THE EFFECT OF SELECTIVE PROTEIN BINDING ON THE ENZYMATIC FORMATION OF TESTOSTERONE GLUCURONIDE

R. HAMPL, M. KUDOVÁ and L. STÁRKA
Research Institute of Endocrinology, 116 94 Prague 1, Czechoslovakia

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1. Introduction

Sex hormone binding globulin (SHBG) is a selective plasma carrier protein, binding both testosterone and estradiol-17β with high affinity [1]. Although it is assumed that SHBG may influence both the hormone action and the metabolism of steroids bound to it [2], there is little evidence for its effect on enzymatic transformations of steroids. Such an effect was demonstrated only for the 17-oxidoreduction of several C_{18} and C_{19} steroids [3,4] and aromatization of testosterone [5,6]; in both instances the presence of SHBG had an inhibitory effect on the reaction rates.

Quantitatively, the transfer of glucuronic acid onto various hydroxysteroids is among the most important catabolic reactions of steroid hormones. The enzyme responsible, UDPglucuronosyltransferase (EC 2.4.1.17) with non-specific acceptor, has been demonstrated in various human and other animal tissues [7–10]. Its activity is associated mainly with the microsomal cellular fraction [7,9,10], but it is also found in cytosol [11,12].

A high glucuronosyltransferase activity occurs in the liver [7,9,11,12], which is also the probable site of SHBG biosynthesis [13]. Therefore, it seemed to be of interest to investigate the effect of SHBG, as a model binding protein, on the enzymatic glucuronosidation of testosterone in vitro.

In this paper, it is shown that SHBG decreases the yields of testosterone glucuronide formed. This effect, to a lower degree was also demonstrated with the non-specific binder, albumin. The effects of specific and non-specific binding proteins differ in their kinetics.

2. Experimental

All chemicals were of analytical grade, the solvents were distilled before use. 4-[^{14}C]Testosterone, specific activity 58.2 mCi/mmol (Radiochemical Centre, Amersham, England), was purified by paper chromatography in system A (see below). Non-labelled steroids were purchased from Koch-Light, Colnbrook, England. Uridine diphosphoglucuronic acid, disodium salt (UDPGA) was obtained from Boehringer, Mannheim, Germany.

The protein fraction enriched with SHBG was prepared from full term placental serum, by repeated ammonium sulphate precipitations, followed by affinity chromatography on the polyacrylamide based matrix, Enzacryl AA® (Koch-Light) with covalently attached 5α-androstane-3α,17β-diol 3-hemisuccinate. The release of protein from its binding to the affinant was achieved by elution with buffered testosterone solution and the steroid was then removed by treatment with charcoal, NORIT A. The procedure in detail has been described elsewhere [14]. The testosterone binding capacity (TeBC) of purified protein fraction was assessed according to Pearlman et al. [15]. An aliquot corresponding to TeBC of 2 nmol (approx. 8 mg of the total protein) in 100 μl of buffer was used for incubations.

Human serum albumin obtained from Fluka AG, Buchs, Switzerland, 20 mg/sample was used as a non-selective protein.

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The incubations were done in a manner similar to that described by Rao et al. [11,12]. In a typical experiment, [^{14}C]testosterone (200 000 dpm), appropriately diluted with non-labelled steroid, the co-substrate (UDPGA, 0.5 μmol) and the carrier protein or buffer only, were first preincubated at 25°C for 20 min in 0.05 M Tris–HCl buffer, pH 8.0, containing 20 μmol of $MgCl_2$ in a total vol of 1.9 ml. The reaction was then started by addition of microsomal fraction of rat liver (male Wistar rats, 150–200 g) prepared according to [16]. An equivalent of 0.5 g of wet liver tissue in 100 μl of 0.25 M sucrose was added and the mixture was incubated at 37°C for 20 min or 1 hr. Simultaneously, a control series of the same number of samples was incubated, each containing all the above components, except the co-substrate. Extraction and assay of enzymatic activity were exactly as described by Rao et al. [11]: the unconjugated substrate was removed by extraction with ethyl acetate and the glucuronide formed was extracted from the aqueous phase with *n*-butanol. The radioactivity in an aliquot of the butanol phase was measured. The yields of the enzymatic reaction were calculated from the net radioactivity in the butanol, the total radioactivity in the system and the known amount of substrate incubated, respectively.

The radioactivity was measured in a Betaszint BF-500 liquid scintillation spectrometer (Berthold and Frieseke, Wildbad, GFR), using the external standard channels ratio method for dpm computing. The scintillation fluid consisted of 4 g of PPO, 50 mg of POPOP and 100 ml of methanol in 1 litre of toluene.

The following systems were used for chromatography; on paper: A) cyclohexane–toluene–methanol–water 9:1:8:2; B) ethyl acetate–*n*-butanol–10% NH_4OH–water 9:1:3:7; and on thin-layer of silica gel HF_{254} (Merck AG, Darmstadt, GFR): C) ethyl acetate–ethanol–conc. NH_4OH 5:2:1; and D) cyclohexane–ethyl acetate 2:1 v/v. Testosterone and its derivative were localized under UV light. Conjugated steroids were eluted with methanol–water 9:1, and a benzene–methanol 4:1 mixture was used for elution of the free steroid.

3. Results and discussion

3.1. *The effect of binding proteins on glucuronosidation*

[^{14}C]Testosterone (5 nmol) was incubated for 1 hr under conditions described in experimental with rat liver microsomes in the presence of either albumin or SHBG-enriched protein fraction or in the absence of a binding protein. The yields of glucuronide formed in each group are summarized in table 1. The addition of either transport protein resulted in significantly decreased yields; this effect was greater with SHBG.

3.2. *Identification of testosterone glucuronide*

To confirm the identity of the measured radiometabolite as testosterone glucuronide, the remaining butanol extracts from incubations were pooled, evaporated and the dry residue (21 831 dpm) was subjected to the following identification steps which were checked by the radioactivity recovered:

1) Chromatography in system C with testosterone glucuronide as an internal standard (50 μg) yielded 19 932 dpm in the testosterone glucuronide area (R_f = 0.08, R_f of testosterone = 0.90).

2) On rechromatography in system B, 18 848 dpm were found in the testosterone glucuronide area (R_f = 0.06, R_f of testosterone = 0.96).

3) After enzymatic hydrolysis of eluted testosterone

Table 1
The effect of albumin and SHBG on the enzymatic glucuronosidation of testosterone (5 nmol) by rat liver microsomes

Protein added	Yields of glucuronide (nmol) (mean of six determinations ± S.D.)	Significance	
None	0.540 ± 0.059	P < 0.01	P < 0.01
Albumin (290 nmol/sample)	0.417 ± 0.059		
SHBG (TeBC = 2 nmol/sample)	0.252 ± 0.045	P < 0.01	

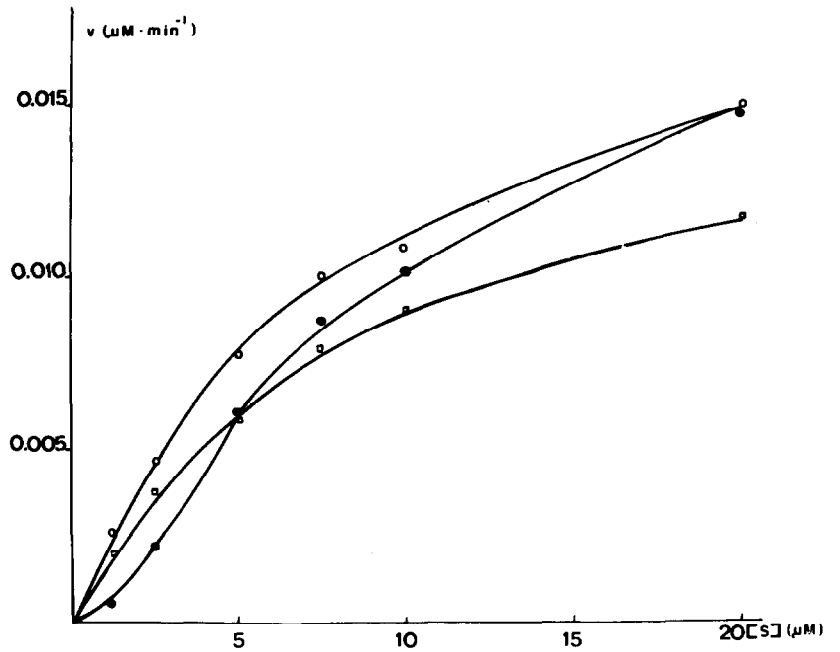

Fig.1. Formation of testosterone glucuronide and the effect of SHBG or albumin. Increasing amounts ([S] = 2.5–40 nmol) of 4-[^{14}C]testosterone were incubated at 37°C in 2.0 ml of Tris–HCl buffer, pH 8.0, containing $MgCl_2$ (20 μmol) and UDPGA (0.5 μmol), with rat liver microsomes (equivalent to 0.5 g wet tissue) in the presence of albumin (290 nmol) or human SHBG (2 nmol) or without addition of protein. (○———○) no protein, (●———●) SHBG, (□———□) albumin.

glucuronide with β-glucuronosidase from *Helix pomatia* (10 000 U), 13 358 dpm were found in the chloroform extract after washing it with 0.1 M NaOH and water.

4) Chromatography in system D with testosterone (20 μg) as internal standard separated 11 149 dpm in the testosterone spot (R_f = 0.28).

5) This isolated radioactive material was admixed with testosterone (50 mg) and crystallized to constant specific radioactivity from acetone–*n*-hexane. The following values of specific radioactivities (in dpm/mg) were obtained:

Crystallization	Crystals	Mother liquors
1st	268	504
2nd	213	120
3rd	206	91

3.3. *Kinetics of glucuronosidation*

To compare the inhibitory effect of SHBG with that of albumin, the reaction rates at increasing concentrations of the substrate (2.5–40 nmol/sample) and a constant excess amount of co-substrate were measured. Preliminary experiments showed that the yields increased linearly up to approx. 30 min incubation, consequently a 20 min incubation time was chosen for estimation of reaction rates. In fig.1 the reaction rates (V) are plotted against the substrate concentrations [S]. In the presence of SHBG, an S-shaped velocity–substrate dependence was obtained. This reflects the fact that the binding capacity of SHBG used is approx. 1 μM and at higher concentrations of testosterone an unbound steroid prevails. When [S]/V was plotted versus [S], the addition of albumin resulted in kinetics resembling non-competitive inhibition, with K_m being 8.4 μM, whereas a straight line could not be obtained when SHBG was added. We have observed kinetic pattern similar to that found in the presence of SHBG when investigating the effect of this protein on the enzymatic oxidation of 17β-hydroxysteroids by bacterial 17β-hydroxysteroid dehydrogenase [4]. At substrate concentrations up

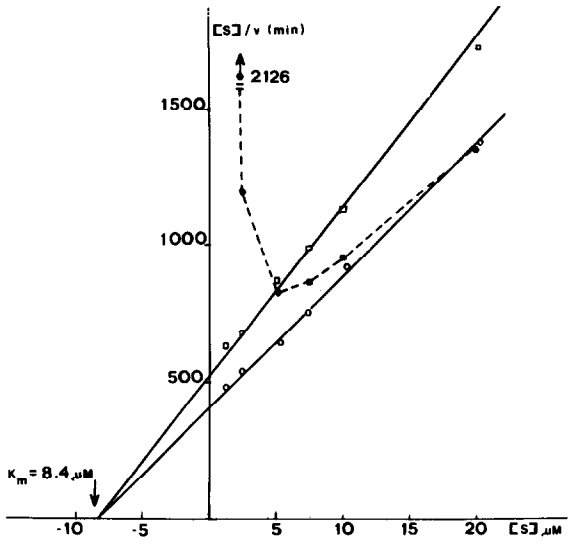

Fig.2. The dependence of $[S]/V$ on the substrate concentration. $[S]/V$ was plotted against $[S]$ using values obtained under conditions described in fig.1. (○———○) no protein, (●———●) SHBG, (□———□) albumin.

to that sufficient to saturate the binding sites of SHBG, the rate of enzymatic glucuronosidation, as well as oxidation, increased only a little, but at higher substrate concentrations a rapid increase of the reaction rate occurred, reaching the value found in the absence of SHBG. It may be concluded that in both cases competition for a substrate between the binding protein and the particular enzyme takes place.

The system SHBG–UDPglucuronosyltransferase–testosterone is only an artificial model for interaction between a binding protein, enzyme and steroid. Nevertheless, the findings demonstrate that binding proteins may be involved in the regulation of the metabolic fates of hormonal steroids.

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