Effect of the Carbohydrate Moiety on the Secondary Structure of β_2-Glycoprotein I. Implications for the Biosynthesis and Folding of Glycoproteins†

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ABSTRACT: By use of six highly purified exoglycosidases with well-defined specificity, the oligosaccharide units of human plasma β_2-glycoprotein I (β_2I) were modified by sequential enzymatic degradation. The released monosaccharides (NeuAc, Gal, GlcNAc, and Man) were quantified, and the carbohydrate compositions of the resulting glycoprotein (gp) derivatives were determined. The gp was found to be both partially sialylated and galactosylated. These findings which are in agreement with earlier reports suggest that the carbohydrate moiety of β_2I possesses more bi- than tri-antennas, probably three of the former and two of the latter carbohydrate units. Circular dichroic (CD) spectra of native β_2I and its derivatives were measured in aqueous buffer and 2-chloroethanol (2-CE). Analysis of these spectra for elements of secondary structure showed β_2I and most of the derivatives to contain predominantly β-sheet and β-turn structures. The lack of α-helical structures in aqueous buffer was noted. Removal of a large portion of the carbohydrate moiety did not alter the CD spectra or secondary structure of β_2I in either aqueous buffer or in 2-CE. However, after enzymatic removal of approximately 96% of the carbohydrate moiety, large significant changes in the spectra and secondary structures were observed. In aqueous buffer a shift in the wavelength minimum occurred, accompanied by an increase in the magnitude of the molar ellipticity and the amount of β-turn, with a reduction in random coil. One-third of the amino acids which were originally in random coil conformation assumed β-turns after removal of 96% of the carbohydrate moiety. In 2-CE the position of the wavelength minimum was unaltered after each enzymatic step; however, a large increase in the magnitude of the molar ellipticity was seen as well as a 10% increase in α-helix and a 5% increase in random coil with corresponding reductions in both β-sheet and β-turn. Analysis of the primary structure of β_2I by secondary structure prediction methods which were modified to include the elements of secondary structure estimated from the CD spectra showed the presence of 31 β-turns and 13 regions of β-sheet and showed the remainder to be random coil. These results suggest that the transfer of large-size Man-type (oligomannose) *N*-glycans during biosynthesis of gp may ensure the proper folding of their polypeptide chains and, thus, the presence of glycans may be a requirement for the biological activity of β_2I.

β_2-Glycoprotein I (β_2I)[1] is a glycoprotein (gp) which has been purified from human plasma and characterized in terms of its major chemical and physicochemical properties (Schwick & Haupt, 1975). Recently, its amino acid sequence has been determined and revealed five N-glycosylation sites (Lozier et al., 1984). Investigation of the structure of the oligosaccharide chains by ^{1}H NMR spectroscopy showed that this protein possesses primarily biantennary (Bürgi et al., 1983) and some triantennary glycans (Bürgi et al., 1984). As to its possible biological roles,[2,3] β_2I binds to platelets, interacts with heparin, and may play a role in blood coagulation (Schousboe & Rasmussen, 1988; Carson & Ross, 1988; Nimpf et al., 1986). This protein, also designated apolipoprotein H (Lee et al., 1983), is found in plasma in association with lipoproteins, such as chylomicrons, very low density lipoproteins, and high-density lipoproteins (Polz & Kostner, 1979) of patients experiencing acute-phase reactions.

The aim of the present study was to assess the effect of the carbohydrate moiety upon the secondary structure of β_2I. For this purpose the protein was sequentially treated with several highly purified exoglycosidases, and the gp derivative obtained at each step was analyzed by circular dichroic (CD) spectroscopy in aqueous buffer and 2-chloroethanol (2-CE), a solvent which stabilizes α-helical regions (Wallace et al., 1984). This study thus presents an opportunity to examine the effect of sequential sugar removal on the physical properties and particularly the secondary structure of the gp.

Materials and Methods

All chemicals were of analytical grade and were purchased from Merck (Darmstadt, FRG) or Serva (Heidelberg, FRG). Sephadex G-100, CH-Sepharose 4B, and Sepharose 6B were

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[1] Abbreviations: β_2I, β_2-glycoprotein I or apolipoprotein H; 2-CE, 2-chloroethanol; CD, circular dichroism; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; gp, glycoprotein(s); Man, mannose; [θ], molar ellipticity in deg·cm^2/dmol; NeuAc, *N*-acetylneuraminic acid; VCS, *Vibrio cholerae* sialidase.

[2] The glycans of many gp are essential for their biological activities (Bürgi, 1989; Hubbard, 1989; Paulson, 1989).

[3] This rule would apply only to protein with *N*-glycans.

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from Pharmacia (Uppsala, Sweden), and Bio-Gel P-2 was from Bio-Rad (Münich, FRG). Solvents for HPLC were of spectroscopic grade. 2-Chloroethanol was purchased from Aldrich (Milwaukee, WI) and redistilled over P_2O_5 prior to use. β_2I was the generous gift of Prof. H. Gerhard Schwick, Behringwerke AG, Marburg/Lahn, FRG.

(*1*) *Glycosidases. Vibrio cholerae sialidase* (specific activity 28 units/mg of protein) was purchased from Behringwerke AG (Marburg/Lahn, FRG). *β-Galactosidase* (specific activity 8 units/mg of protein) isolated from bovine testes was further purified by a modification of the procedure described by Distler et al. (1973). Except for the presence of sialidase (approximately 1% of the total activity), no other glycosidases could be detected. Jack bean *β-N-acetylglucosaminidase* and *α-mannosidase* were obtained from Sigma (Deisenhofen, FRG) (specific activity 50 and 17 units/mg of protein, respectively). *β-Mannosidase* derived from *Aspergillus niger* was purified by a modification of Bouquelet et al. (1978), yielding an enzyme preparation with a specific activity of 12 units/mg of protein and containing traces of proteolytic activity which could be inhibited by 1% pepstatin. All other exoglycosidases were free of proteolytic activity as determined by the procedure described below and other glycosidases except for the galactosidase as mentioned above. The activity of the enzymes was measured by using 4-methylumbelliferyl glycosides as substrates. One unit of enzyme is defined as the amount of enzyme that cleaves 1 μmol of substrate/min at 37 °C. *Endoglycosidase D* from *Diplococcus pneumoniae* was a product of Boehringer Mannheim (Frankfurt, FRG). The specificity of this enzyme cannot be indicated because of lack of a proper substrate.

For the preparation of the β_2I derivatives, all enzymatic digestions were performed at 37 °C under the conditions specified below and in buffers containing 0.02% NaN_3 and 1% pepstatin.

(*2*) *Affinity Matrix.* NeuAc was immobilized on Sepharose 6B using divinyl sulfone. Details of this procedure will be described elsewhere (Watzlawick and Brossmer, unpublished data). 4-Aminophenyl-thiogalactoside was coupled to CH-Sepharose 4B employing *N*-ethyl-*N'*-[3-(dimethylamino)-propyl]carbodiimide as described in the Pharmacia Affinity Chromatography Instructions.

(*3*) *Chemical Analysis.* (*a*) *Acid Hydrolysis Conditions.* For determination of its carbohydrate content, the gp and its partially deglycosylated derivatives were hydrolyzed under different conditions depending on the type of sugar to be quantified. A standard sugar solution (containing 0.5 mg of monosaccharide/mL) was "hydrolyzed" under identical conditions in order to be able to calculate corrections for destruction of each sugar due to acid hydrolysis. The conditions were as follows. For NeuAc, gp (0.2 mg) was hydrolyzed in 200 μL of 0.05 N H_2SO_4 at 80 °C for 3 h. The amount of NeuAc was determined at 30-min intervals; thus, the maximum NeuAc value could be determined (see below). For Gal, gp (0.2 mg) was hydrolyzed in 100 μL of 1 N HCl at 100 °C for 2 h. After neutralization with 1 N NaOH, Gal was determined as indicated below. For Man, gp (0.2 mg) was hydrolyzed in 100 μL of 1 N H_2SO_4 at 100 °C for 8 h and neutralized with 1 N NaOH for subsequent Man determination. For amino sugars, gp (0.2 mg) was hydrolyzed in 500 μL of 4 N HCl at 100 °C for 3 h, resulting in optimal recovery of GlcNAc. After removal of HCl, the amino sugars were reacetylated with acetic anhydride in methanol at room temperature for 2 h followed by removal of excess reagent. The error of these methods was estimated to be ±5%.

(*b*) *Analytical Methods. NeuAc* was quantified by HPLC on an Aminex HPX-87H column (Bio-Rad, 300 × 7.8 mm). This sugar, detected at 200 nm, eluted at a retention time of 7.8 min (Silver et al., 1981). The amount of NeuAc was calculated by comparison with appropriate standards (1.0–5.0 nmol). *Gal* was determined by a microadaptation (total volume 0.6 mL) of the coupled enzymatic–optical procedure of Kurz and Wallenfels (1974). *Man* was measured by a microadaptation (total volume 0.6 mL) of the coupled enzymatic–optical procedure of Warburg (Gawehn, 1974). *GlcNAc* was quantified by HPLC on an Aminex HPX-87H column (Bio-Rad, 300 × 7.8 mm) (Preinl, 1987). GlcNAc was detected at 200 nm and eluted at a retention time of 11 min. Suitable standards (0.1–1 μg) were used for the calculation of this sugar. The tetrasaccharide $(Man)_3GlcNAc$ was also analyzed by HPLC using an aminopropyl column. The tetrasaccharide content was monitored at 200 nm and calculated with respect to a GlcNAc standard.

(*4*) *Protease Assay.* Protease activity was determined essentially as described earlier (Williams & Lin, 1971) using [^{14}C]methemoglobin as substrate. For the detection of minute proteolytic activity present in the exoglycosidase preparations, the same incubation conditions (up to 10 days) were used as described below for the digestion of gp.

(*5*) *High-Performance Liquid Chromatography.* The HPLC system consisted of a Model 655A-11 pump and UV monitor (Merck-Hitachi, Darmstadt, FRG) with a variable-wavelength detector and a Rheodyne injection valve. The Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad) was run isocratically with 0.01 N H_2SO_4 at a flow rate of 0.6 mL/min and at 100 kg/cm^2, while the aminopropyl column (250 × 4 mm, Zinsser Analytik, Frankfurt, FRG) was run isocratically with a mixture of acetonitrile (grade S) and 15 mmol of KH_2PO_4 in double-distilled water (25:75 v/v) at a flow rate of 1 mL/min at 150 kg/cm^2.

(*6*) *Evaluation of the Purity of Protein Derivatives.* Each gp derivative was analyzed by SDS–PAGE (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue. N-Terminal sequence analysis was performed with the aid of a Beckman liquid-phase sequencer (Model 890C).

(*7*) *Circular Dichroic Spectra and Secondary Structural Analysis and Prediction.* CD spectra of native β_2I and its enzymatically prepared derivatives were recorded at ambient temperature on a Cary 61 CD spectropolarimeter (Varian, Palo Alto, CA) calibrated with *d*-10-camphorsulfonic acid. A 0.1-cm quartz cell was used for measurements in the wavelength region 250–200 nm. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. Sample concentrations were in the range of 0.3–0.6 mg of protein/mL. On the day the CD measurements were conducted, lyophilized samples were dissolved in 5 mM sodium phosphate, pH 7.4, containing 0.02% NaN_3, or in 2-CE.

The spectrum reported for each sample represents the average of three individual spectra and has been corrected for base-line contribution due to solvent. It has been shown that the carbohydrate moiety of the protein does not contribute significantly to the ellipticities that were recorded from 250 to 200 nm (Schmid et al., 1978; Johnson, 1987). As control, a glycopeptide mixture isolated from an enzymatic digest of human plasma α_1-acid glycoprotein was used. This mixture contained approximately 90% sugar. The CD spectrum of the glycopeptide was not altered after enrichment of the carbohydrate content. Molar ellipticity values, $[\theta]$, were calculated according to the following equation (Greenfield & Fasman,

1969): $[\theta]$ (deg·cm^2/dmol) = (θ)(MRW)/10lc, where θ represents the displacement from the base-line value times the full range in degrees, MRW equals the mean residue weight of the amino acids, l is the path length of the cell in cm, and c equals the concentration of protein in g/mL. The $[\theta]$ values reported are ±1% for each sample after averaging over multiple preparations and scans.

Data points were analyzed at 1-nm intervals between 250 and 200 nm by nonlinear, constrained least-squares curve-fitting procedures to obtain estimates of each type of secondary structure. CD spectra were analyzed for α-helix, β-sheet, β-turn, and random coil according to the reference data sets of Greenfield and Fasman (1969), Chang et al. (1978), and Brahms and Brahms (1980) by using the computerized LINEQ program of Cynthia Teeters (Mao & Wallace, 1984).

Secondary structural predictions for α-helix, β-sheet, and β-turn using the amino acid sequence (Lozier et al., 1984) were performed by the computerized method of SEQ (Whitlow, 1986). SEQ is a protein primary sequence analysis program which is based on the frequency tables of Chou and Fasman (1978), with the additional constraint of including an estimate of the percentage of each secondary structural element (α-helix, β-sheet, β-turn, and random coil) which results from analysis of the CD spectra. The program first assigns the most probable β-turn regions. Next, either α-helix or β-sheet regions are assigned, depending on which predominates in the protein's secondary structure. Since the secondary structure of β_2I is, on the basis of the CD data which are presented below, predominantly β-sheet, β-sheet regions are next assigned.

RESULTS

(1) Preparation of Partially Deglycosylated β_2I. *(a) Asialo-β_2I.* β_2I (50 mg) was incubated for 24 h with 80 milliunits of VCS in 1 mL of 50 mM NaOAc buffer, pH 5.5, containing 7 mM $CaCl_2$, cleaving 98% of NeuAc. The enzymatic activity remained essentially unchanged (95%) during the experiment. Following dilution of the digest with 10 mL of the same buffer, the sialidase was removed by affinity chromatography on immobilized NeuAc (10 mL bed volume). Asialo-β_2I was detected in the effluent by monitoring the absorption at 280 nm and desalted subsequently by chromatography on Bio-Gel P-2. The derivative was recovered after lyophilization (40.5 mg, yield 84%). No residual NeuAc could be detected by the "acid hydrolysis" procedure.

(b) Agalacto-β_2I. Asialo-β_2I (35 mg) was digested with 2 units of β-galactosidase in 1 mL of 0.1 M sodium citrate/phosphate buffer, pH 4.3. After 24 h, 95% of Gal was cleaved and 90% of the enzymatic activity remained. The enzyme was separated from the incubation mixture by affinity chromatography on 4-aminophenyl thiogalactoside–CH-Sepharose 4B (10 mL bed volume). Gal and salts were removed by chromatography on Bio-Gel P-2. Subsequent lyophilization afforded 29.5 mg of agalacto-β_2I (yield 87%) which contained 0.11% Gal by weight determined after acid hydrolysis.

(c) A-N-acetylglucosamino-β_2I. Agalacto-β_2I (16.5 mg) was treated with 1.5 units of β-*N*-acetylglucosaminidase in 1 mL of 0.1 M sodium citrate buffer, pH 4.7. After 5 days 75% of GlcNAc was cleaved. Following addition of further enzyme (1 unit) and by extension of the digestion to a total of 10 days, cleavage of GlcNAc increased to 87%. The enzyme activity was 70% at the end of the experiment. A-*N*-acetylglucosamino-β_2I was separated from enzyme, salt, and pepstatin chromatographically on Sephadex G-100. Lyophilization yielded 13.9 mg (88% yield) of this derivative.

(d) A-α-mannosino-β_2I. A-*N*-acetylglucosamino-β_2I (15 mg) was incubated with 12 units of α-mannosidase in 1 mL of 0.1 M sodium citrate, pH 4.7, containing 5 mM $ZnAc_2$. The release of Man was followed over an incubation period of 3 days (Figure 1). Although during incubation the enzyme activity fell to 50%, additional enzyme did not release further Man. The gp derivative was isolated from the digest by Sephadex G-100 gel filtration. Subsequent lyophilization yielded 13.1 mg (89% yield).

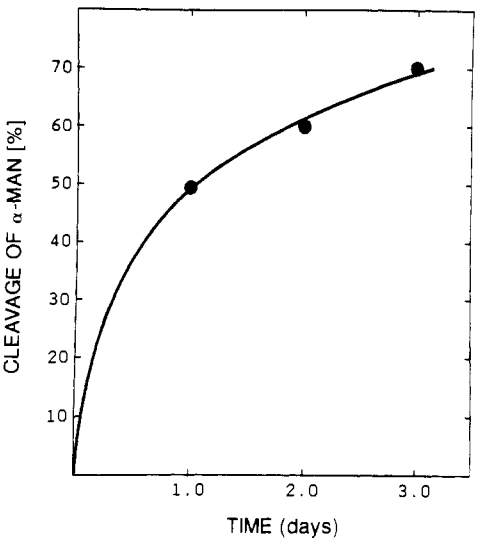

FIGURE 1: Time course of liberation of Man during digestion of a-*N*-acetylglucosamino-β_2I with α-mannosidase. The released Man was determined as described under Materials and Methods and is expressed as percent of total α-Man.

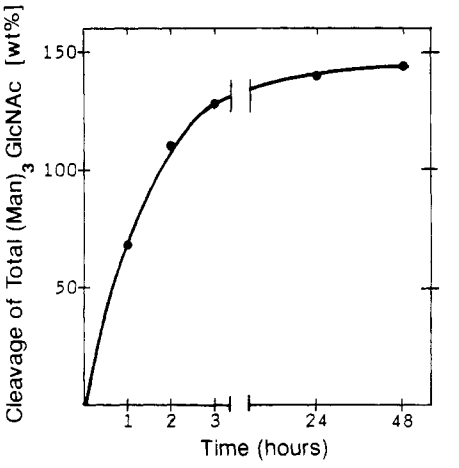

FIGURE 2: Time course of the digestion of a-*N*-acetylglucosamino-β_2I with endoglycosidase D. Released $(Man)_3$GlcNAc was determined as indicated under Materials and Methods and is expressed as weight percent $(Man)_3$GlcNAc to protein.

(e) A-β-mannosino-β_2I. A-α-mannosino-β_2I (5.2 mg) was treated with 300 milliunits of β-mannosidase in 200 μL of 0.1 M sodium acetate, pH 4.7. After 1 day of incubation 3.2 mol of Man residues/mol of gp derivative was cleaved, an amount that was not increased on further incubation, although the enzyme remained active (90%). After chromatography on Sephadex G-100 and lyophilization, 3.1 mg of a-β-mannosino-β_2I was obtained (70% yield).

(f) Endo-N-acetylglucosaminidase D Digested β_2I. A-*N*-acetylglucosamino-β_2I (3.6 mg) was incubated with 40 milliunits of endoglycosidase D in 0.2 M sodium citrate, pH 6.3. Liberation of sugar was followed over a period of 2 days (Figure 2). Additional enzyme did not increase the release of the tetrasaccharide. Subsequently, the digest was chromatographed on Sephadex G-100 and afforded 2.5 mg of the derivative (83% yield).

Table I: Monosaccharides Released from β_2I by Sequential Treatment with Exoglycosidases

step of treatment	enzyme used	mol of residues released/mol of protein	total residues
a	VCS	7.0 NeuAc[a]	7.0[a]
b	β-galactosidase	8.0 Gal	8.0[b]
c	β-*N*-acetylglucosaminidase	9.3 GlcNAc	12[c]
d	α-mannosidase	7.0 α-Man	10
e	β-mannosidase	3.2 β-Man	5
f	endoglycosidase D	3.5 $(Man)_3GlcNAc$	5

[a] β_2I is known to be partially sialylated (see text). [b] β_2I is partially galactosylated. [c] These are the peripheral GlcNAc residues of the three N-bi- and two N-triantennary glycans. These *N*-glycans contain in their cores two additional GlcNAc each.

(*2*) *Homogeneity and Integrity of the β_2I Derivatives.* As to the homogeneity and integrity of the above-described preparations, no cleavage products of the native gp nor of the derivatives could be detected on SDS–PAGE (Figure 3) even when the gel was overloaded (Figure 3, lane 7). Further, N-terminal sequencing did not reveal any N-terminal amino acids in addition to those of native β_2I.

(*3*) *Carbohydrate Composition of β_2I and the Monosaccharides Sequentially Cleaved by Glycosidases.* The amount of monosaccharide released by the enzyme employed in each step of the degradation of β_2I (Table I) agrees essentially with the carbohydrate composition of the native protein determined by the acid hydrolysis procedure (Table II). *NeuAc* was probably completely cleaved as deduced from comparable investigations with other human plasma gp (unpublished data). Further, as shown by ^{1}H NMR spectroscopy of the glycopeptides isolated from β_2I, this protein is partially sialylated (J. Vliegenthart, unpublished data). This finding is not unusual as many gp have been shown to be partially sialylated (Montreuil, 1980; Kornfeld & Kornfeld, 1976). The *Gal* content of β_2I accounts for only two thirds of the theoretically possible Gal residues. The β-galactosidase, which was used in the present study and which is known to cleave this sugar completely from gp, released an amount of Gal that was equal to the Gal content determined by the acid hydrolysis procedure. From these data it was concluded that this protein is partially galactosylated. The *GlcNAc* content of β_2I determined by the acid hydrolysis procedures was found to correspond to the theoretical number of residues. However, in step c (Table I) only 12 residues are available for cleavage by β-*N*-acetylglucosaminidase. These accessible residues are peripherally located, in contrast to the additional 10 GlcNAc residues that are in the core of the five *N*-glycans. The latter enzyme cleaved 87% of the accessible residues. It is also important to note that this value is higher than that of Gal residues cleaved by β-galactosidase or determined by the acid hydrolysis procedure (Table II). Part of the incomplete release of the peripheral GlcNAc residues may be due to the presence of Fuc which is assumed to be linked to the GlcNAc of the branches of the glycans. Hence, on average only four of the five glycans could be de-*N*-acetylglucosaminated, and thus, a value of 9.3 residues of liberated GlcNAc appears reasonable. These results suggest that β_2I may possess terminal GlcNAc which have been shown previously with other gp (Montreuil, 1980; Kornfeld & Kornfeld, 1976). *Man* could be cleaved to the expected extent by α- and β-mannosidase, namely, to a molar percentage of 70 and 64, respectively. The determination of Man of the native protein and its derivatives presented a unique problem in that the Man content (of the native and asialo-β_2I derivative) appeared to be very low, probably because, under the conditions for hydrolysis for Man in the presence of NeuAc and/or Gal, Man is largely destroyed. Analysis of the a-*N*-acetylglucosamino-β_2I derivative afforded the correct Man value of 15 mol of residues/mol of protein. As shown in the data of Table I, step f, 3.5 mol of the tetrasaccharide $(Man)_3GlcNAc$ was cleaved, corresponding to a yield of approximately 70%.

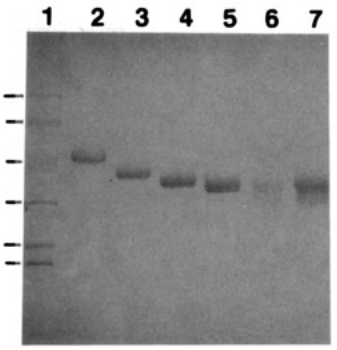

FIGURE 3: SDS–PAGE of native human plasma β_2-glycoprotein I and its deglycosylated derivatives. (Lane 1) Molecular weight standards; the lines to the left of Lane 1 mark the migration of the protein standards. Molecular weights (top to bottom): phosphorylase *b*, 97 400; bovine serum albumin, 66 200; ovalbumin, 42 700; carbonic anhydrase, 31 000; trypsin inhibitor, 21 000; α-lactalbumin, 14 000. (Lane 2) Native protein; (lane 3) agalacto derivative; (lane 4) a-*N*-acetylglucosamino derivative; (lane 5) a-α-mannosino derivative; (lanes 6 and 7) a-β-mannosino derivatives. Lane 7 contains twice the amount of protein as lane 6.

(*4*) *CD Spectroscopy.* Far-UV CD spectra of the native β_2I and the above-described derivatives of this protein in aqueous buffer and organic solvent are shown in Figures 4 and 5. In phosphate buffer (Figure 4), native β_2I and its asialo, agalacto, a-*N*-acetylglucosamino, and a-α-mannosino derivatives exhibited their minima at the same wavelength, with their maxima varying over a narrow wavelength range, with minor differences in magnitude. The positive maximum was centered between 228 to 230 nm ($[\theta]$ = +800 to +1100 deg·cm^2/dmol) with the negative minimum at 210 nm ($[\theta]$ = −1200 to −800). Removal of β-mannose yielded a spectrum which was different from those described above in that the magnitude of the minimum at 210 nm increased ($[\theta]$ = −2100). Cleavage of $(Man)_3GlcNAc$ (see step f, Table I; see also Endo D derivative, Table II) yielded a spectrum that differed from those described

Table II: Carbohydrate Composition[a] of β_2I and Its Enzymatically Prepared Derivatives

β_2I or derivative	M_r[b]	NeuAc	Fuc	Gal	GlcNAc	Man
native	48 000[c]	7.0[a] (4.1)	–[d]	8.0 (2.6)	21.0[e] (9.5)	1.0 (3.7)
asialo	46 042	–	1[c]	8.0 (2.8)	20.3 (9.0)	3.8 (4.8)
agalacto	44 746	–	–	0.3 (0.1)	21.0 (9.5)	14.0 (5.1)
a-*N*-acetylglucosamino	42 919	–	–	0.3 (0.1)	10.3 (4.8)	15.3 (5.8)
a-α-mannosino	41 737	–	–	–	10.7 (5.2)	7.3 (2.8)
a-β-mannosino	41 251	–	–	–	10.5 (5.2)	5.0 (2.0)
Endo D	40 380	–	–	–	6.0 (3.0)	3.0 (1.2)

[a] Determined by the acid hydrolysis procedure; values are expressed in mol of residues/mol of protein. In parentheses, these values are expressed in weight percent. Each sugar was determined after acid hydrolysis as described under Materials and Methods. [b] Calculated from the amount of sugar cleaved enzymatically. [c] Taken from Schwick and Haupt (1975). [d] (–) Not determined. [e] β_2I contains a total of 22 GlcNAc residues.

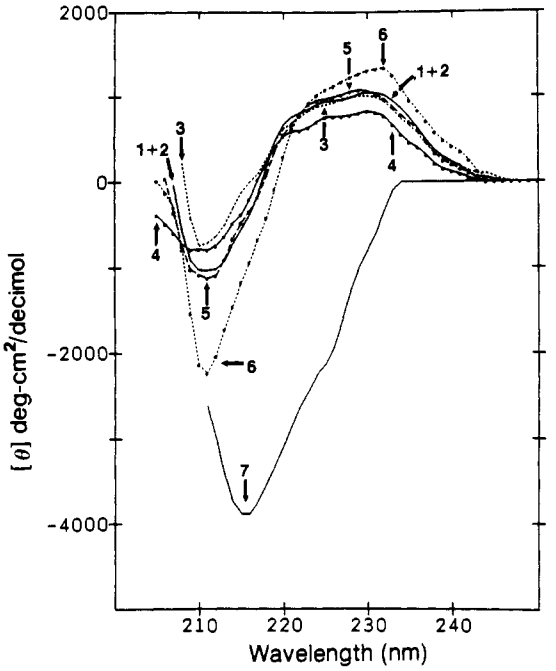

FIGURE 4: Circular dichroic spectra of β_2I and its partially deglycosylated derivatives in 5 mM sodium phosphate, pH 7.4: (1) (—) Native protein; (2) (---) asialo derivative; (3) (···) agalacto derivative; (4) (—·—) a-*N*-acetylglucosamino derivative; (5) (---•---) a-α-mannosino derivative; (6) (···•···) a-β-mannosino derivative; (7) (—) Endo D derivative. Note: The molar ellipticities reported in Figures 4 and 5 are calculated with respect to protein (not glycoprotein) concentration.

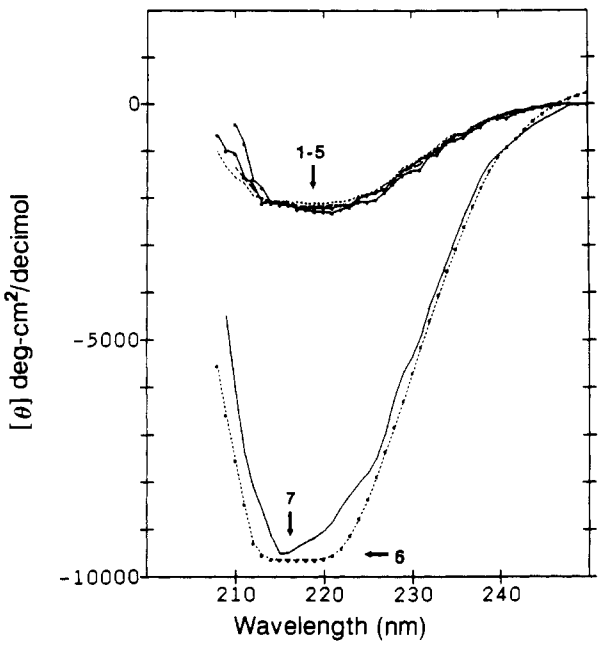

FIGURE 5: Circular dichroic spectra of β_2I and its partially deglycosylated derivatives in 2-CE. (1) (—) Native protein; (2) (---) asialo derivative; (3) (···) agalacto derivative; (4) (—•—) a-*N*-acetylglucosamino derivative; (5) (---•---) a-α-mannosino derivative; (6) (···•···) a-β-mannosino derivative; (7) (—) Endo D derivative.

above and was typical of a protein that has a significant amount of β-structure. The minimum occurred at 217 nm ([θ] = −3805) with a slight shoulder at 228 nm and no positive maximum, unlike the spectra of the other derivatives.

In 2-CE (Figure 5), the CD spectra of native β_2I and its asialo, agalacto, a-*N*-acetylglucosamino, and a-α-mannosino derivatives were very similar. All were characterized by a wide negative minimum (from 214 to 224 nm) centered at 221 nm ([θ] = −2400). However, while the spectra of the a-β-mannosino and Endo D derivatives were similar to each other, they were quite different from the spectra of the others in that they exhibited a negative minimum centered at approximately 215 nm ([θ] = ∼−9750), suggesting that cleavage of β-mannose and subsequently GlcNAc as $(Man)_3GlcNAc$ leads to a change in the secondary structure of β_2I.

Table III: Percentage of Secondary Structural Components of β_2I in Phosphate Buffer, pH 7.4

	α-helix (%)	β-sheet (%)	β-turn (%)	random coil (%)
native	0	40	30	30
asialo	0	40	30	30
agalacto	0	40	28	32
a-*N*-acetyl glucosamino	0	40	28	32
a-α-mannosino	0	40	30	30
a-β-mannosino	0	40	35	25
Endo D	0	40	**42**[a]	**18**[a]

[a] These two numbers are in bold type in order to emphasize the major changes in the secondary structure occurring upon enzymatic cleavage of 96% of the carbohydrate moiety of the gp.

Table IV: Percentage of Secondary Structural Components of β_2I in 2-Chloroethanol

	α-helix (%)	β-sheet (%)	β-turn (%)	random coil (%)
native	5	55	20	20
asialo	5	55	20	20
agalacto	5	55	20	20
a-*N*-acetyl glucosamino	5	55	20	20
a-α-mannosino	5	55	20	20
a-β-mannosino	15	50	10	25
Endo D	**15**[a]	50	**10**[a]	**25**[a]

[a] These three numbers are in bold type in order to emphasize the major changes in the secondary structure occurring upon enzymatic cleavage of 96% of the carbohydrate moiety of the gp.

Analysis of the CD spectra for elements of secondary structure of the samples in aqueous solvent is summarized in Table III. In phosphate, pH 7.4, all spectra were indicative of a mixture of β-sheet, β-turn, and random with no α-helix. Native β_2I and the derivatives that have similar CD spectra have approximately 70% β-structure (sheet regions plus turns) and 30% random coil. Removal of β-mannose resulted in a small increase in the amount of β-turn with a reduction in random coil. Further cleavage of the carbohydrate (Endo D derivative, 96% of the carbohydrate removed) resulted in an increase in β-turn accompanied by an additional reduction in random coil.

In 2-CE (Table IV), native β_2I and its asialo, agalacto, a-*N*-acetylglucosamino, and a-α-mannosino derivatives exhibited similar secondary structures, characterized by a small amount of α-helix, a large amount of β-sheet, 20% β-turn, and 20% random coil. However, for the a-β-mannosino and Endo D derivatives, α-helix and random coil increased with respect to the other derivatives, and β-sheet and β-turn decreased.

(*5*) *Prediction of Secondary Structure.* The secondary structure predicted from analysis of the primary amino acid sequence of β_2I by the Chou–Fasman algorithm (1978) and weighted with the secondary structure calculated from analysis of the CD data (Whitlow, 1986) of β_2I in aqueous buffer is shown in Figure 6. β-Sheet and β-turn predominate, with 13 predicted regions of β-sheet and 31 β-turns. The remaining structure is random coil. Four of the five N-linked glycosylation sites are located in regions predicted to be β-turns, while the fifth is in a region of β-sheet. The Cys residues, which

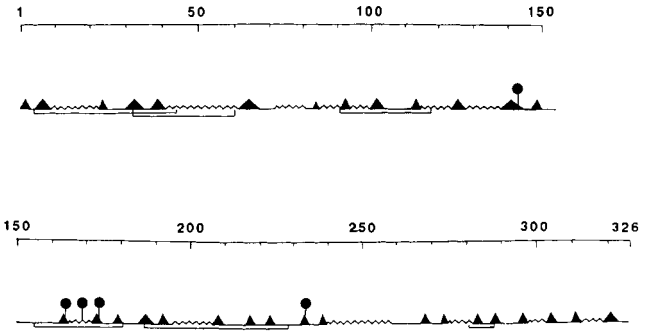

FIGURE 6: Predicted secondary structure of native β_2I. The Chou–Fasman (1978) probability parameters together with the secondary structure estimated from analysis of CD spectra of native β_2I in aqueous buffer were used to predict the secondary structure of β_2I (Whitlow, 1986) from its known primary structure (Lozier et al., 1984). (〰) β-sheet; (▲) β-turn; (📍) Asn, N-linked glycosylation site; (—) disulfide bonds that have been determined to date as described by Lozier et al. (1984); (—) random coil.

are known to be linked through intermolecular disulfide bonds (Lozier et al., 1984), are as shown in Figure 6.

Discussion

The advantage of the above-described multienzyme procedure which uses in sequence various highly purified glycosidases, is as follows: it affords, on a preparative scale, several β_2I derivatives which are deglycosylated to increasing degrees. These gp derivatives were well-defined in terms of their sugar compositions. The natural state of the polypeptide chain with its five glycosylation sites is not altered by the enzymes but by the removal of specific carbohydrate residues. More precisely, although the primary structure of the polypeptide chain is maintained, certain changes in the secondary structure occurred after removal of at least 90% of its sugar.

The availability of β_2I derivatives has allowed an investigation, by physical chemical methods, of the secondary structure of native gp and the mentioned derivatives, as well as the effect of the removal of specific sugar residues on their conformation. Such derivatives would also allow studies on the biological properties and the effect of the various sugars of the glycans, when unmasked, on the biological function as certain biological properties may be masked by other sugars (Bennett & Schmid, 1980). This investigation may then allow identification of the monosaccharides that are associated with such a biological property.

The CD spectra of β_2I and its asialo, agalacto, a-*N*-acetylglucosamino, and a-α-mannosino derivatives in an aqueous solvent are similar to one another and are characteristic of a protein that contains no α-helical regions but large amounts of β-structure (40%) and β-turns ($\sim$30%), the remainder being random coil ($\sim$30%). However, the CD spectra of the a-β-mannosino and Endo D derivatives are different from those mentioned above and are characteristic of a protein that has a similar amount of β-sheet but larger amounts of β-turn (42%) than the native β_2I. This finding suggests that only after extensive removal of the carbohydrate moiety is the secondary structure of β_2I altered. This leads to a greater number of β-turns, accompanied by reduction in random coil structures.

In 2-CE, the CD spectra of native β_2I and its asialo, agalacto, a-*N*-acetylglucosamino, and a-α-mannosino derivatives are also similar to one another. The major structural component is still β-sheet with considerable amounts of β-turn and random coil; but in this solvent a small amount of α-helix is formed. Enzymatic removal of β-mannose and $(Man)_3GlcNAc$ leads to significant changes in both the CD spectra and secondary structure, as expressed in an increase from 5 to 15% of the α-helical content, a decrease in β-sheet and β-turn, and a moderate increase in random coil. This observation may be explained in that, after the largest part of the carbohydrate moiety is removed, certain amino acid residues are exposed to the solvent and form α-helical regions.

Table V: Comparison of Number of Pro and Cys Residues in β_2I with That in Other Apolipoproteins

	no. of amino acid residues	Pro (% of total)	Cys (% of total)
AI[a]	243	10 (2.4)	0 (0)
B100[b-d]	4536	200 (4.4)	25 (0.6)
E[e]	299	8 (2.7)	2 (0.7)
β_2I[f]	326	31 (10.0)	22 (7.0)

[a] Brewer et al., 1978. [b] Knott et al., 1986. [c] Yang et al., 1986. [d] Cladaras et al., 1986. [e] Rall et al., 1982. [f] Lozier et al., 1984.

Comparison of the primary amino acid sequence of native β_2I to those of other human apolipoproteins (Table V) shows that β_2I contains a large number of Pro (a conformationally rigid residue) and Cys (commonly forming intermolecular disulfide bonds) residues, both of which impose structural constraints on the way in which β_2I folds. Also remarkable in the secondary structure of native β_2I when studied in aqueous buffer is the absence of α-helical regions, particularly amphipathic α-helices, which are strongly implicated as lipid-binding regions in apolipoproteins (Segrest et al., 1974). Thus, the mode of interaction of β_2I with lipid may well be mediated by another type of structural interaction than by the amphipathic α-helix, perhaps by the β-sheet.

Chou–Fasman analysis of the primary sequence of β_2I which has been weighted with the secondary structure estimated from analysis of the CD spectra of the native gp in aqueous solution predicts the secondary structure as shown in Figure 6. Remarkable is the large number of β-turns (31 regions). Of 31 Pro residues in β_2I, 15 occur within predicted β-turns, 3 within 2 residues of a predicted β-turn, and 13 within regions predicted to be β-sheet. Of the 22 Cys residues, 15 are at or in close proximity to β-turns. Chou–Fasman analysis, when not weighted with the secondary structural elements, predicts two regions of α-helix, residues 103–111 and 226–231. The second region contains a Cys residue which is known to be involved in a disulfide bond (Lozier et al., 1984). The first region contains 2 Cys residues which may also be involved in disulfide bonds, although this remains to be determined. These predicted α-helical regions appear to be absent in native β_2I, as judged by the shape and analysis of the CD spectra.

The apparent disparity between the basic Chou–Fasman analysis in which two α-helical regions are predicted and that by the modified SEQ program which includes weighting for the secondary structure as estimated by CD, where CD indicates the absence of α-helical regions, may possibly be explained by secondary and tertiary structural constraints (extensive disulfide bonding, large number of β-turns, and the presence of N-linked glycans). However, at least one of these predicted α-helical regions may well have a tendency to form an α-helical conformation in 2-CE, suggesting that a surface-located site in the folded native protein may be accessible to the solvent and has a propensity to be in a helical conformation.

As the secondary and tertiary structures of a protein determine its native, biologically active form, it appears of interest to explore the question pertaining to the relationship of the carbohydrate moiety and the secondary structure of β_2I. The study presented above demonstrates that NeuAcGalGlcNAcMan from the glycans can be cleaved without altering

the secondary structure. The carbohydrate remaining on the polypeptide chain consists of the trisaccharide ManGlcNAcGlcNAc, which, however, is still too large and/or has too many hydroxyl groups both of which may sterically hinder a change in the secondary structure. The secondary structure can only be altered after removal of the Man and one GlcNAc from this trisaccharide. The last monosaccharide, GlcNAc, which remains on the polypeptide chain is probably too small and/or has an insufficient number of hydroxyl groups to prevent conformational changes. As shown in this study, after enzymatic cleavage of 96% of the carbohydrate moiety approximately one-third of the random coil of the native protein assumes β-turns in aqueous solution.

The implication of this finding for the biosynthesis of glycoproteins is of particular interest. In this biosynthetic process high molecular weight (high-Man-type) glycans are transferred onto the nascent and—more importantly—not yet completely synthesized polypeptide chain. It is conceivable that the transfer of large-size glycans is required in order to assure proper folding of the polypeptide chain. In contrast, if the biosynthesis of the *N*-glycans were carried out by stepwise addition of each monosaccharide, attachment of the first GlcNAc might not ensure proper folding and subsequent addition of monosaccharides might not effect refolding of the secondary structure. Therefore, the biologically active form may not be obtained. In this regard, it is also noteworthy that, in the transformation of the high-mannose-type to the complex-type glycan, the pentasaccharide trimannosylbiacetylchitobiose remains unchanged, ensuring that the conformation of the polypeptide chain remains unaltered, thus maintaining the state necessary for biological activity.

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Conformation of Membrane Fusion-Active 20-Residue Peptides with or without Lipid Bilayers. Implication of α-Helix Formation for Membrane Fusion

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ABSTRACT: Fusion of small unilamellar vesicles of egg phosphatidylcholine can be triggered with synthetic 20-residue peptides. Taking the N-terminal amino acid sequence of HA-2 polypeptide of influenza virus as a guideline, we designed and synthesized several peptides having amphiphilic structures. Among the peptides so far studied, those active to induce membrane fusion took an α-helical conformation in the presence of phospholipid bilayers, while a peptide which was unable to induce membrane fusion was in a β-structure. Mixing of a pair of positively and negatively charged peptides, which had a complementary arrangement of electric charges to each other, resulted in α-helix formation at neutral pH, the condition of forming a randomly coiled conformation for each peptide. We concluded that α-helix formation was one of the necessary conditions to trigger a process of membrane fusion, at least in the present set of peptides. Characteristic features of these amphiphilic peptides are also described.

Hemagglutinin (HA)[1] of influenza virus is a glycoprotein responsible for the virus' ability to catalyze fusion of the membranes of the virus and a targeting cell (White et al., 1983). In vitro studies showed the HA-induced liposome fusion was triggered at a pH as low as 5.5 (Maeda & Ohnishi, 1980), the pH of the mildly acidic environment in endocytic vacuoles where in vivo fusion takes place to release the viral genetic material into the host cell cytoplasm. HA protein is made of two components, HA-1 and -2, and an inspection of the amino acid sequence of the protein revealed a hydrophobic region at the N-terminus of HA-2, which suggested the region as an interaction site with lipid membranes. We synthesized an eicosapeptide having the same amino acid sequence (1–20) of HA-2 [virus strain A/PR/8/34(H-1)] and found that the peptide actually induced membrane fusion of small unilamellar vesicles of egg PC, with a similar dependence on pH (active below pH 6) as the virus-mediated fusion (Murata et al., 1987a). Also, a closely related peptide, which had the amino acid sequence of the N-terminal 20 residues of influenza HA-2 strain B/Lee/40, has been reported to induce fusion of PC vesicles (Lear & DeGrado, 1987).

The structure of lipid bilayers is not rigid, and we expect that rather than the amino acid sequence itself, secondary or higher structures trigger the process of membrane fusion. We have synthesized HA-2-related peptides (Figure 1), and pH-dependent liposome fusion activities have been found for peptides II–VI (Murata, personal communication). In this paper, we describe the conformations of our synthetic peptides in solution, with or without phospholipid bilayers, to elucidate a correlation between peptide structure and the peptide activity of membrane fusion.

[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; CD, circular dichroism; Fmoc, fluorenylmethyloxycarbonyl; HA, hemagglutinin; IR, infrared; MES, 2-(*N*-morpholino)ethanesulfonic acid; PC, phosphatidylcholine.

Materials and Methods

Peptides. Peptides I–III were synthesized by a Boc methodology with [[(phenylacetamido)methyl]amino]polystyrene (prepared from Bio-Rad SX-1). Peptide coupling was carried out with an amino acid symmetric anhydride and monitored with the Kaiser test (Kaiser et al., 1970). Peptides were cleaved and deblocked with HF [a low–high procedure (Tam et al., 1983)] or with trifluoromethanesulfonic acid (Tam et al., 1986). Peptides III–VII were prepared by an Fmoc methodology (Eberle et al., 1986) using Ultrosyn A polyacrylamide–Kieselgel resin (Pharmacia–LKB) and Fmoc amino acid pentafluorophenyl esters (Kisfaludy & Schoen, 1983). Peptide cleavage from resin (1 g) was achieved by keeping the resin in a mixture of anisole (3 mL), 2-mercaptoethanol (1.5 mL), and trifluoroacetic acid (10 mL) at room temperature for 6 h. Each peptide was purified by a successive application of Sephadex G25 gel filtration, ion-exchange chromatography (DEAE-Toyopearl for acidic peptides, CM-Toyopearl for peptide VI), and reversed-phase HPLC [Cosmosil 300C4 for peptides I and II, elution with acetonitrile–2 mM phosphate (pH 7.1); Cosmosil C18-P or YMC A343-S5 C18 for the others, elution with acetonitrile–5 mM ammonium acetate]. The purity of the peptides was confirmed in every aspect of amino acid composition (Table I) and analytical HPLC. The samples of peptide III prepared by Boc and Fmoc methodology were not distinguished on HPLC.

Spectroscopy. CD spectra were obtained with a JASCO J-20 spectropolarimeter modified as to have a quartz stress-modulator, and the ellipticities are expressed as a mean residue weight basis, $[\theta]_{MRW}$, with units of degrees centimeter squared per decimole. Cells having 0.2–10-mm optical path lengths were used, depending on peptide concentrations; most measurements were carried out with a 0.2-mm cell. All the peptides, except VI, precipitated below about pH 5, CD mea-

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