INTERACTION OF PURIFIED HUMAN TRANSCORTIN WITH SPIN LABELLED STEROIDS

M. BASSET, G. DEFAYE and E. M. CHAMBAZ
Biochimie Endocrinienne, CERMO, Université Scientifique et Médicale, Grenoble, France

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1. Introduction

Since the introduction of stable paramagnetic spin labels [1], electron spin resonance (ESR) spectroscopy has been applied to the study of various molecular interactions, especially in the case of enzymes, immunoglobulins and membrane structures [2]. Meanwhile, advances in the knowledge of steroid–protein interactions have mostly been obtained by biochemical methods. Specific, high-affinity binding macromolecules have been characterized in serum and in target tissues [3,4]; specific immunoglobulins can be obtained and are widely used for steroid immunoassays [5].

In a previous paper, we demonstrated the potential value of e.s.r. for the investigation of steroid–protein interaction using a synthetic steroidal nitroxide and bovine serum albumin as a model protein [6]. Recently, similar results have been reported using the same protein and a spin labelled androstanol [7,8] and the principle of spin immunoassay has been extended to progesterone and testosterone using the corresponding antibodies [9,10]. However, to our knowledge, the spin labelling technique has not yet been used in the study of specific, high-affinity, naturally occurring steroid binding proteins. This may be due to inherent difficulties: (i) suitable spin labelled steroids must be available and must behave as their natural counterparts as far as binding is concerned. (ii) sufficient amount of pure stable binding protein must be obtained [11,12].

In this work, we report the synthesis of several steroid spin labelled derivatives. These compounds were studied with respect to their binding to human transcortin (corticosteroid binding globulin: CBG). In addition a homogeneous preparation of human CBG was prepared and its interaction with suitable spin labelled cortisol derivatives is reported.

2. Materials and methods

N.m.r. spectra were recorded using a JEOL C-60HL spectrometer, in deuteriochloroform, with tetramethylsilane as the internal standard. Optical rotations were measured in chloroform with a Jouan, Quick, polarimeter. Radioactivity measurements were carried out using an Intertechnique SL-30 liquid scintillation counter. ESR spectra were recorded on a VARIAN E-12 spectrometer, at room temperature, in calibrated glass capillary tubes (1.5 mm i.d., Corning).

(a) 2,2,5,5-tetramethyl-3-carboxypyrroline-1-oxyl was prepared from triacetonamine according to Dupeyre et al. [13].

(b) 10-hydroxy undecanoic acid was obtained from 10-undecenoic acid (Fluka) by hydratation of the double bond [14] and recrystallization from methanol. F = 48–49°C (lit. [15]: 49–49,5°C); n.m.r. spectrum: δ (ppm) 1.18 (d, 3 H, CH_3-CHOH J = 6 Hz), 8.7 (s, 1 H, –COOH).

(c) 10-oxo undecanoic acid. Compound (b) (4.5 g), dissolved in acetone (100 ml) and kept at 0°C, was oxidized by addition of 10 ml of Jones' reagent [16] over 5 min. After dilution with water, the product was extracted with ethyl ether. F = 58–60°C (lit. [14]: 59–60°C); NMR spectrum: δ (ppm), 2.12 (s, 3 H, $C\underline{H}_3$-CO), 8.7 (s, 1H, –COO$\underline{H}$).

(d) Methyl-10-oxo undecanoate was obtained as an oil by treatment of (c) with diazomethane in ether. N.m.r. spectrum: δ (ppm), 2.12 (s, 3 H, CH_3–CO), 3.12 (s, 3 H, –O–$C\underline{H}_3$).

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(e) 10-nitroxyl undecanoic acid. Compound (d) was treated according to Kfana [17] and Waggoner [18] to form an oxazolidine, which was oxidized to afford a nitroxide. F = 48–50°C; mass spectrum: molecular ion at 300 a.m.u. Hydrolysis of the methyl ester (300 mg) was carried out in 40 ml of methanolic NaOH (1 N) for three days at 30°C. Hydrochloric acid (1 N) was added to pH 3.0 and the product was extracted with chloroform and purified by thin layer chromatography on silica gel with ethyl ether as the eluant (R_f. = 0.4; F = 58–60°C).

Spin labelling of steroids by esterification at the 17β (testosterone) or at the 21-(cortisol) positions was carried out with the nitroxyl acid (a) and/or (e) as follows: to a solution of the nitroxyl acid (1 mmol) was added dropwise, with constant stirring, 0.09 ml of thionyl chloride*. After 45 min, the solution was filtered, the filtrate was concentrated under vacuum to about 1/4 of its vol and the steroid (0.7–1 mmol) was added in 10 ml of a benzene–pyridine (v:v) mixture. After 24 h at room temperature the solvent was evaporated under vacuum and the resulting product was purified by t.l.c. on silica gel in the system hexane–ethyl acetate (3:7) and cristallization from methanol.

The nitroxyl acid (a) was coupled to cortisol (F) to yield the spin labelled derivative F-NO, compound 1 (fig.1): F = 249–250°C; $[\alpha]_D^{20}$ + 165°: (c = 0.5); e.s.r. a_N (10^{-5} M, H_2O): 15.9 G.

Esterification of testosterone (T) with (a) gave the spin labelled steroid T-NO (3, fig.1): F = 214–215°C; $[\alpha]_D^{20}$ = +18° (c = 0.5); e.s.r. a_N = 15.9 G.

Reaction of (e) with cortisol led to the cortisol derivative 2 (fig.1), bearing the spin label at C-10 of the added side chain (F-O,8-NO) $[\alpha]_D^{20}$ = 163° (c = 0.5); e.s.r. a_N = 15.8 G.

Human transcortin (HCBG) was purified from human plasma using the affinity-chromatography technique as described by Le Gaillard et al. [21]. In this case, the purified binding protein was equilibrated with a large excess of spin labelled steroid in order to displace the bound cortisol used in the elution step; the remaining unbound steroids were then removed by gel filtration through Sephadex G-25. In most cases the elution of the affinity-chromatography column was carried out with the nitroxyl steroid so as to obtain in a single step the pure protein saturated with the spin labelled derivative.

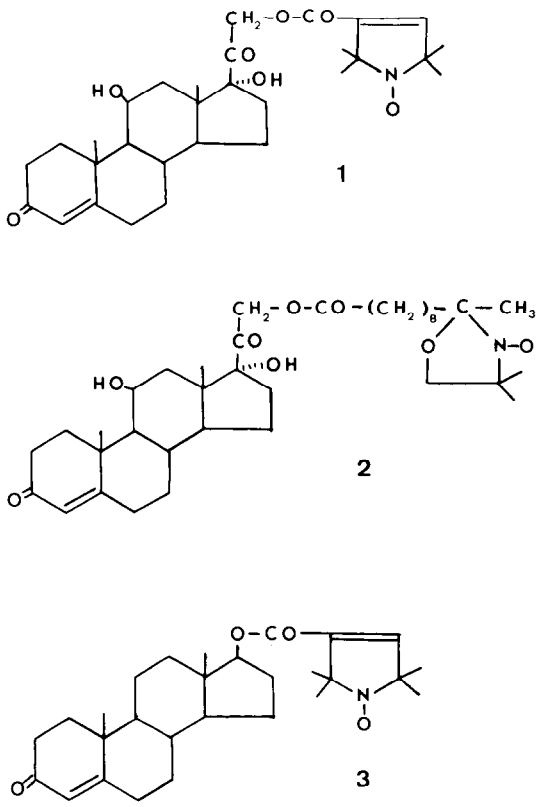

Fig.1. Structures of the spin labelled steroid derivatives obtained by synthesis and used in this work. (1,2) Cortisol derivatives ((1): F-NO; (2): F-0,8-NO) and (3) Spin labelled testosterone (T-NO).

Binding experiments. The binding affinity of the nitroxyl derivatives to HCBG was evaluated using human plasma, diluted 100-fold with 0.01 M Tris–HCl buffer, pH 7.4. The diluted plasma was incubated with [1,2-^{3}H]cortisol (C.E.A.; 50 Ci/mmol; $2 \cdot 10^{-10}$ M), to which was added various amounts (0 to 10^{-7} M) of non-radioactive steroid or spin labelled compounds. After 20 min at 37°C, the mixture was left 2 h at 4°C. The bound radioactivity was measured after separation from the unbound form using an adsorbent suspension [22].

* Recently Dodd et al. [19] reported an unsuccessful attempt to use the acid chloride of (a) in a similar type of coupling; however, preparation [20] and use of this reagent with satisfactory yields had already been described.

3. Results and discussion

3.1. *Binding of spin labelled steroids to HCBG*

The binding affinity of the synthetic spin labelled steroids to HCBG was evaluated by their ability to displace tritiated bound cortisol from the binding protein. An addition of unlabelled cortisol at a concentration of $0.6 \cdot 10^{-8}$ M gave a 50% displacement, whereas $1.1 \cdot 10^{-8}$ M and $1.5 \cdot 10^{-8}$ M were necessary for the same result in the case of F-NO and F-0,8-NO respectively. No displacement was observed upon addition of T-NO, even in large excess. Therefore, the spin labelled F-NO exhibited a high affinity for the HCBG cortisol binding site, which should reflect an association constant of similar magnitude ($\simeq 10^8$ M^{-1} for cortisol). The cortisol derivative bearing the C_{11} side chain at C-21 was still firmly bound, whereas no binding at all was obtained with T-NO. Thus, the bulky nitroxide ring did not greatly impair the binding process, providing it was separated from the steroid nucleus by a minimal side chain at C-17. Further lengthening of this side chain diminished only slightly the binding ability.

3.2. *E.s.r. spectroscopy studies*

When a mixture of F-NO and HCBG was incubated overnight and then analysed by ESR spectroscopy, the recorded spectrum (figure 2 (1)) clearly appeared as the result of two superimposed spectra. One spectrum could be attributed to the free motion of the spin label in aqueous medium by its isotropic splitting constant (a_N = 15.9 G); the other trace was attributed to the spin label form immobilized by binding and characterized by the splitting between the low and high field extrema 2T = 56.3 G. Since the 2T value increases as the freedom of the spin label decreases, it thus reflects the degree of immobilization of the spin label. However, 2T can also be influenced by the polarity of the spin label local environment i.e., the binding site nature [1,2].

When the F-NO-HCBG mixture was treated with 1 vol of ethanol, denaturation of the protein gave an

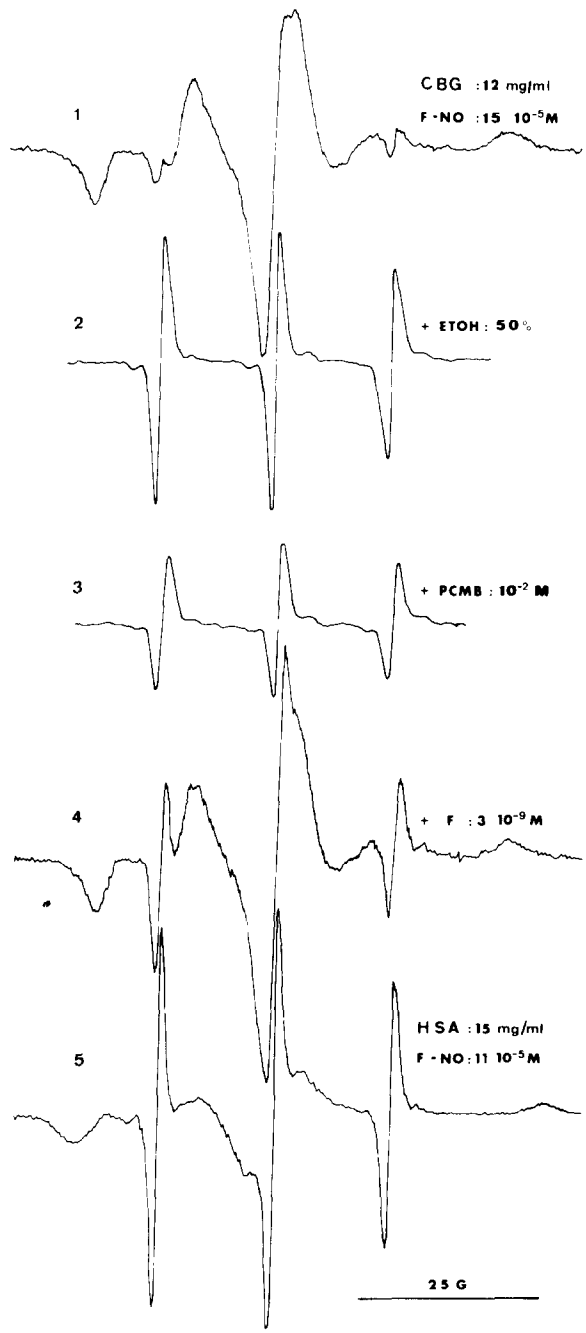

Fig.2. (1–4): ESR spectra given by pure HCBG ($24 \cdot 10^{-5}$ M) in the presence of the spin labelled cortisol F-NO ($15 \cdot 10^{-5}$ M). (1) After overnight incubation in buffer. (2) The same after denaturation of HCBG by ethanol. (3) Binding inhibition by an SH blocking agent (*p*-chloromercuribenzoate: PCMB). (4) Displacement of the bound F-NO upon addition of cortisol (F) led to a large increase in the free spin label signal. (5) Human serum albumin ($23 \cdot 10^{-5}$ M) interaction with F-NO. 2T value: 64.4 G, as compared to 56.3 G for the interaction with HCBG (fig.2 (1)).

e.s.r. spectrum that showed a spin label totally free in solution (fig.2 (2)). This effect was thus used to measure by ESR the concentration of the total spin label present in the mixture.

It has been established that blocking of the thiol groups of CBG results in inhibition of its binding properties [23]. This was readily confirmed by the ESR spectrum given by a mixture of F-NO and HCBG previously treated by *p*-chloromercuribenzoate (fig.2(3)): only unbound steroid could be detected.

Displacement of the bound F-NO upon addition of unlabelled cortisol was readily detected by the increase in the intensity of the unbound spin label signal (fig.2 (4)). These displacement experiments indicate that the spin labelled F-NO did bind to the HCBG cortisol binding site. In addition, the increase in the free radical signal could be measured by e.s.r., thus this result may constitute the basis of a spin label competitive binding assay for cortisol.

Fig.2 (5) shows the ESR spectrum when the interaction of F-NO with human serum albumin (HSA, Calbiochem.) was studied. The ratio of bound steroid was much lower than in fig.1 (1); this could be expected due to the lower affinity of HSA for corticosteroids as compared to HCBG [3]. The 2T value obtained for the HSA-F-NO interaction (64.4 G) indicates a marked difference in the binding site character between HSA and HCBG. This can be related either to a difference in the degree of immobilization of the spin label or to a difference in the binding site polarity. This question cannot be solved at present and because the e.s.r. spectrum reflects the local environment of the spin label, which is only partially involved in the interaction of the steroid moiety with the binding site structure. However, these observations are of interest in view of a potential application to steroid binding studies in crude biological systems where high and low affinity bindings are usually present and might be distinguished and characterized by e.s.r. spectroscopy.

When the interaction of HCBG with F-0.8-NO was studied by e.s.r. (data not shown), no immobilized spin label could be detected. However, F-0,8-NO was able to bind to HCBG (table 1). This suggests that the steroid nucleus was able to enter the HCBG binding site and leave the spin label, attached to a long side chain, outside. Thus both its motion and its local environment were not drastically affected. This result represents an approach to the study of binding site topography using ESR evaluation of the binding site depth [24]. This work is currently being undertaken with the synthesis of cortisol derivatives bearing the spin label radical on side chains of increasing length.

In conclusion, with suitable spin labelled steroids, ESR spectroscopy can be applied to the study of specific, (high affinity, low capacity) purified steroid binding systems. This methodology offers several advantages of potential value:

(i) Bound and unbound forms of the ligand can readily and simultaneously be detected in a binding system at equilibrium without the need of any physical separation which can disturb the equilibrium. Quantitative measurements can be made to determine binding parameters under various experimental conditions (e.g. thermodynamic studies at variable temperatures) [6].

(ii) Being most sensitive to the local environment of the spin label, the ESR parameters can characterize a binding site (2T value). Thus, two simultaneous binding systems may be distinguished (as in the case of HSA and HCBG); this approach might be developped into a powerful tool by a mathematical treatment of the superimposed mixed spectra [25].

(iii) An approach to binding site mapping is possible by using spin labels that are attached differently to the binding ligand.

(iv) The principle of a spin competitive protein binding assay may be developed into a practical method for cortisol determination.

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