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GEL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STUDIES ON THE ELUTION BEHAVIOR OF CHEMICALLY DEGLYCOSYLATED HUMAN CHORIONIC GONADOTROPIN AND ITS SUBUNITS

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SUMMARY

The elution behavior of human chorionic gonadotropin and its subunits, and their desialylated and deglycosylated derivatives was studied on gel high-performance liquid chromatography. Using three TSK G-SW columns, excellent separations were achieved for eleven human chorionic gonadotropin derivatives. In a comparison of the native (30% carbohydrate content), asialo (24%) and HF-deglycosylated (8%) subunits, the estimated values of molecular weights on the basis of a series of reference globular proteins deviated in various degrees from the actual molecular weight. The extent of deviation depended on the carbohydrate content and possibly the location of carbohydrate chains. The data from recombination studies suggest that the β-subunit is the dominant determinant for their expanded molecular size.

INTRODUCTION

The molecular weights of proteins can be estimated on the basis of the correlation of mobilities between the unknown and a series of reference proteins by gel chromatography on a media such as soft-gels from cross-linked dextran or polyacrylamide[1], or by gel electrophoresis on sodium dodecyl sulfate–polyacrylamide[2]. Both procedures have been widely employed because they are simple and accurate, especially if the protein molecule is globular. However, when applied to glycoproteins, these methods often give a high estimate of molecular weight. Although this anomaly is attributed to the presence of carbohydrate and the effect of its hydration on the molecular size[3–5], systematic studies on the direct relationship between carbohydrate content and chemical structure or physical properties are lacking. Until recently, the chemical structures of the carbohydrate moieties were poorly understood, and efficient methods for their specific modification were not available.

Human chorionic gonadotropin (HCG) is a glycoprotein hormone, containing approximately 30% carbohydrate, and is composed of two dissimilar α- and β-subunits[6]. In gel chromatography[6] or gel electrophoresis[7], HCG has shown apparent molecular weights 2–3 times larger than the actual values. Recently, the chemical structures of carbohydrates in HCG have been elucidated[8–10]; namely, two different

types of N-Asn-linked groups (n = 11 or 12) and O-Ser-linked groups (n = 4). Progressive removal of carbohydrate residues by a series of exoglycosidases[11] and by chemical deglycosylation with anhydrous hydrofluoric acid–anisole[12,13] has recently been described. Employing HCG and its two subunits as model glycoproteins, we have investigated the role of carbohydrate moieties in the apparent chromatographic anomaly of glycoproteins. We have taken advantage of gel high-performance liquid chromatography (HPLC) on silica-based TSK G2000SW and G3000SW columns[14], allowing rapid, reproducible and sensitive size-exclusion chromatographic studies. The elution behavior of HCG, its subunits before and after deglycosylation, and their recombinants have been studied in an attempt to evaluate the contribution of carbohydrate moieties to the overall size of these protein molecules.

MATERIALS AND METHODS

Proteins and reagents

Purified HCG and its α- and β-subunits (lot CR119) were obtained from the Center of Population Research, National Institute of Child Health and Human Development. Proteins and amino acid were purchased (sources in parentheses, all U.S.A.): bovine thyroglobulin and soybean trypsin inhibitor (Sigma, St. Louis, MO), bovine serum albumin (Miles, Elkhart, IN), ovalbumin (Pharmacia, Piscataway, NJ) and phenylalanine (Pierce, Rockford, IL). Water, ammonium acetate (Fisher; Fairlawn, NJ), and acetic acid (J. T. Baker, Phillipsburg, NJ) were of HPLC grade. The 0.1 *M* ammonium acetate–acetic acid buffer was filtered through a 0.45-μm filter (Millipore, Bedford, MA) before use.

Deglycosylation of HCG subunits

The HCG α- and β-subunits (lot CR119) were highly purified preparations, containing less than 0.2% of intact HCG molecules. The removal of the terminal sialic acid residues in the carbohydrate chains was carried out by incubation (2 h, 37°C) of each subunit (50 mg) with neuraminidase (EC 3.2.1.18., from *Glostridium perfringens*, 92 μg of Type X; Sigma) in 0.5 *M* potassium acetate (pH 6.0), as previously described[15]. The pure, desialylated subunits (20 mg of each) were further treated with anhydrous liquid hydrofluoric acid (10 ml) for 1 h at 0°C in the presence of anisole as a scavenger. The complete removal of hydrofluoric acid and anisole was achieved by high-vacuum evaporation over potassium hydroxide pellets in a desiccator, followed by extraction of an aqueous solution of the product with diethyl ether.

Sephadex G-100 purification of deglycosylated HCG subunits

The purification of deglycosylated products (20–50 mg) was carried out by Sephadex G-100 gel chromatography on a series of four connected columns (343 × 1.6 cm), previously equilibrated with 0.1 *M* ammonium hydrogencarbonate (pH 7.9). In the case of desialylation, the reaction mixture (*ca.* 2.5 ml) was directly applied to the column and eluted in order to remove the enzyme. The fractions containing subunits were pooled and twice lyophilized (*ca.* 40 mg). The hydrofluoric acid-treated asialo subunits (*ca.* 20 mg) were dissolved in 0.1 *M* ammonium hydrogencarbonate (2 ml) and chromatographed on the same Sephadex G-100 columns. The first peak to emerge (Fig. 1) was pooled and twice lyophilized (*ca.* 10 mg).

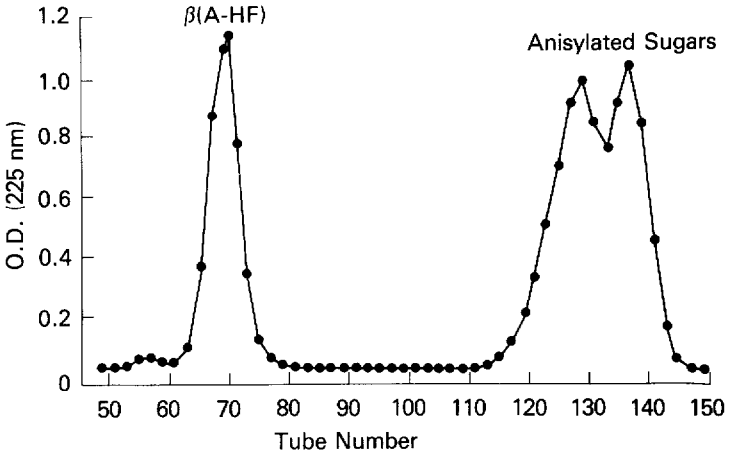

Fig. 1. Elution pattern of hydrofluoric acid-treated asialo HCGβ on Sephadex G-100. Column, 343 × 1.6 cm; eluent, 0.1 *M* ammonium hydrogen carbonate (pH 7.9); flow-rate, 3.5 ml/h; fractions, 5.7 ml per tube; detection, 225 nm; temperature, 4°C; sample, 20 mg of hydrofluoric acid-deglycosylated product.

Gel HPLC analysis of deglycosylated HCG subunits

Three TSK-type columns (each 60 × 0.75 cm: Beckman, Palo Alto, CA, U.S.A.) were connected in series: one G2000SW plus two G3000SW (total length, 180 cm). For gel HPLC, these columns were connected to a Hewlett-Packard 1084B liquid chromatograph (Hewlett-Packard, Geneva, Switzerland), equipped with a processor-controlled sampling and UV monitoring systems. The elution conditions employed were as follows: buffer, 0.1 *M* ammonium acetate–acetic acid pH 7.0; flow-rate, 1.0 ml/min; sample size, 100 μl; temperature, 25°C; UV detection, 278 nm.

The samples of native HCG, its subunits and their deglycosylated derivatives (100–150 μg) were dissolved in 0.1 *M* ammonium acetate (150 μl). The solution was briefly centrifuged for 5–10 min in a rotary concentrator (Savant, Hicksville, NY, U.S.A.) and the clear supernatant was applied to the column with the automatic sampling system.

Gel HPLC purification of HCG recombinants

Deglycosylated HCG subunits were recombined with each native complementary subunit by incubation (4°C, 24 h) in 0.1 *M* ammonium hydrogencarbonate (pH 7.9). The same amount of each subunit was employed for this recombination as reported previously[15]. The reaction mixture was lyophilized in a high-vacuum rotatory concentrator (Savant) in order to achieve the maximal recombination of subunits by gradual concentration. The residue was redissolved in 0.1 *M* ammonium hydrogencarbonate (150 μl) and purified in the gel HPLC system described above. The fractions showing receptor-binding activity were collected and lyophilized to yield 3–5 mg of pure products.

Analytical procedures

After hydrolysis of the samples in constant-boiling hydrochloric acid (110°C, 24 h), the amino acids were analyzed on a Beckman Model 121MB analyzer in con-

junction with a Beckman Model 126 Data System integrator. Amino sugars (glucosamine and galactosamine) were also determined by the amino acid analyzer, after hydrolysis in 3 *M* hydrochloric acid (100°C, 3 h). Quantitative analysis of sialic acid acid was carried out by the procedure of Warren[16]. Neutral sugars (mannose, galactose and fucose) were determined according to the procedure of Boykins and Liu[17].

RESULTS

The HCGα- and HCGβ-subunits were desialylated by neuraminidase and then deglycosylated by anhydrous hydrofluoric acid in the presence of anisole. The purification of these modified subunits was achieved by Sephadex G-100 gel chromatography. Fig. 1 shows a representative chromatogram of hydrofluoric acid-treated asialo HCGβ-subunit, β(A-HF), on Sephadex G-100. Only the first peak was found to contain protein, whereas peaks near the total permeation volume (Fraction 130) contained the anisole–carbohydrate conjugates derived from the hydrofluoric acid-catalyzed Friedel Crafts-type reactions, as demonstrated by mass spectroscopy and ^1H nuclear magnetic resonance (to be published). Hydrofluoric acid–anisole treatment under the conditions (0°C, 1 h) employed did not affect the polypeptide moiety[12,18]. Amino acid analyses of all modified subunits after purification, indeed, revealed that their compositions were in close agreement with the expected stoichiometry of unmodified subunits (data not shown).

When the elution position of HCG subunits on a Sephadex column was compared with respect to the degree of deglycosylations, it was obvious that these modifications considerably affected the molecular size of glycoproteins[13]. The partition coefficients for the β-subunits were 0.33 (asialo) and 0.53 (asialo-HF), and those for the β-subunits were 0.29 (asialo) and 0.38 (asialo-HF). The increased values of hydrofluoric acid-treated asialo subunits suggest that the molecular size of the glycoprotein was significantly reduced by deglycosylation.

The results of sugar content analyses are shown in Table I. The thiobarbituric quantitation method of Warren[16] revealed the complete removal of sialic acid residues after the neuraminidase treatment. The complete desialylation corresponds to

TABLE I

MONOSACCHARIDE COMPOSITION OF DEGLYCOSYLATED HCG SUBUNITS

Values are expressed as moles of monosaccharide per mole of protein and those in the parentheses represent percent of carbohydrate remaining after deglycosylations. Amounts of NeuNAc in native HCGFα and HCGβ were 4.2 and 6.3 moles/mole protein, respectively.

Sugar	*HCGα*		*HCGβ*	
	Asialo	*Asialo-HF*	*Asialo*	*Asialo-HF*
NeuNAc	0	0	0	0
Gal	3.0	0	6.3	0
Man	5.6	1.6	5.2	1.5
Fuc	–	–	1.2	0
GlcNAc	7.3	3.0	7.1	2.6
GalNAc	–	–	2.8	2.1
Total	15.9 (79)	4.6 (23)	22.6 (78)	6.2 (21)

a reduction of 20–21% in carbohydrate content, and of 9% in molecular weight of both α- and β-subunits. Reductions caused by hydrofluoric acid deglycosylation were more drastic: a 77–79% reduction of carbohydrate content and a 23–24% reduction of molecular weight of the two subunits (Table II). No cleavage of N-Asn-linked N-acetylglucosamine or O-Ser-linked N-acetylgalactosamine was evident under the conditions employed for the hydrofluoric acid treatment. As previously reported[13,15], hydrofluoric acid appears to cleave predominantly the saccharide linkages at the mannose residues of the N-Asn-linked chain and at the galactose residue of the O-Ser-linked chain. Neither galactose nor fucose were detected in the two subunits after HF treatment.

TABLE II

CALCULATED MOLECULAR WEIGHTS OF NATIVE AND DEGLYCOSYLATED HCG SUBUNITS

The % values in the parentheses represent the carbohydrate content. All values were calculated on the basis of compositions of polypeptide and carbohydrate from Table I.

Source	*HCGα*	*HCGβ*
Native	14,500 (30%)	22,200 (30%)
Asialo	13,200 (23%)	20,300 (24%)
Asialo-HF	11,000 (7.4%)	17,100 (9.3%)

The elution behavior of these modified glycoproteins and standard globular proteins was studied by using TSK G-SW columns in an HPLC apparatus. When one TSK G2000SW and two G3000SW columns were connected in series (total length, 180 cm), the isolation of native HCG and its subunits became feasible. The performance of this set of columns is further illustrated by the excellent separation of the following reference proteins (Fig. 2): bovine thyroglobulin (mol.wt. 669,000), bovine serum albumin dimer (134,000) and its monomer (67,000), ovalbumin (43,000), soybean trypsin inhibitor (21,000) and phenylalanine (169). At a flow-rate of 1 ml/min, the peak representing each component was symmetrical and they were almost completely resolved. The elution time in five replicate analyses was highly reproducible, with a standard deviation of 0.05 min.

When the native and deglycosylated HCG subunits were analyzed by gel HPLC, all subunits exhibited a single, symmetrical peak. Fig. 3 shows the elution positions of the α- and β-subunits before and after deglycosylation. The two subunits with the same modification were well separated from each other, the β-subunit being eluted first because of its larger molecular weight. By progressive deglycosylations, the elution position of each subunit distinctly shifted to greater elution volumes: the differential change in partition coefficients, ΔK, caused by desialylation was 0.02 (α) and 0.09 (β), and ΔK caused by hydrofluoric acid deglycosylation was 0.07 (α) and 0.17 (β). The terms for distribution coefficient (K) and ΔK were determined by use of the equations

$$K = (V_e - V_0)/(V_t - V_0)$$

$$\Delta K = K_{(\text{deglycosylated})} - K_{(\text{native})}$$

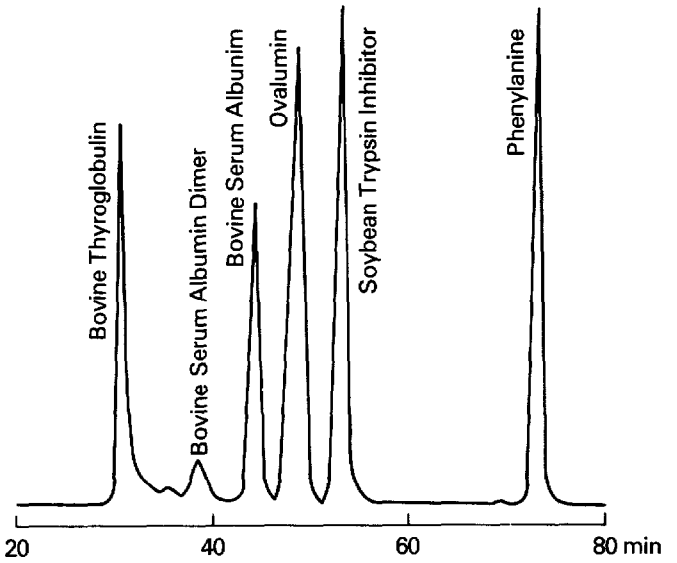

Fig. 2. Standard gel HPLC elution pattern of globular proteins. Columns, TSK-G2000SW + G3000SW + G3000SW (in that order, 180 × 0.75 cm); eluent, 0.1 *M* ammonium acetate–acetic acid (pH 7.0); flow-rate, 1.0 ml/min; sample, proteins (1.0 mg each) in 100 μl.

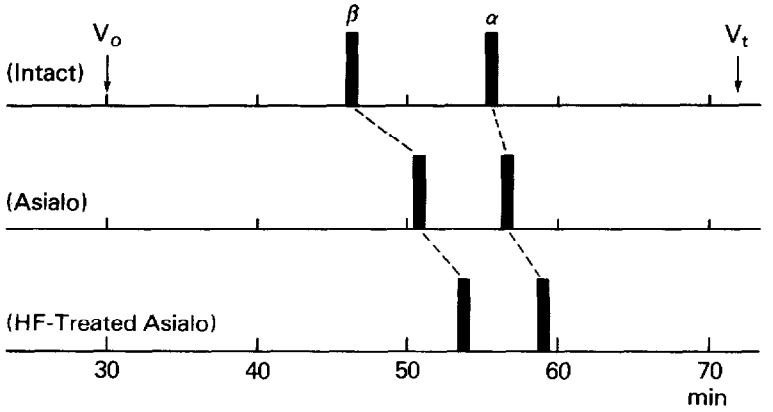

Fig. 3. Elution positions of HCG subunits before and after deglycosylations (for conditions see Fig. 2).

where V_e is the solute elution volume. The void volume (V_0) and total volume (V_t) were determined as the elution times of bovine thyroglobulin and of amino acids or water, respectively. It should be noted that the shifts caused by each modification are more marked for the β- than for the α-subunit. The ΔK values for the β-subunit as compared to those of the α-subunit were larger by a factor of 0.07 and 0.10 after desialylation and hydrofluoric acid deglycosylation, respectively. Moreover, the large shifts presumably become apparent after hydrofluoric acid deglycosylation because

of a drastic reduction of carbohydrate content or molecular weight, as indicated above.

Since the progressive deglycosylation of HCG subunits reduces the molecular size accordingly, we have examined the effect of carbohydrate modifications of one subunit in the recombinant after association with its native complementary subunit. The separations by gel HPLC were excellent for the α- and β-subunits. However, the separation of HCG from the β-subunit was less satisfactory and worsened further when the HCG recombinants contained the deglycosylated subunits. In order to eliminate contamination of the free native or modified β-subunit in the purified recombinants, the recombination reactions were carried out in the presence of excess α-subunits[15]. This resulted in the formation of recombinants having an amino acid composition in close agreement with that of the intact HCG. As shown in Fig. 4, all recombinants were well separated from excess α-subunits. The greater shift in elution time was evident for the recombinants having hydrofluoric acid deglycosylated subunits. The downshifts in elution time due to modifications of the β-subunit appear

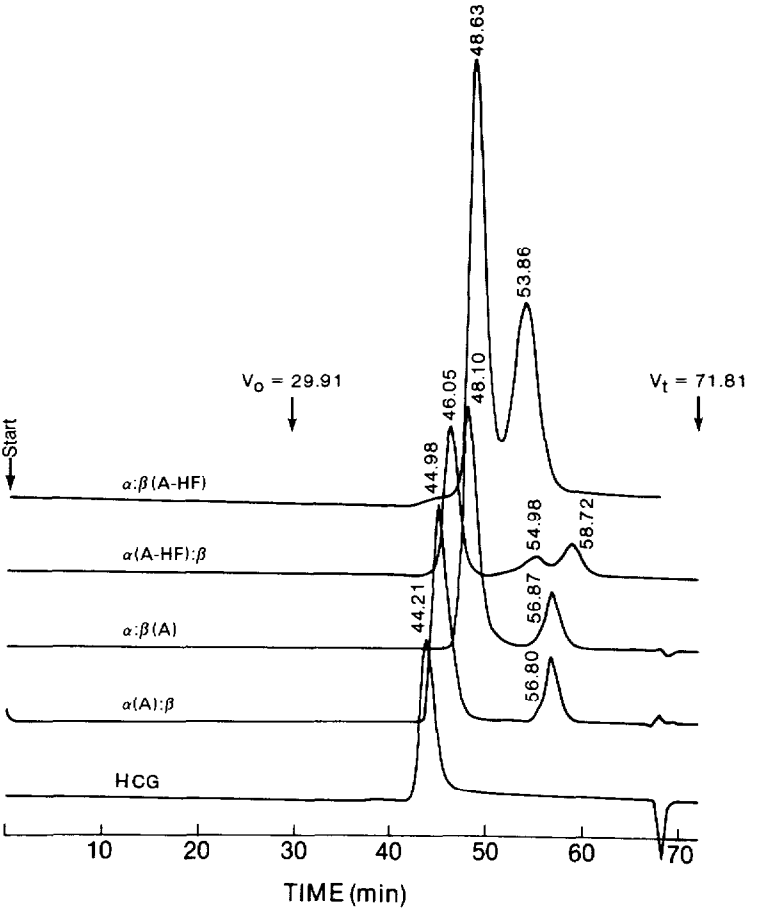

Fig. 4. Gel HPLC purification of HCG recombinants. Samples, products of recombination (3 mg of each subunit) in 150 μl (for conditions see Fig. 2).

to be larger than those due to α-subunit modifications (Fig. 5). Overall, we have observed that the β-subunit exhibits the most pronounced change in elution behavior induced by the deglycosylations.

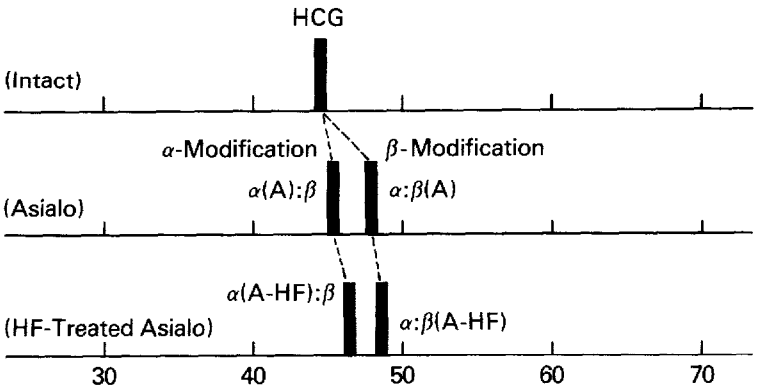

Fig. 5. Elution positions of HCG recombinants with α- and β-subunit modifications (for conditions see Fig. 2).

Like conventional gel chromatography, gel HPLC can also be applied to molecular weight determinations[14]. Employing a series of proteins with known molecular weights as references, a correlation of elution volume and molecular weight can be established[1,3]. Fig. 6 shows that an excellent straight-line relationship was ob-

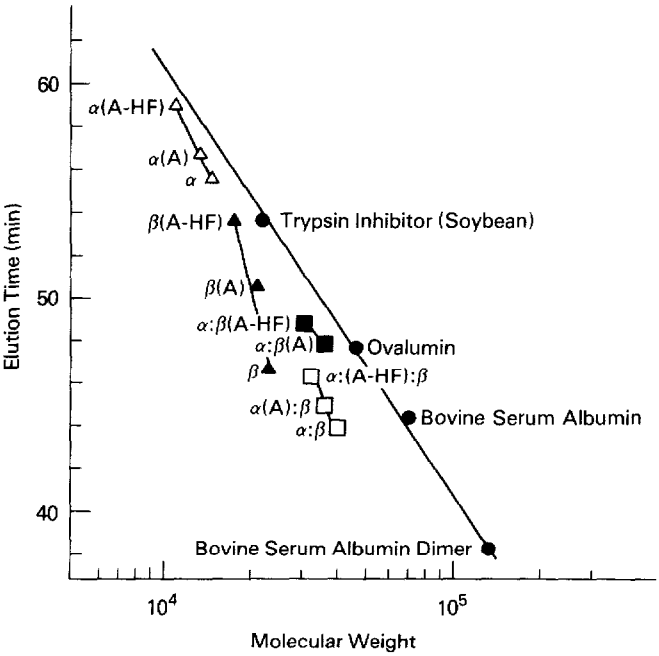

Fig. 6. Correlation between molecular weight and elution time in gel HPLC. Molecular weights of HCG recombinants were calculated on the basis of those of the subunits shown in Table II.

tained from the present gel HPLC system when the molecular weight on a logarithmic scale was plotted against the elution time of a series of standard globular proteins. The total analysis time was *ca.* 75 min. Molecular weights (M_0) of the derivatives of HCG and its subunits were calculated on the basis of their carbohydrate and amino acid compositions (Tables I and II). When the elution times of HCG proteins were plotted against their molecular weights, all subunits and HCG considerably deviated from the standard correlation (Fig. 6). Such deviations are most notable for the β-subunits. Apparent molecular weights (M) estimated by the standard calibration were larger than the calculated ones (M_0). The ratios of M/M_0 were 2.27 (native), 1.58 (asialo) and 1.30 (asialo-HF) for the β-subunit. For the α-subunit, the M/M_0 ratios, 1.26 (native), 1.21 (asialo), and 1.13 (asialo-HF), were lower than the corresponding β-subunits. These results clearly indicate that the differences between the calculated and apparent molecular weights were attributable to the change in carbohydrate content. The progressive removal of carbohydrate residues significantly reduced the deviation of molecular weight from the standard calibration curve, as shown by the ratio of M/M_0. When the differential deviation was calculated by the equation

$$\Delta(M/M_0) = M/M_{0(\text{native})} - M/M_{0(\text{deglycoslated})}$$

the values indicated the extent to which the reductions in carbohydrate content cause the values to approach the standard calibration. The calculated values of differential deviation were 0.05 (α) and 0.69 (β) after desialylation, and 0.13 (α) and 0.97 (β) after hydrofluoric acid deglycosylation. It is noteworthy that such reversals of molecular weight by deglycosylation are quite dramatic for the β-subunit.

Similarly, native HCG had a ratio of 1.83, which was reduced to 1.6–1.7 by α-subunit modifications, and to 1.2–1.3 by β-subunit modifications (Fig. 6). These results indicate that the apparent molecular weight of recombinants containing the native β-subunit are uniformly higher than those for the α-subunit, and are influenced to a lesser extent by the α-subunit modifications. These data further suggest that the effective molecular size of HCG is predominantly determined by the β-subunit.

DISCUSSION

The usefulness of gel HPLC on TSK G-SW[19] and Synchropak GPC[20] columns in size fractionation and molecular weight determination has been demonstrated. The advantages of these procedures over the conventional soft-gel permeation columns, such as Sephadex and Bio-Gel, are their dramatic reduction in both analysis time and sample size, decreased bandspreading, increased reproducibility and high recovery values. Although the gel used in HPLC contains a large number of theoretical plates per unit volume, the currently available columns have one notable limitation, namely, a small gel volume. In order to improve the resolution, decreases in the flow-rate have been advantageous[20]. In addition, increases in column length can be also advantageous, and thus we conducted the gel HPLC by using three connected TSK G-SW columns in series (G2000SW + G3000SW + G3000SW, in that order). We have been able to improve resolution considerably, relative to previous methods[19,20]. At a flow-rate of 1 ml/min, baseline separation of standard globular proteins was obtained (Fig. 2). A chromatogram can be completed in 75 min and the

reproducibility is excellent. Furthermore, this gel HPLC system can serve as a preparative method for protein purifications. After recombination reactions of the α- and β-subunits of HCG, submilligram quantities of the recombinants were easily separated from the excess α-subunits by this system. The purity of the products, as judged by amino acid analysis is highly acceptable. Thus, this method is useful for both the estimation of molecular weights and the purification of proteins.

As to the molecular weight determination with soft-gels or gel electrophoresis, it is well known that glycoproteins give a significantly higher value if the estimation is based on the standard calibration curve derived from glubular proteins[1–6]. For example, HCG, containing *ca.* 30% carbohydrate, was eluted at the position of globular proteins with an apparent molecular weight of 65,000–70,000, a considerable deviation from the value of 36,700[6]. This anomalous elution behavior was also observed in the gel HPLC system, where the molecular weight of HCG appeared to be 1.83 times larger than the true value obtained from the composition analyses. Similarly, the α- and β-subunits gave values 1.26 and 2.27 times, respectively, higher than the actual molecular weight.

In the present studies, the reduction of the carbohydrate content in the two subunits from 30 to 8% by two deglycosylation processes resulted in a decrease of the M/M_0 ratio from 1.26 and 2.27 to 1.13 and 1.30 for the α- and β-subunits, respectively. These data directly demonstrated the effect of carbohydrates on the anomalous elution behavior of glycoproteins. However, the question can be raised why the two subunits, both of which contain 30% of carbohydrate and had been deglycosylated to the same extent, exhibited such a difference in the reduction of the M/M_0 ratio after the deglycosylation. Previously, Andrews[3] found no apparent correlation between the extent of deviation and the content of carbohydrate in glycoproteins. Moreover, ovalbumin showed no deviation from the reference globular proteins, as illustrated in Fig. 6, even though it contains 3.5% carbohydrate. Perhaps not only the carbohydrate content but also the location of carbohydrate chains in the spatial arrangement of the protein molecule determines the overall structural dimensions of glycoproteins. If this is the case, the high values obtained from HCGβ and its derivatives may be the result of the contribution by the unique carboxyl-terminal peptide, which contains four additional O-Ser-linked carbohydrate chains not found in the α-subunit as shown in Fig. 7. It is likely that the carboxyl-terminal

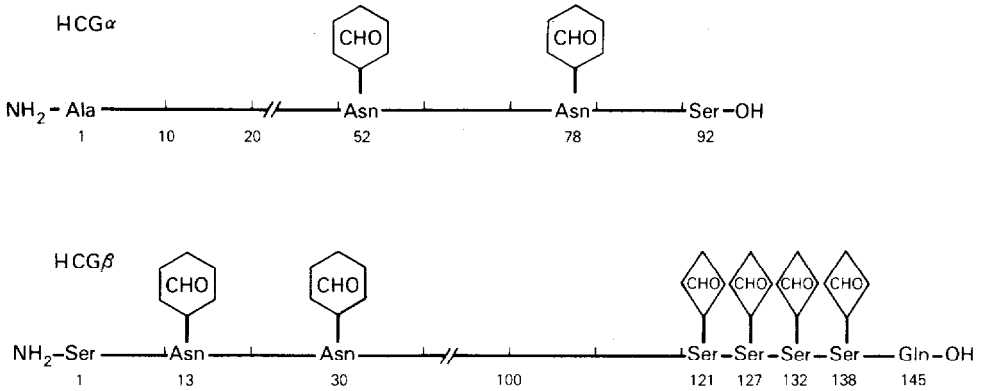

Fig. 7. Location of carbohydrate moieties (CHO) in the α- and β-subunits of HCG.

glycopeptide extends outward as an appendage[21], and thus amplifies the overall molecular size. Our data also illustrate that the β-subunit is the dominant factor in determining the carbohydrate effect on the molecular dimensions of recombinants. It is intriguing that the carbohydrate moieties in the β-subunit also play a dominant role in maintaining the hormonal activities of the native and recombined molecule[15].

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