ELECTRON SPIN RESONANCE STUDY OF HUMAN TRANSCORTIN BINDING PROPERTIES. INTERACTION OF MONOMERIC AND POLYMERIC FORMS WITH SPIN LABELED CORTICOSTEROIDS

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SUMMARY

Corticosteroids bearing a nitroxide radical on the side chain were shown to bind with a high affinity to purified human serum transcortin. ESR spectroscopy data allowed calculation of the thermodynamic parameters (ΔH, ΔS) of the interaction and characterization of a hydrophobic spin label binding site. Transcortin spontaneously associated upon storage into reversible polymeric forms which partly retained the steroid binding properties. Apparent rotational correlation times of 34 and 70 n sec were obtained by ESR analysis at 20°C for the transcortin monomer and dimer respectively.

INTRODUCTION

The potential value of electron spin resonance spectroscopy (ESR) in the study of steroid-protein interaction using ligand bearing a stable paramagnetic spin label has been previously investigated with serum albumin (1, 2) as well as with the high affinity Human Corticosteroid Binding Globulin (HCBG or transcortin) (3).

This paper reports further study of spin labeled corticosteroid binding with purified HCBG which allowed determination of the thermodynamic parameters of the interaction and characterization of the steroid binding site polarity. During these investigations, the propensity of purified HCBG to spontaneously aggregate into polymeric forms was encountered (4, 5). This led us to evaluate the binding properties of these HCBG molecular associations. ESR spectroscopy data were used to calculate the rotational correlation time of the monomer and dimer HCBG complexes in solution, after spin labeled corticosteroid binding.

EXPERIMENTAL PROCEDURES

. *Spin labeling of cortisol and corticosterone* with a stable nitroxide

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radical was performed by esterification of the 21 OH group with 3-carboxy-2,2,5,5-pyrrolin-1-oxil, as previously described (3, 6), to yield cortisol nitroxide (F-NO) and corticosterone nitroxide (B-NO) respectively. The corresponding tritiated compounds were prepared with the same procedure from 1,2,6,7 [^{3}H]cortisol (Amersham, 95 Ci/mmole) and 1,2 [^{3}H] corticosterone (CEA, Saclay, 26 Ci/mmole) respectively yielding [^{3}H]F-NO (4.3 Ci/mmole) and [^{3}H]B-NO (6.5 Ci/mmole).

. *ESR spectra* were recorded with VARIAN E-12 or 104 spectrometers in calibrated capillary tubes (1.5 mm ID, CORNING). The microwave power was set at 5 mW for recording above 0°C whereas the setting was 2 mW when samples were studied at liquid nitrogen temperature. A variable temperature accessory was used for thermodynamic studies. ESR spectra parameters were determined as previously described (2).

. *Human serum transcortin* (HCBG) was isolated from serum by the affinity chromatography procedure of LE GAILLARD *et al.* (7). When the elution was carried out with cortisol, the protein was obtained with saturating amount of bound steroid (∿ 8 µg/mg protein). For ESR studies, the protein was usually obtained upon addition of F-NO to the elution buffer and exhibited a lower degree of saturation (∿ 1 µg steroid/mg protein).

. *Equilibrium dialysis* was performed with 0.2 ml of HCBG preparation inside each dialysis bag (VISKING) against 3.8 ml of 0.05 M Tris HCl buffer, pH 7.4, containing increasing amounts of tritiated steroid, at 4°C for 48 hours.

. *Polyacrylamide gel electrophoresis* (PAGE) was carried out according to ORNSTEIN (8) and DAVIS (9) in 7.5 % gels. Bromphenol blue was added to the sample as a tracking dye and used for evaluation of Rf values. After separation, the protein bands were localized by staining with amidoschwartz. For steroid binding studies, the protein was labeled by incubation with [^{3}H]corticosteroid prior to PAGE. The gel was then cut into 2 mm thick slices which were counted for radioactivity. PAGE in the presence of 1 % sodium dodecyl sulfate (SDS) was performed as described by FAIRBANKS (10). Evaluation of HCBG apparent molecular weight by SDS PAGE was performed using cytochrom C (MW 13.500), chymotrypsinogen A (MW 25,000), egg albumin (MW 45,000) and bovine serum albumin (MW 67,000) as marker proteins (Boehringer).

RESULTS AND DISCUSSION

1. Polymerisation of purified HCBG and binding properties

Freshly purified HCBG preparations yielded a single homogeneous band upon PAGE analysis with a Rf of 0.55 with regard to the tracking dye. After labeling with [^{3}H]corticosterone, a well defined radioactive peak of bound steroid was detected with the same electrophoretic mobility. This bound radioactivity could be totally displaced by incubation in the presence of an excess of unlabeled corticosterone (peak 1, figure 1-A). An apparent MW of 52,000 was found for this binding moiety which was therefore attributed to the monomeric form of HCBG as characterized by other workers (5, 11). The binding properties of HCBG monomer was examined by equilibrium dialysis in the presence of

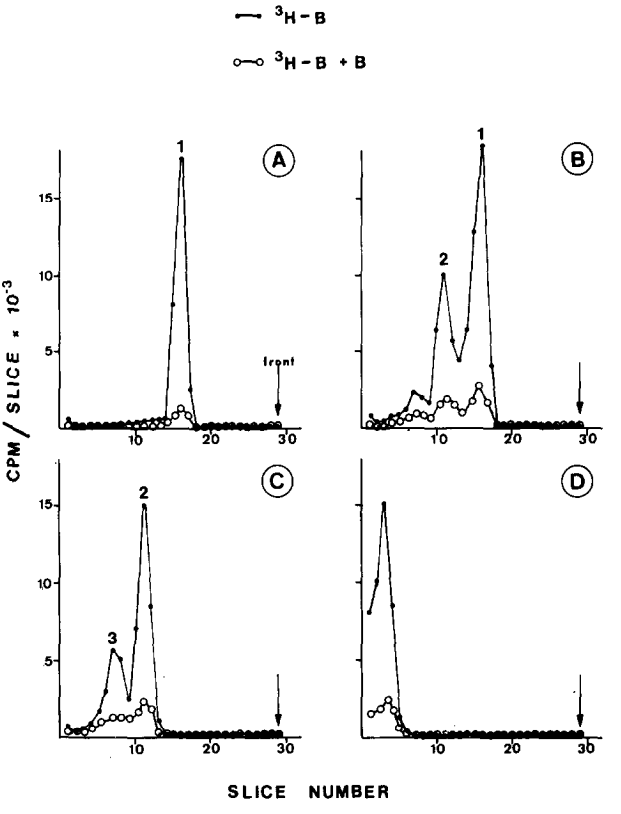

Figure 1

Polyacrylamide gel electrophoresis of HCBG preparations (100 µl) after labeling with ^{3}H corticosterone in the absence (•——•) and in the presence (o——o) of a 100 fold excess of unlabeled corticosterone.
A = freshly purified HCBG ; B = HCBG after 5 days at 4°C ; C = HCBG after 4 weeks at 4°C ; D = HCBG after 3 months of storage at 4°C. The arrow indicates the location of the tracking dye (bromphenol blue).

[^{3}H]cortisol as well as tritiated F-NO and B-NO. In both cases, the Scatchard plots (12) showed an apparent single set of binding sites. The calculated association constants (Ka) and binding capacity (N, number of steroid binding sites/mole protein) are listed in table I and are in agreement with previously reported figures for cortisol (13). The synthetic spin labeled compounds exhibited a somewhat lower affinity whereas their binding capacity remained the same as for the natural glucocorticoid.

TABLE I

Association constant (Ka) and binding capacity (N, number of steroid binding sites/mole protein) of monomeric HCBG for cortisol and spin labeled corticosteroids at 4°C.

	Cortisol	Cortisol-nitroxide	Corticosterone-nitroxide
Ka (M^{-1})	$1.5 \cdot 10^8$	$1.2 \cdot 10^7$	$0.8 \cdot 10^7$
N	0.92	0.94	-

Within a few days of storage at 4°C, the HCBG preparation exhibited upon PAGE an additional protein component which was detected after staining as well as after [^{3}H]corticosterone labeling (peak 2, figure 1-B). This slower moving moiety (Rf 0.38) was attributed to a spontaneously aggregated HCBG most probably representing a dimeric association. It may be noticed that this HCBG dimer was still able to specifically bind corticosterone. Within a few weeks, the preparation had totally lost the native monomeric HCBG moiety and the dimer became the major protein component, while additional polymeric forms appeared (figure 1-C). This preparation mostly representing the HCBG dimer was examined with regard to its binding properties. An apparent Ka of 2.10^7 M^{-1} was found for cortisol ; assuming a MW of about 100,000 for the dimer, a binding capacity of 0.92 mole steroid/mole dimeric HCBG was calculated. This indicated that the HCBG dimer remained able to bind cortisol although with a somewhat impaired affinity, whereas the molecular HCBG association led to the disappearance of one steroid binding site. Continued storage of the HCBG preparation led to further molecular aggregation giving rise to larger protein components. After several months, an ill defined protein zone was observed upon PAGE, however still able to retain corticosterone (figure 1-D).

Possible reversal of the aggregation process was studied by PAGE after various treatments of the aged HCBG preparation. Treatment

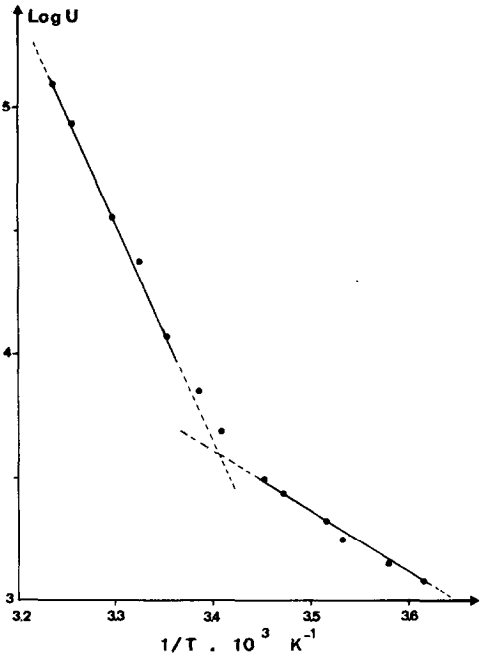

Figure 2

Arrhenius plot of HCBG-corticosterone nitroxide interaction at equilibrium.

with 1 % SDS + 6 M urea with or without 1 % dithiothreitol led to the complete recovery of monomeric HCBG (apparent MW 52,000). Identical result was obtained with 1 % SDS alone. These observations suggested that by aging, HCBG spontaneously aggregated into physical protein-protein associations, without involving covalent disulfide bridges as observed upon HCBG polymerisation promoted by elevated temperature (5).

2. *ESR spectroscopy*

ESR studies were performed with freshly prepared, monomeric HCBG eluted from the affinity chromatography column with F-NO and concentrated by ultrafiltration to about 5 mg protein and 3 µg steroid/ml. The ESR spectrum of such a preparation was the result of the addition of two spectra i.e. the narrow band signal attributed to the unbound, free moving spin labeled steroid and a widened spectrum (2 T value = 56.3 Gauss) given by the immobilized spin label bound to the protein, as previously described (3).

TABLE II

Thermodynamic parameters calculated for HCBG-FNO interaction at different temperatures.

Temperature range	5 - 20°C	20 - 40°C
Ka (average)	10^7 M^{-1}	10^6 M^{-1}
ΔH Kcal/mole	- 3.8	- 17.5
ΔS cal/deg./mole	30	- 20

The binding equilibrium was studied by ESR at various temperature using F-NO and B-NO as the ligand. When the temperature was increased from 4°C, the unbound steroid signal increased in a reversible fashion up to 40°C. This phenomenon was identical with B-NO and F-NO and was in agreement with the expected binding dissociation occuring when the temperature was increased (13). Since the ESR signal of the unbound spin label (U) was very small as compared to the bound form (B), the latter may be considered as constant and changes in [U] taken as directly reflecting variations in Ka. By plotting Log [U] versus 1/T, two distinct straight lines were obtained with an obvious break around 22°C (figure 2). From the slopes of these two straight lines, entropy and enthalpy of the steroid-protein interaction could be calculated within the corresponding temperature intervals. The obtained values are given in table II. Whereas the negative enthalpy figures are in line with data reported by others (13), a striking change in the entropy value was observed depending upon the temperature range studied. This suggests that the interaction may involve different intimate mechanisms possibly related to a different behaviour of the protein hydration water or/and a conformational transition of HCBG around 20-22°C (16). However, no break in the 2 T value was observed in this temperature range.

. *Steroid binding site polarity*

HCBG-FNO interaction was examined by ESR in a medium of increasing viscosity (η) obtained by addition of increasing (5-55 %) sucrose concentrations (14). Extrapolation of the straight line obtained by plotting 2 T values versus $(\eta/T)^{2/3}$ to an infinite viscosity yielded the maximum value of the ESR hyperfine splitting constant $2A_{zz}$ (15). At 10, 20 and 30°C, values of 62.0, 61.2 and 59.6 Gauss were respectively calculated in the case of the monomeric HCBG. Identical figures were obtained with the HCBG dimer (61.2 Gauss at 20°C). These values were compared to the width of the ESR spectrum given by F-NO immobilized by freezing in various solvents. Values of 65.4 and 70.8 Gauss were obtained for $2A_{zz}$ in toluene and methanol, respectively. This indicated that the surrounding environment of the spin label probe in the HCBG monomer and dimer binding site had a strong hydrophobic character. However, this property concerns the environment of the cortisol side chain nitroxide moiety and may not reflect the overall polarity of the HCBG binding site, especially the area involved in the specific interaction with the steroidal nucleus. In addition, a 2 T value of 69 Gauss was observed when the HCBG-FNO complex was frozen at - 50°C in aqueous medium. Such a discrepancy with the $2A_{zz}$ value calculated for infinite viscosity (61.2 Gauss at 20°C) has already been observed in the case of serum albumin (2) and should reflect drastic modification of the steroid binding site polarity by the freezing process, probably involving hydration water. This may be related with the break observed between - 4 and - 10°C, in the plot of 2 T versus temperature ; a similar phenomenon has been reported in the case of leucine aminopeptidase (17).

. *Rotational correlation time of HCBG-FNO complexes*

The ESR method developped by the Mc CONNELL group (14) was applied to evaluate the apparent rotational correlation time of the spin label bound to HCBG. Both monomeric HCBG and a mostly dimeric HCBG preparations (figure 1-C) were labeled with F-NO and variation of the spectrum width was examined in aqueous sucrose (5-55 %) solutions of increasing viscosity. Values of 48, 34 and 24 n sec were obtained for the HCBG monomer at 10, 20 and 30°C respectively whereas 70 n sec was calculated for the dimer at 20°C. These data confirm the HCBG protein-protein association and show that ESR spectroscopy may provide an interesting new approach for the direct study of steroid-protein dynamics in solution.

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