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HORMONE-ASSOCIATED VARIATION OF THE GLYCAN MICROHETEROGENEITY PATTERN OF HUMAN SEX STEROID-BINDING PROTEIN (hSBP)*

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Summary—hSBP is a steroid-binding protein (human) whose serum concentration is increased by estrogens and decreased by androgens. This regulation is independent of a direct effect on the hSBP gene transcription. The purpose of this work was to study the glycan microheterogeneous composition of the mature protein under physiological estrogen stimulation, by means of crossed affinoimmunoelectrophoresis using concanavalin-A. In men hSBP always divided into 2 fractions, both retarded. In women hSBP showed two other components, still more retarded. An explanation for these differences is given and the role of the glycan moiety of hSBP is discussed.

INTRODUCTION

The interaction of concanavalin-A with plasma proteins during electrophoresis was first described by Nakamura *et al.* [1] and then widely used to study the carbohydrate moiety of glycoproteins [2]. Crossed affinoimmunoelectrophoresis is a semi-quantitative method which combines an electrophoretic step through an immobilized lectin in the first dimension, followed by electrophoresis in an antibody-containing gelose in the second dimension. So it is possible to calculate the dissociation constants of concanavalin-A–glycoprotein complexes [2]. Bøg-Hansen *et al.* [3–8] used this technique to detect biospecific interaction of several human plasma proteins with free concanavalin-A, and to calculate their affinities.

Human sex steroid-binding protein (SBP) is a dimeric plasma steroid-binding glycoprotein, the carbohydrate moiety of which is composed of two biantennary *N*-linked oligosaccharide chains of the *N*-acetyllactosamine type and one *O*-linked oligosaccharide chain per monomer [9], and presents a microheterogeneity [10, 11]. Both monomers are identical but are differentially glycosylated [10]. The role of this carbohydrate moiety has not yet been elucidated, but seems to be important in the cellular targeting of SBP in hormone-dependent tissues [12], and is implicated in the intracellular traffic of the protein during its maturation and its secretion, as well as in its metabolic clearance rate [13]. The SBP serum concentration is increased by estrogens and decreased by androgens. This regulation seems to be independent of a direct effect on the gene transcription as shown recently by semi-quantitative analyses of mRNA with specific cDNA probes [14, 15]. As the circulating concentration of SBP does not seem to be sex hormone-regulated at the transcription level, different maturation steps could be involved in the secretion and/or the metabolic clearance rate of the protein. If such a mechanism occurs, one could observe different types of glycosylation in different hormonal states.

In this study, the heterogeneity of the carbohydrate moiety of SBP, as demonstrated by crossed affinoimmunoelectrophoresis using free concanavalin-A and specific antibodies, was studied in individual human serum samples under various estrogenic conditions, and dissociation constants of concanavalin-A–SBP complexes were calculated for the variants of this binding protein.

EXPERIMENTAL

Serum samples

Human serum samples from men ($n = 10$), women ($n = 10$), and pregnant women ($n = 10$), were allowed to clot at room temperature and

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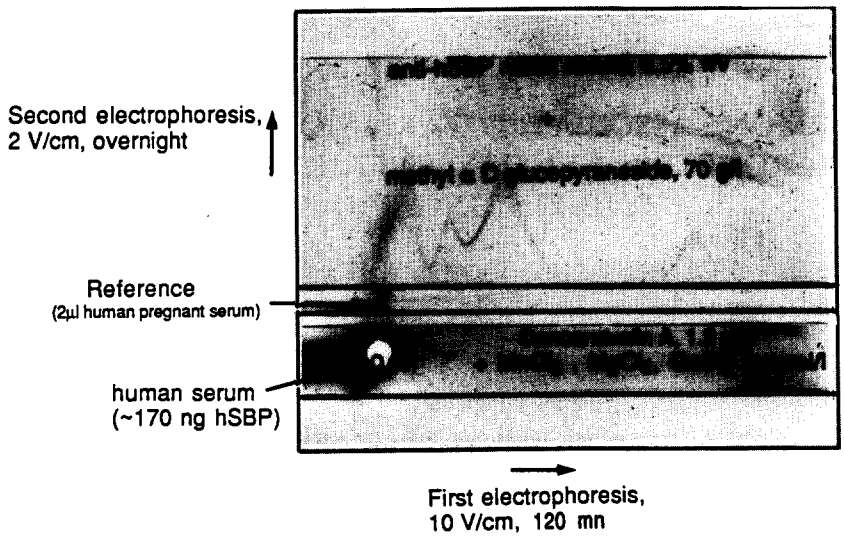

Fig. 1. Crossed affinoimmunoelectrophoresis technique run with pregnant woman serum.

then frozen at −30°C until use. SBP was quantified using electroimmunodiffusion [16] before affinoimmunoelectrophoresis.

Affinoimmunoelectrophoresis

This method was carried out essentially as described by Bøg-Hansen *et al.* [5, 7]. The gel was 1% (w/v) agarose HSB (from Litex, Denmark) in 36.6 mmol/l Tris, 12 mmol/l veronal, 0.17 mmol/l calcium lactate and 0.1 g/l sodium azide, pH 8.6. The electrode solution was the gel buffer, twice more concentrated. In the first dimension SBP (170 ng, 0.19 µmol/l) was electrophoresed 120 mn at +15°C, 10 V/cm, in a gel lane containing 1.2 g/l (46.15 µmol/l) concanavalin-A (from IBF, France), 1 mmol/l calcium chloride, 1 mmol/l magnesium chloride and 1 mmol/l manganese chloride. The reference sample (2 µl pregnant woman serum) was run in a parallel lane without additives. In the second dimension proteins were electrophoresed overnight at room temperature, 2 V/cm, in a gel containing 70 g/l 1-*O*-methyl-α-D-glucopyrannoside (from Serva, Fed. Rep. Germany), and 0.5% (v/v) rabbit antiserum directed against pure human SBP (from our laboratory, [16]). The gels were then rinsed, dried, and stained in 0.04% Coomassie blue R250. All chemicals were from Merck (Fed. Rep. Germany).

Calculations

The calculation of dissociation constants used the following equation given by Bøg-Hansen and Takeo [4]:

$$1/R_{mi} = 1/R_{mo} \cdot (1 + C/K) \qquad (1)$$

where K is the dissociation constant of the concanavalin-A–SBP variant complex, C is the concentration of the concanavalin-A (mol/l, $M_r = 26{,}000$), R_{mo} is the mobility of SBP without concanavalin-A (reference, relative mobility = 1), and R_{mi} is the relative mobility of SBP variant in the presence of the lectin. This equation is derived from Horejsi's equation [2]:

$$d/(d_0 - d) = (K_i/c_i) \times (1 + c/K) \qquad (2)$$

where d is the migration distance of the protein in the affinity gel (="mobility"), d_0 is the migration distance of the protein (reference) in the non-interacting control gel, K_i is the dissociation constant of the protein–immobilized ligand complex, c_i is the total concentration of the immobilized ligand in the affinity gel, K is the dissociation constant of the protein–free ligand complex, and c is the total concentration of the free ligand in the affinity gel. In the chosen electrophoretic conditions, concanavalin-A is considered as a non-migrating species and assumed as completely "immobilized" ligand; so we may consider $c = O$.

Dissociation constants were also determined graphically by linear regression (mean squares method) using various concanavalin-A concentrations in the affinity gel. A plot of $(1\text{-}R_{mi})^{-1}$ as a function of C^{-1} produces a straight line, whose intercept on the abscissa is $-K^{-1}$.

RESULTS

The different glycosylated variants of human SBP appeared as distinct immunoprecipitates (Fig. 1). The distances (in mm) between the

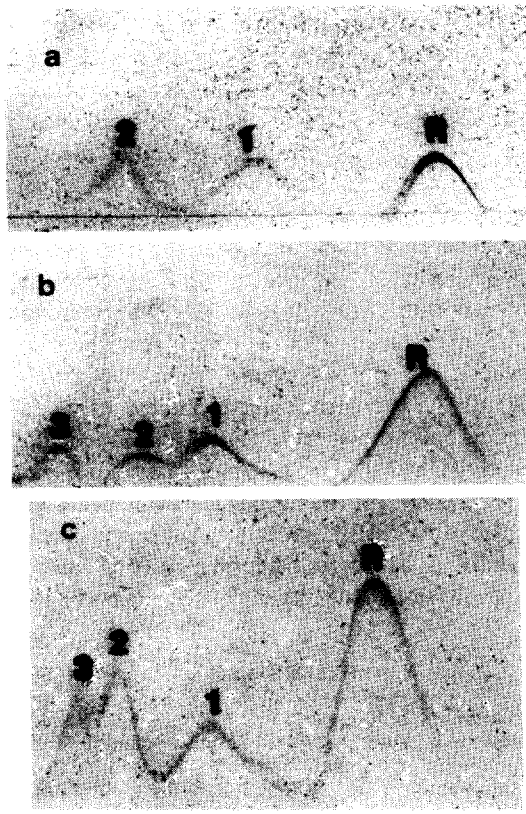

Fig. 2. Microheterogeneity pattern of hSBP in men (a), women (b) and pregnant women (c). The shoulder on peak 3 is more discernible in Fig. 1.

sample well and the orthogonal projections of the top of the precipitates were considered as the mobilities of the respective variants, and the calculation of the affinities of the concanavalin-A–SBP variants complexes was made according to Horejsi [2] and Hansen *et al.* [6]. The relative mobility of each component was calculated in reference with the mobility of SBP electrophoresed in the lane without concanavalin-A.

Table 1. Dissociation constants (K_d) calculated and graphically determined for the four components of human SBP

	Calculated K_d ($\times 10^{-5}$)	Graphically determined K_d ($\times 10^{-5}$)
Component 1	5.3	8.7
Component 2	2.8	7.1
Component 3	1.5	2.2
Component 4	0.3	0.5

In human males, SBP always divided into two fractions called component 1 and 2, respectively, both retarded [Fig. 2(a)]. In women there appears systematically one more fraction still more retarded [Fig. 2(b)], and relatively more important during pregnancy [Fig. 2(c)]; a faint shoulder (more discernible on Fig. 1) appeared on the cathodic side of this third fraction, indicating it was likely to be composed of at least two molecular species called component 3 and 4, respectively. The relative mobilities of the different variants ($\pm$range) were 0.51 ± 0.05, 0.30 ± 0.07, 0.12 ± 0.03 and 0.05 ± 0.03 in women, and 0.55 ± 0.06 and 0.30 ± 0.05 in men, respectively when they were electrophoresed through 46.15 μmol/l concanavalin-A.

Dissociation constant of each component–concanavalin-A complex was calculated using equation (1) (see Experimental section) and by graphical estimation (Fig. 3). For these calculations we considered concanavalin-A as a monomer ($M_r = 26{,}000$) but we cannot exclude it was in the dimeric or tetrameric form. Both

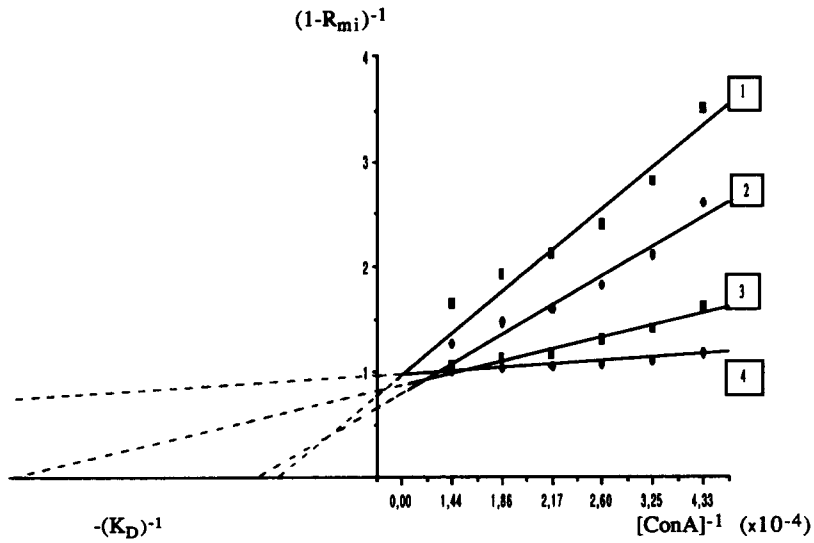

Fig. 3. Graphic determination of K_d. Plots are made according to equation (1), with varying concentrations of concanavalin-A; 1, 2, 3 and 4 are the graphs for component 1, 2, 3 and 4, respectively; the intercepts with the abscissa give the dissociation constants (drawn after computed values, $r^2 = 0.97$).

determination methods gave similar results as shown in Table 1. The differences observed in individual values are due to the statistical adjustment of the linear regression used for the graphical estimation and to the lack of precision of the abscissa intercept evaluation.

DISCUSSION

The aim of this study was to detect differences in the microheterogeneity of human SBP from men, women and pregnant women representing three very different estrogenic status types. Crossed immunoelectrophoresis of SBP always produces a single precipitate. As our tool, we have used crossed affinoimmunoelectrophoresis with concanavalin-A, and we have found that native SBP exhibits a large degree of microheterogeneity, different in each estrogenic status; these data result only from interaction of SBP with concanavalin-A, different mobilities of SBP–concanavalin-A complexes being due to differences in the number of concanavalin-A molecules bound to each SBP molecule. This is likely the consequence of differences in the antennary structures at the glycosylation sites of SBP [6].

Human SBP is known to have one *O*-branched and two *N*-branched oligosaccharide chains per monomer [10, 12]. The *N*-branched chains can be of bi- or tri-antennary type, with consequent different affinities for concanavalin-A (high affinity for bi, none for tri). If, in the native protein, the glycosylation of each monomer is independent, it is possible to find up to five different oligosaccharide combinations (= five different SBP variants): 1bi/3tri, 2bi/2tri, 3bi/1tri, 4bi and 4tri, the latter having no affinity for the lectin. Our results show there is no SBP variant 4tri (with the same mobility with or without concanavalin-A). But we have provided evidence for the existence of the four other types. In men there are two retarded types. In women there are two more types with lower mobility, and proportionally more important in pregnant women.

The bi-antennary type glycans, which bind to concanavalin-A, are the least modified in the glycosylation process during the maturation of secreted glycoproteins in the Golgi system. So we can expect that a high proportion of bi-antennary type oligosaccharide chain (in pregnant women) corresponds to a high degree of maturation, and that a lower proportion of this type of glycan (in men) corresponds to a lower degree of maturation. In serum the SBP levels are dramatically increased by estrogens, and decreased under androgenic influence. Recently we have demonstrated *in vivo* in the monkey [15] that serum SBP variations are not directly governed by estrogens at the mRNA level. Others [14] had the same results *in vitro* in human cells. So it is likely that the modulation of SBP action is not due to a difference in the synthesis rate of this protein but to a difference of its maturation (for a review on the biosynthesis of protein-bound oligosaccharides, see [14]). As the carbohydrate moiety of glycoproteins plays an important role in cellular targeting [18–20], in intracellular processing [17, 20] and in metabolic clearance rate via hepatic uptake [13], modulation of the maturation of glycans could be the means of control of the physiological impact of the plasma SBP.

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