_f[e]	0.14	0.16	0.14

[a] Based on *S*-aminoethylcysteine. [b] Molecular weight calculated from composition. [c] Experimental value obtained by thin-layer gel filtration (Figure 2). [d] Calculated from hexose recovery. [e] Mobility in paper electrophoresis at pH 6.4 measured relative to DNP-alanine. [f] Not determined.

from the majority of peptides by chromatography on a Dowex 50-X2 column (Figure 3A). The glycopeptide fraction, accounting for greater than 95% of the carbohydrate of transferrin, was further purified on a DEAE-cellulose column (Figure 3B). Two glycopeptide fractions (I and II) were obtained using a gradual gradient and a third fraction was obtained upon elution with 0.5 M ammonium bicarbonate. Fraction I represented 85% of the hexose applied to the column, while fractions II and III represented 8% and 3%, respectively.

The homogeneity of fractions I and II was examined by paper electrophoresis and by amino acid composition. Fraction I gave a single component upon electrophoresis (inset, Figure 3B) and contained a single basic amino acid, *S*-aminoethylcysteine (Table III), indicating homogeneity. Electrophoretic analysis of fraction II revealed two components (inset, Figure 4), having R_f values of 0.64 and 0.16 relative to DNP-alanine. These components were resolved by gel filtration chromatography using a Bio-Gel P-4 column (Figure 4). All the carbohydrate was recovered in fraction IIa which gave a single component upon electrophoresis (inset, Figure 4), and contained a single basic amino acid, *S*-aminoethylcysteine (Table III), indicating homogeneity.

The amino acid and carbohydrate compositions of glycopeptide fractions I and IIa are shown in Table III. The values are expressed relative to *S*-aminoethylcysteine assuming one basic amino acid residue for a tryptic peptide. The molecular weight for fraction I calculated from the composition data (2785) is in excellent agreement with the value determined experimentally (2700) by thin-layer gel filtration (Figure 2), thus validating the composition and size of the glycopeptide. These data further substantiate that the carbohydrate of rabbit transferrin is present in the form of two heteropolysaccharide units having identical size and monosaccharide compositions (3 Man, 2 Gal, 4 GlcNAc, 2 AcNeu), since intact transferrin contains twice the number of monosaccharide residues per

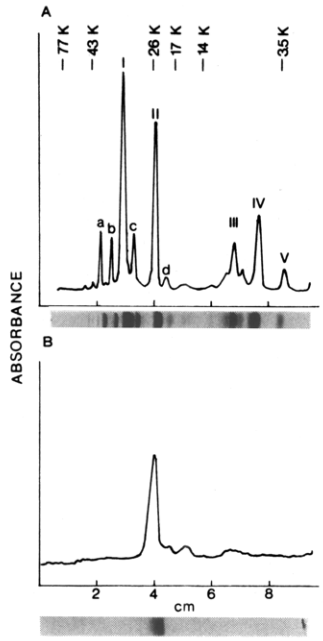

FIGURE 5: Spectrophotometric scans and polyacrylamide gels of the cyanogen bromide digest of reduced and aminoethylated rabbit transferrin. The gels were run in the presence of sodium dodecyl sulfate and 6.25 M urea. (A) Thirty micrograms of the digest was applied to the gel which was stained for protein with Coomassie blue. The standards used were: human transferrin (77 000), ovalbumin (43 000), chymotrypsinogen (25 700), myoglobin (17 200), lysozyme (14 300), and the B chain of insulin (3500). (B) One hundred micrograms of the digest was applied to the gel which was stained for carbohydrate with periodic acid–Schiff stain.

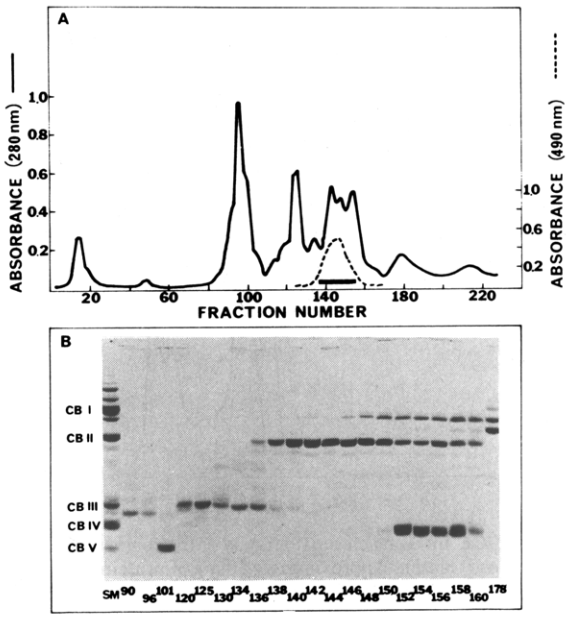

FIGURE 6: (A) Fractionation of 100 mg of a cyanogen bromide digest of reduced and alkylated rabbit transferrin on a CM-cellulose column. The column (1.6 × 20 cm) was equilibrated with 0.02 M sodium citrate (pH 3.6) and eluted with a linear gradient consisting of 400 mL of 0.02 M sodium citrate (pH 3.60) and 400 mL of 0.02 M sodium citrate (pH 3.60) containing 0.50 M sodium chloride. Peptides were detected by measuring the absorbance at 280 nm, and aliquots of 100 μL were removed from each fraction and assayed for hexose content. The bar indicates those fractions pooled for further study. (B) Analysis of selected fractions by sodium dodecyl sulfate–urea gel electrophoresis. A sample containing 10–15 μg of peptide was applied to each gel. SM indicates the material applied to the column, while numbers refer to the fraction number from A. The gels were stained for protein using Coomassie blue.

polypeptide chain. In addition, both units have identical amino acid residues in the immediate vicinity of the glycopeptide bond, i.e., 1 Asp, 2 Ser, 1 Leu, and 1 Cys.

Fraction IIa has an identical carbohydrate composition as fraction I (Table III) but has an additional residue each of glutamic acid and leucine. The origin of this glycopeptide, which accounts for 8% of the carbohydrate of transferrin, is unknown but could arise from either (a) a genetic variant of transferrin that was not detected by any of the electrophoretic systems used to establish homogeneity of the transferrin preparation or (b) an anomalous cleavage of the polypeptide chain by trypsin. The latter is a more likely explanation in view of several reports in the literature describing nonspecific cleavages by trypsin (Walsh, 1970; Bachmayer et al., 1968; Plapp et al., 1967).

Fraction III (Figure 3B), representing 3% of the hexose of transferrin, was shown to be incomplete tryptic digestion products. It contained several basic amino acids and contained two major components which could be further degraded by redigestion with trypsin.

Cyanogen Bromide Cleavage of Rabbit Transferrin. In a further attempt to separate the two heteropolysaccharide units, reduced and aminoethylated transferrin was cleaved with cyanogen bromide to yield polypeptide segments that were larger than those obtained upon tryptic digestion. The cleavage products were analyzed by sodium dodecyl sulfate–urea polyacrylamide gel electrophoresis (Figure 5) and shown to consist of five distinct polypeptides (I–V), having molecular weights of 33 000, 24 000, 10 000, 8000, and 3000, respectively. The sixth peptide predicted on the basis of 5 methionine residues per chain was not observed possibly because of its low molecular weight. Alternatively, one of the cyanogen bromide polypeptides could be duplicated in the primary sequence of the molecule. The four protein bands designated a–d (Figure 5A) were generated by non-specific hydrolysis with the formic acid solvent and accounted for 10–15% of the total protein. Only polypeptide CBII stained positive for carbohydrate using the periodic acid–Schiff reagent (Figure 5B). Fractionation of the cleavage products on CM-cellulose (Figure 6A) and analysis of column fractions by sodium dodecyl sulfate–urea gel electrophoresis (Figure 6B) and for hexose content also revealed that this peptide contained all the carbohydrate found in transferrin. The column fractions containing CBII were further purified on a Sephacryl S-200 column equilibrated in 6 M urea to remove traces of CBI and CBIV (Figure 7). The column fractions containing CBII were rechromatographed on the same column (inset B, Figure 7) to remove completely any remaining CBI. CBII isolated by this procedure was shown to be homogeneous by electrophoresis (Figure 8). The amino acid and carbohydrate compositions of purified CBII are presented in Table IV and are compared with whole transferrin. Its molecular weight (25 156) calculated from amino acid composition, assuming one residue of homoserine, is in excellent agreement with the experimentally determined value of 24 000 (Figure 5), indicating that CBII accounts for about one-third of the transferrin polypeptide chain. Moreover, the carbohydrate composition indicates that CBII contains all the monosaccharide residues found in whole transferrin and, therefore, contains two heteropolysaccharide units. In different preparations of CBII, the sialic acid content varied slightly. This was attributed to hydrolytic removal of sialic acid during the cleavage reaction in the presence of 70% formic acid.

Tryptic Digestion of CBII and Isolation of Glycopeptides. A tryptic digest of CBII was chromatographed in a Dowex 50-X2 column (Figure 9A), and the resulting glycopeptide

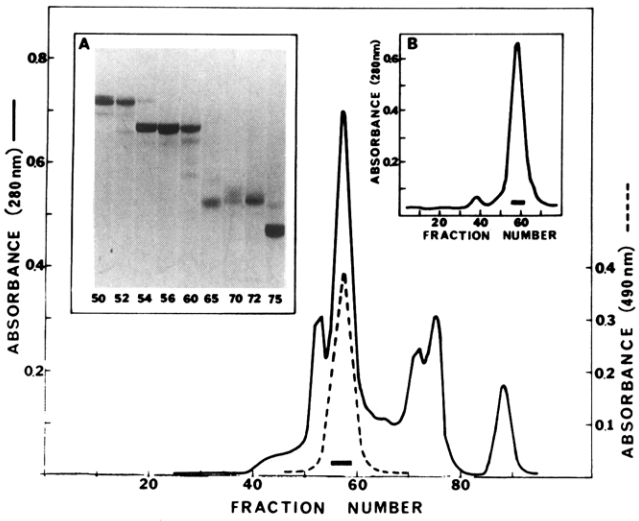

FIGURE 7: Gel filtration chromatography of the pooled fraction from the CM-cellulose column. Approximately 50 mg of protein was applied to a Sephacryl S-200 column (2.0 × 90 cm) equilibrated with 0.10 M sodium phosphate (pH 6.0) containing 6.0 M urea. A flow rate of 15 mL/h was maintained and fractions of 3.7 mL were collected. Peptides were detected by monitoring the absorbance at 280 nm and 50-μL aliquots from each fraction were assayed for hexose content. Inset: (A) indicated samples containing 10–15 μg of protein were analyzed for polypeptide content by sodium dodecyl sulfate–urea polyacrylamide electrophoresis; (B) those fractions containing CBII were pooled and rechromatographed on the same column.

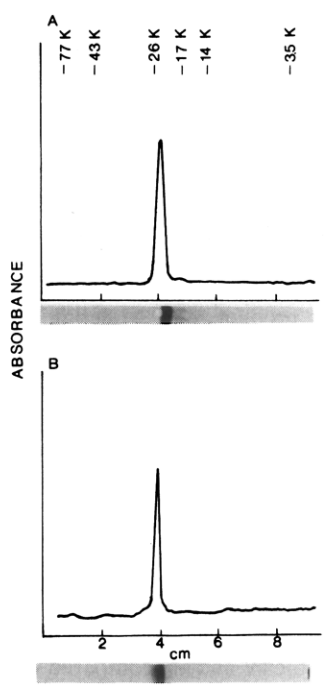

FIGURE 8: Spectrophotometric scans and polyacrylamide gels of purified CBII. The gels were run in the presence of sodium dodecyl sulfate and 6.25 M urea. (A) Ten micrograms of CBII was applied to the gel which was stained for protein with Coomassie blue. (B) One hundred micrograms of CBII was applied to the gel which was stained for carbohydrate using the periodic acid–Schiff reagents.

fraction further purified on a DEAE-cellulose column (Figure 9B). A single glycopeptide fraction was obtained which has an identical elution position (Figure 3B) as well as an identical chemical composition (Table III) as the tryptic glycopeptide fraction I isolated from whole transferrin, indicating that fraction I originates from CBII. In some instances, two fractions were observed that differed only in their content of sialic acid, owing to some hydrolysis during cyanogen bromide cleavage.

TABLE IV: Amino Acid and Carbohydrate Composition of Rabbit Transferrin, Reduced and Alkylated Rabbit Transferrin, and Purified CBII.

components	residues/mole RbTf[a]	AERbTf[a]	CBII[b]
Lys	54	51	13
His	18	18	8
Arg	26	26	11
S-aminoethyl Cys		34	7
Asp	70	70	22
Thr	22	23	6
Ser	39	39	13
Glu	61	55	20
Pro	35	31	6
Gly	46	47	16
Ala	50	50	16
Val	37	37	13
Met	5	5	
Hse			1
Ile	14	17	2
Leu	55	54	18
Tyr	21	20	6
Phe	25	22	9
½-cystine	34		
Trp	9	ND	ND
mannose	5.4	5.3	5.2
galactose	3.9	3.9	3.9
glucosamine	7.5	7.6	7.8
sialic acids	3.8	3.8	3.3
mol wt[c]			25 156
mol wt[d]			24 000

[a] All amino acid values based on a molecular weight of 77 000 and expressed to nearest integer. [b] Based on homoserine content and expressed to nearest integer. [c] Calculated from composition. [d] Estimated by sodium dodecyl sulfate–urea gel electrophoresis.

Discussion

The structure of rabbit transferrin was investigated with regard to number, size, and composition of the carbohydrate units and their relative location on the polypeptide chain. This was accomplished by isolation and characterization of glycopeptides produced by Pronase and tryptic digestions and by cyanogen bromide cleavage of transferrin. The composition and molecular weight of the Pronase glycopeptides revealed that the carbohydrate moiety of rabbit transferrin consists of two heteropolysaccharide units which have identical size and monosaccharide composition (Tables I and II). These units are of the complex type (Kornfeld & Kornfeld, 1976) being composed of 2 sialic acid residues, 2 galactose residues, 3 mannose residues, and 4 *N*-acetylglucosamine residues and are probably linked to the polypeptide chain by an *N*-glycosidic linkage between *N*-acetylglucosamine and the amide nitrogen of asparagine. Their composition and size appear similar to the two heteropolysaccharide units of human transferrin (Spik et al., 1975) and thus may be similar in monosaccharide sequence. However, the exact structure of the heteropolysaccharide units of human transferrin is not resolved since discrepancies exist as to their composition and monosaccharide sequence (Spik et al., 1975; Jamieson et al., 1971; Graham & Williams, 1975).

The composition and molecular weight of the tryptic glycopeptide (fraction I, Table III) provide additional evidence for the existence of two heteropolysaccharide units of identical

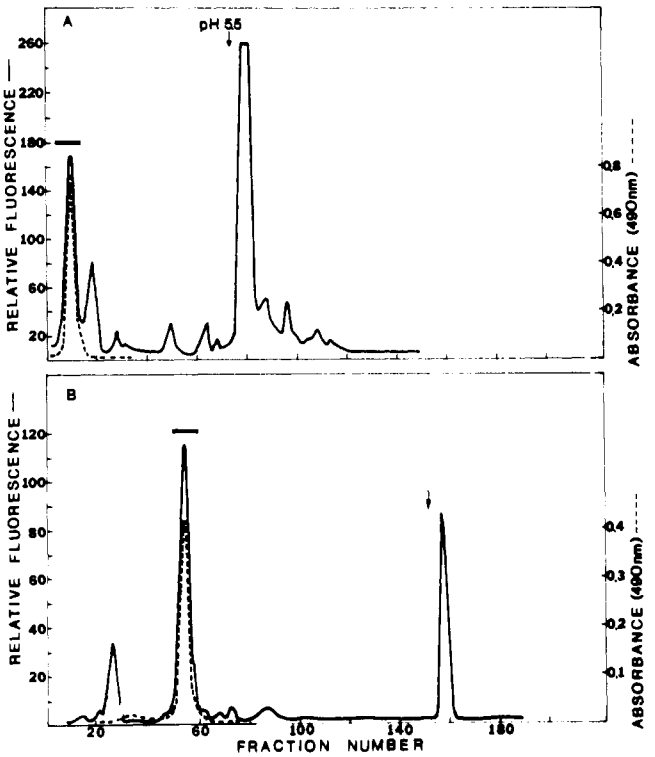

FIGURE 9: Purification of the glycopeptides resulting from a tryptic digest of purified CBII. (A) Chromatography of 20 mg of CBII digest on a Dowex 50-X2 column. Conditions for elution of the glycopeptides were exactly the same as described for Figure 3A. Aliquots of 200 μL were analyzed for hexose and peptide content. The bar indicates those fractions pooled for further study. (B) Chromatography of the glycopeptide fraction on a DEAE-cellulose ion-exchange column. Conditions for elution of the glycopeptides were the same as described for Figure 3B. Aliquots of 100 μL were analyzed for hexose and peptide content.

size and monosaccharide composition. In addition, these data indicate that both heteropolysaccharide units have identical amino acid residues in the immediate vicinity of the carbohydrate attachment sites on the transferrin molecule, namely, 1 aspartic acid residue, 2 serine residues, 1 leucine residue, and 1 half-cystine residue, suggesting identical amino acid sequences in these regions.

Both heteropolysaccharide units are located on a single polypeptide fragment (CBII) representing approximately one-third of the transferrin molecule. This was demonstrated by the compositional analysis of purified CBII and confirmed by the subsequent demonstration that this cyanogen bromide polypeptide yielded a tryptic glycopeptide that was identical with the tryptic glycopeptide obtained from intact transferrin. Although the alignment of the cyanogen bromide fragments of rabbit transferrin is not known at this time, it appears likely that CBII is found in the carboxy-terminal region of this molecule, since both of the glycosylation sites in human transferrin (MacGillivray et al., 1977) and ovotransferrin (Williams, 1975) are found in the carboxy-terminal region of the molecule. Finally, it is noteworthy that this segment of the transferrin chain containing both heteropolysaccharide units also contains 30% of the tyrosine and 45% of the histidine residues found in the intact molecule. In view of the studies implicating tyrosine and histidine residues as ligands in the iron binding sites (Rogers et al., 1977; Komatsu & Feeney, 1967), these data suggest the possibility that at least one of the two iron binding sites is located in this segment.

Amino acid sequence studies of human transferrin (MacGillivray & Brew, 1975; MacGillivray et al., 1977) have suggested that this molecule is composed of two homologous domains and that certain regions within these domains appears to be highly conserved. The results of this study have further substantiated that internal sequence homology is found in the transferrins. Of particular interest is the fact that the amino acid sequence immediately surrounding the two glycosylation sites of rabbit transferrin appear to be identical. This sequence homology surrounding the glycosylation sites has not been observed with other species of transferrin (Graham & Williams, 1975) and, thus, reflects a symmetry in rabbit transferrin not found in other transferrins. It is of interest in this regard that the two iron binding sites in rabbit transferrin appear to be functionally similar. In studying iron release from rabbit transferrin mediated by organic phosphate compounds, Morgan (1977) has observed that iron was released at the same rate from both iron binding sites, suggesting that these binding sites are similar. Furthermore, each iron binding site possesses similar acid-base iron-binding properties (Princiotto & Zapolski, 1978). Finally, these studies are further substantiated by the work of Harris & Aisen (1975a) who examined the iron-donating properties of rabbit transferrin and found that both iron binding sites appeared to be functionally equivalent.

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