THERMAL ACTIVATION OF STEROID BINDING PRINCIPLE IN COMMERCIAL BOVINE SERUM ALBUMIN

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1. Introduction

It has long been recognized that serum albumin is a microheterogeneous protein [1,2] capable of forming a wide spectrum of polymers [3] that could be attributed to changes in the affinity of albumin molecules for a given ligand in question [4]. The low ($\sim 10^{-4}$ M) association constant of albumin–glucocorticoid complex [4], and the low free energy change for the binding (21–26 kJ M^{-1}, is indicative of a relatively weak bond formation that decreases directly with increased polarity of the steroid [4], despite the evidence that as many as 20 steroid molecules may be bound to each albumin vector [5].

On the other hand, the high affinity ($\sim 10^{-8}$ M) corticoid binding globulin (CBG), present in human plasma (26–60 mg/ml), involving a free energy change of –46 kJ M^{-1}, is endowed with only one binding site for the steroid [4], but may exhibit polymorphism [6].

This report adds a new parameter that must be borne in mind while speculating on the role of serum albumin in relation to steroid hormone action. Due to widespread use of commercial bovine serum albumin (BSA) in diverse types of studies, the results reported here emphasize the need to exercise caution in the interpretation of data obtained with such preparations.

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2. Methods

Bovine serum albumin (Sigma lot 57 C-8003) and blood serum, obtained from male, adrenalectomized, Wistar rats, were used throughout this study. Protein concentrations were determined by the Biuret method. The protein preparations were equilibrated with the desired concentration of the steroid of choice (see figure legends for details) and the reaction was stopped with activated charcoal (Sigma 27-0022) which was thereafter eliminated by centrifugation (5 min, 5000 × *g*). Aliquots of 0.5 ml or 1 ml (see legends) were mixed with 10 ml Scintix (Isotec, France) and counted in a Packard, Tricarb Scintillation Spectrometer equipped with automatic background subtraction and external standardization.

[1,2-^{3}H]Dexamethasone (20 Ci/mM; lot B16); [1,2-^{3}H]cortisol (40 Ci/mM; lot B45); and [1,2,6,7-^{3}H]corticosterone (80 Ci/mM; lot B13) were purchased from Amersham. [1,2-^{3}H]Cortexolone (47 Ci/mM; lot TMM 55) was a product of CEA, France. [1,2-^{3}H]Triamcinolone acetonide (11 Ci/mM; lot ZT 2194) was obtained from Schwarzman, Orangeburg, NJ. Purity of these products exceeded 97% in thin-layer chromatography.

Nonradioactive steroids: corticosterone (114 C-0003), dexamethasone (54 C-0375, triamcinolone acetonide (53 C-2710), and cortisol (25 C-0318) were purchased from Sigma. All other chemicals were high purity reagent grade from Merck.

Chromatographic procedures are detailed in [6,7] and further recalled in the figure legends.

3. Results and discussion

Data in fig.1a show that charcoal-resistant binding to BSA (bovine serum albumin) increased linearly with temperature between 0–60°C at a time when rat serum–cortisol binding decreased under otherwise identical conditions. Both proteins coagulated beyond temperatures >60°C. In other experiments, albumin–cortisol binding reached a plateau within 40 min at 60°C. Furthermore, binding at 60°C increased linearly as BSA was raised progressively from 5–40 mg/ml in the assay mixtures. In other studies, heating at 60°C prior to equilibration with the radioactive steroid effectively destroyed the binding principle in bovine serum albumin.

From the results in fig.1b it is clear that activation of binding at 60°C was inversely proportional to the quantity of the steroid bound at 4°C. Thus, maximum %activation at 60°C: cortexolone > dexamethasone > cortisol was associated with the binding order at

Fig.1a Fig.1b

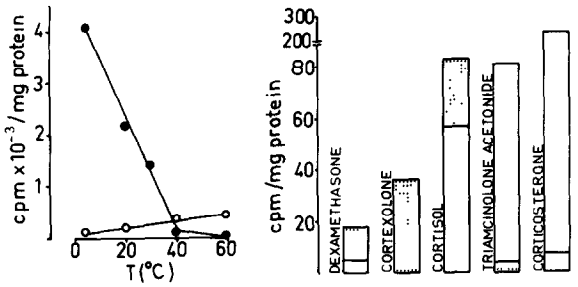

Fig.1. Evidence for thermal activation of steroid binding to bovine serum albumin (BSA). BSA (50 mg/ml) and rat serum (58 mg/ml) were incubated (0.5 ml aliquots) with 10^{-8} M [^{3}H]cortisol and 10^{-5} M nonradioactive cortisol for 60 min at the indicated temperature (fig.1a). For fig.1b, albumin was incubated at either 4°C (□) or 60°C (■) with 10^{-8} M of either of the indicated tritiated steroid in presence of 1000-fold excess of homologous, nonlabelled molecule. In all cases, reaction was stopped by the addition of 0.5 ml activated carbon (50 mg/ml), additional incubation (10 min, 4°C) and centrifugation. Further details are given in the text. To account for nonspecific binding, counts obtained in presence of radiolabelled steroid alone were corrected by subtracting those obtained in presence of the tritiated steroid + cold, homologous molecule. All values are average of 3 separate determinations. The experiments were repeated thrice but only one is shown here. Albumin (○——○); serum (●——●).

Fig.2a Fig.2b

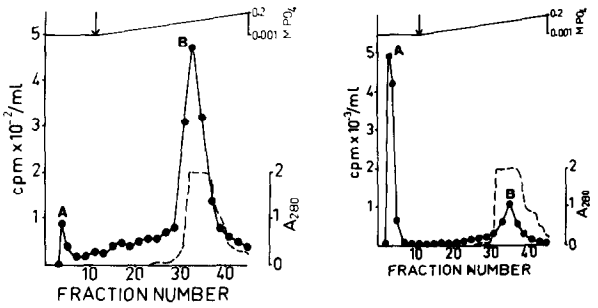

Fig.2. Ion-exchange separation of albumin binders by chromatography on cellulose-DEAE-52 columns. 300 mg BSA in 5 ml initial buffer (0.001 M phosphate, pH 7.5) was incubated (60 min) with 10^{-8} M [^{3}H]cortisol at either 4°C (2a) or 60°C (2b). Free radioactivity was removed by additional incubation (10 min, 4°C) in presence of 100 mg/ml activated charcoal, centrifugation (5 min, 5000 × *g*) and passage through glass wool. The clear solutions were loaded onto DEAE-52 (Whatman) columns (1 × 25 cm) equilibrated with 0.001 M phosphate, pH 7.5. After passage of 60–70 ml initial buffer (fraction vol. 6–7 ml), protein was eluted (at arrow) with a linear gradient of 60 ml 0.001 M phosphate and 60 ml 0.2 M PO_4, pH 7.5, at a flow rate of 60 ml/h (fraction vol. ~ 3 ml). All manipulations were carried out at 4°C. Aliquots (1 ml) were mixed with 10 ml Scintix and counted as before. A_{280} values were recorded manually. (– – – –) A_{280}; (●——●) ^{3}H.

4°C: cortisol > dexamethasone > cortexolone. In addition, corticosterone and triamcinolone binding to BSA actually decreased at 60°C as compared to 4°C. These would argue against polarity or affinity as factors determining thermal activation of steroid binding to BSA.

Due to the heterogeneous nature of BSA [1,2], attempts were made to determine whether changes in qualitative abundance of a given subspecies could be correlated with the activation at 60°C or whether this activation was an expression of proportional increase in steroid bound to all subclasses of the albumin molecule. Cortisol was chosen for this purpose since the bound quantity of other steroids was too weak to be quantitated after physical separation by chromatography.

From fig.2 it is clear that peaks A and B increased 50- and 2-fold, respectively, when incubation was at 60°C instead of 4°C. Thus, the majority of increased binding was limited to the weakly-charged, basic com-

Fig.3a

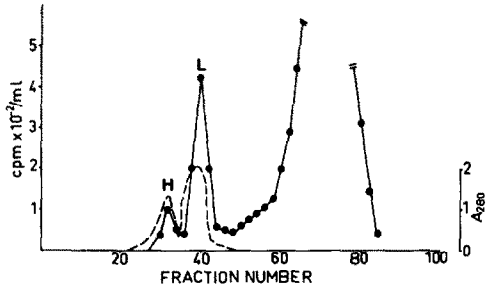

Fig.3b

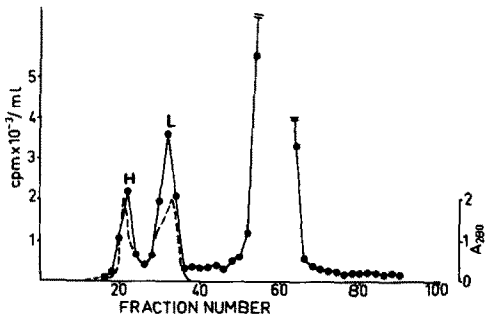

Fig.3. Molecular filtration of albumin–steroid binders. 100 mg BSA in 2 ml initial buffer (0.01 M phosphate + 0.1 M NaCl, pH 7.4) was incubated (60 min) with 10^{-8} M [^{3}H]cortisol either at 4°C (3a) or 60°C (3b). The solutions, without charcoal treatment, were loaded onto Ultrogel-44 (Pharmacia) columns (1 × 130 cm) equilibrated and eluted with the initial buffer. Fractions (~ 1.3 ml) were collected at a flow rate of 10–12 ml/h. For other details see above. (– – – –) A_{280}; (●——●) ^{3}H.

ponent of the albumin molecule. It is clear that activation was not due to appearance of new subspecies of the complex albumin molecule. In addition, binding activity was lost if BSA was chromatographed prior to equilibration with the tritiated steroid.

Results in fig.3 reveal that the high (H) and the low (L) molecular weight peaks increased 25- and 8-fold, respectively, when incubation was at 60°C prior to chromatography on Ultrogel at 4°C. Quantitation of the column revealed that these would correspond to mol. wt 130 000 (H) and 70 000 (L), respectively. Again, there was no indication of the formation of aggregates of intermediate molecular weights as a result of BSA heating at 60°C. Furthermore, as with DE-52, the activation process was irreversible and did not decrease during chromatography at 4°C.

Separation based both on molecular weight and charge density was next attempted on DEAE–Sephadex A-25 columns. Results in fig.4 show that the peak in 0.6 M KCl (fig.4a) was replaced by another in 0.2 M KCl, followed by a shoulder in 0.35 M KCl region, as a result of activation at 60°C, confirming earlier results on Ultrogel and DEAE-52 columns.

Collectively, heat activation of corticoid hormone binding to bovine serum albumin does not appear to depend upon either the presence of CBG, the polarity of the steroid molecule, or change in the affinity of the protein for the hormone, since the process was irreversible, charcoal resistant and was not observed if albumin was preheated in absence of the steroid. The binding principle appears to be a protein capable of forming relatively stable, weakly charged, high molecular weight complexes in presence of selected steroid hormones. This activation of high capacity, low affinity BSA is to be contrast-

Fig.4a

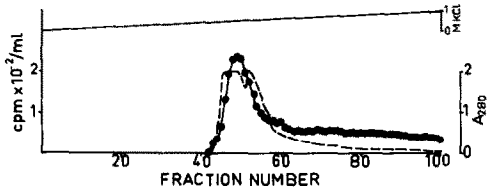

Fig.4b

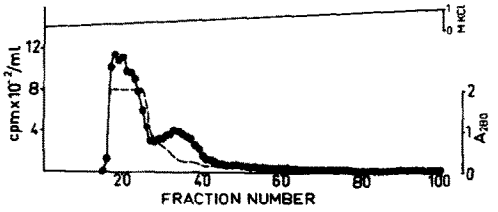

Fig.4. Separation of albumin–steroid binders by weight and charge. 400 mg BSA was dissolved in 6 ml Tris–HCl, pH 7.5, and incubated with 10^{-8} M [^{3}H]cortisol either at 4°C (4a) or 60°C (4b). After charcoal treatment, they were chromatographed, separately, on DEAE–Sephadex A-25 columns (1 × 90 cm) eluted with a linear gradient between 0–1 M KCl in the initial buffer at a rate of 5–6 ml/h at 4°C. Fractions of 3 ml were collected and processed for radioactivity and absorbance as before. (– – – –) A_{280}; (●——●) ^{3}H.

ed with inactivation, under similar conditions, of low capacity, high affinity CBG–steroid binding. Multiplicity in serum CBG [6], tissue steroid hormone receptor [7] and albumin binding components as reported here, makes it possible to visualize a process where spatial stereospecificity in various classes of binders may explain varying degrees of physiological response to a given hormone under diverse conditions. Although high temperatures required for activation in vitro make it difficult to conceive a physiological role for this type of phenomemon of activation, the wide use of BSA as a marker calls for caution in the eventual interpretation of such studies.

Acknowledgements

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