STEROID–PROTEIN INTERACTIONS

Kinetics of binding of cortisol and progesterone to human corticosteroid-binding globulin

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1. Introduction

Human corticosteroid-binding globulin is the first high-affinity steroid binding protein that has been studied in detail [1–5]. Although its general physicochemical parameters (molecular weight, absorptivity, carbohydrate and amino acid content) have been defined, further information related to steroid binding of CBG^{2+} is needed. It is noteworthy that the rather unusual progesterone binding globulin (PGB), obtained from pregnant guinea pig serum, has been more thoroughly characterized with respect to specificity [6–8] and with respect to mechanism of binding [9–11] than has human CBG. The sensitive method of stopped-flow fluorescence has been applied to explore the mechanism of binding of steroids to human CBG.

The binding of steroids to various proteins results in quenching of the intrinsic fluorescence of the protein [9]. The large decrease in fluorescence which occurs upon the binding of 3-oxo,4-ene steroids to PBG has been used to measure the rates of complex formation and dissociation [11]. We have recently demonstrated the generality of the fluorescence quenching method in determinations of the dissociation rate constants of human CBG-steroid complexes [12]. In the present communication, we report a study on the kinetics of association of the two natural steroid ligands, cortisol and progesterone, with CBG.

This study is of interest for the following reasons. First, CBG is the primary high-affinity binding protein of C_{21} steroids in human blood. Second, fluorescence studies and equilibrium dialysis experiments indicate that, whereas progesterone dissociates from CBG five-times faster than cortisol, the affinity constants of cortisol and progesterone at 20°C are essentially the same. This would predict a faster association of progesterone with CBG than that expected for cortisol [12]. The present study verifies the prediction.

2. Materials and methods

Human CBG was prepared as its corticosterone complex from pregnancy serum by affinity chromatography as in [12]. Bound steroid was partially removed by gel filtration in mildly acidic milieu at room temperature, conditions of less acidic pH and higher temperature than suggested [13]. CBG-corticosterone complex, 5 ml (0.62 mg/ml of 50 mM phosphate buffer, pH 7.4) was titrated to pH 6.15 with 1 M acetic acid and applied to a 2.5 × 40 cm column of Sephadex G-25 equilibrated with deionized, distilled water. Elution was performed at 1 ml/min using water, and the protein-containing fractions were pooled, adjusted to 0.1 M phosphate concentration, pH 7.4, and diluted to total vol. 45 ml with 0.1 M

Abbreviations: CBG, corticosteroid-binding globulin; PBG, progesterone-binding globulin of pregnant guinea pig

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sodium phosphate buffer, pH 7.4. The concentration of available CBG-binding sites was determined by fluorescence-quenching titration [9] to be 0.92 μM or 69% of the original sites. At the equivalence point, CBG fluorescence was quenched about 45%*. The loss of 31% of the original active binding sites may be caused by denaturation of CBG or by incomplete removal of corticosterone. Neither denaturation nor incomplete removal of steroid should interfere with the stopped-flow measurements. In the former case, the denatured protein would not interact with steroid, and in the latter case, cortisol, corticosterone and progesterone quench CBG fluorescence to the same extent.

Stopped-flow fluorometry was performed on a Gibson-Durrum stopped-flow instrument equipped with a fluorescence cuvette and interfaced with a Nova 1200 computer. The procedures used were as in [11]. All measurements were made at 20°C. The CBG stock solution was mixed with an equal volume of aqueous steroid solution; the final CBG concentration was 0.46 μM in 50 mM phosphate of pH 7.4 with steroid concentrations as indicated. Aqueous stock solutions of steroids were prepared gravimetrically and contained about 1% ethanol. The concentrations of the steroid solutions were verified spectrophotometrically.

The initial rate (k_{obs}) of fluorescence quenching was measured as a function of increasing cortisol and progesterone concentrations. The slope of k_{obs} plotted as a function of steroid concentration gives k_{on}, the association rate constant of the steroid. The instrumental conditions, in particular the light source intensity (Xenon lamp), varied from one determination to the other so that the extrapolated total fluorescence change is not constant (see fig.1,2).

3. Results and discussion

Figure 1 gives the initial rate of quenching of CBG fluorescence (Δ Volts) at different cortisol concentrations. Figure 2 gives a similar kinetic study of fluorescence quenching of CBG by complex formation with progesterone. Initial rates were estimated from the semilog plots for both steroids. Figures 1 and 2 illustrate representative runs; each data point is the mean of four identical determinations. At least two sets of four runs were performed at each steroid concentration. It is immediately apparent that progesterone binds to CBG more rapidly than cortisol.

Figure 3 gives the dependence of k_{obs} on steroid concentration at constant CBG concentration. Binding is, as expected, first order with respect to both cortisol and progesterone, implying a bimolecular association between protein and steroid. Least squares analysis of the two sets of data gives association rate constants, k_{on}, of 1.4×10^7 and 5.8×10^7 M^{-1} s^{-1} for cortisol and progesterone, respectively. Table 1 summarizes several parameters of steroid binding to CBG. It is seen that the steroid with the faster association rate has also a faster dissociation rate so that the ratio k_{on}/k_{off} is approximately the same for progesterone and cortisol. The k_{on}/k_{off} values agree with the association constants determined by equilibrium dialysis [12].

The mechanism of steroid binding to human CBG is less well understood than the binding to PBG,

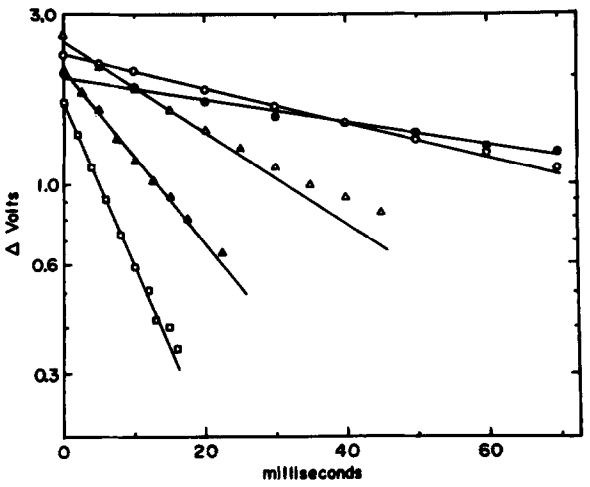

Fig.1. Initial rates of cortisol binding to human CBG at 20°C. CBG at 0.46 μM was reacted with cortisol of (•) 0.46 μM, (○) 1.0 μM, (△) 2.0 μM, (▲) 4.0 μM, and (□) 8.0 μM concentration. Each data point is the mean of 4 separate determinations; the average standard deviation was approx. 30 mV. Δ Volts indicates change of relative fluorescence. Time 0 refers to start of data collection after a mixing time of 3–5 ms.

*The highest quench value observed with various stripped CBG preparations was 65% (unpublished)

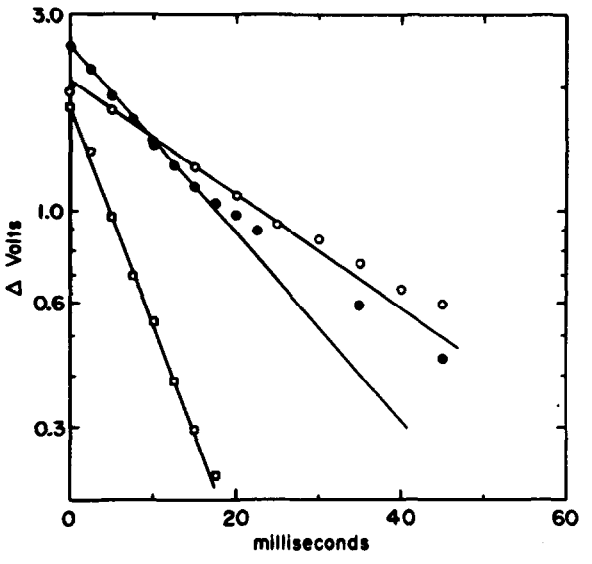

Fig.2. Initial rates of progesterone binding to human CBG at 20°C. CBG at 0.46 μM was reacted with progesterone of ○ 0.46 μM, ● 1.0 μM, and □ 2.0 μM concentration. Each data point is the mean of 4 separate determinations; the average standard deviation was approx. 30 mV. Δ Volts indicates change of relative fluorescence. Time 0 refers to start of data collection after a mixing time of 3–5 ms.

human serum albumin, or α_1-acid glycoprotein. In the latter cases the primary driving force appears to be hydrophobic with affinities generally following the polarity rule. In the case of PBG, all tightly bound steroids associate with the protein at the same rate [11]; the dissociation rate constant thus defines the affinity constant. In contrast, human CBG binds cortisol and progesterone with approximately the same affinity (cortisol slightly more tightly) [12]. However, progesterone has a dissociation rate constant approx. 5-times that of cortisol, implying that the association of progesterone with CBG is more rapid than that of cortisol. This paper confirms the prediction. The k_{on} of progesterone to CBG ($5.8 \times 10^7\ M^{-1}\ s^{-1}$) approaches the k_{on} of steroids to PBG ($8–9 \times 10^7\ M^{-1}\ s^{-1}$ at 20°C [11]); k_{on} for cortisol to CBG is significantly smaller ($1.4 \times 10^7\ M^{-1}\ s^{-1}$).

There are several possibilities to interpret these results which are different from those obtained for the PBG–steroid complexes. However, more information on the physicochemical nature of the CBG complexes is needed before a mechanism can be formulated.

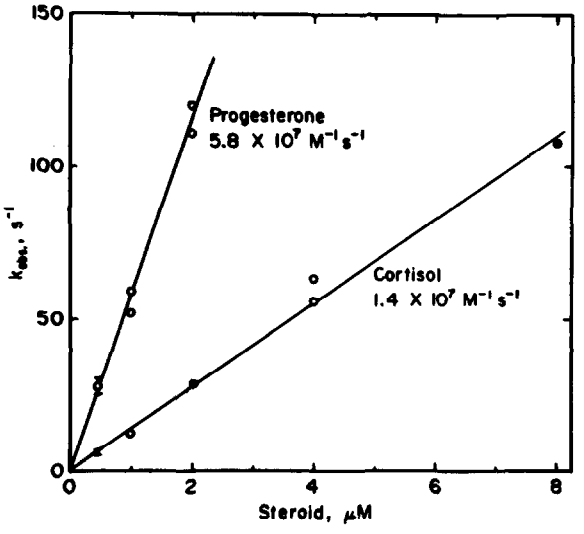

Fig.3. Association rate constants for the binding of progesterone and cortisol to human CBG at 20°C. From the concentration dependence of the initial rates of binding (k_{obs}), progesterone has an association rate constant of $5.8 \times 10^7\ M^{-1}\ s^{-1}$ and cortisol has the value of $1.4 \times 10^7\ M^{-1}\ s^{-1}$. (○) Mean of 4 identical determinations; (●) average of 2 runs of 4 identical determinations each; (I) (at 0.4 μM cortisol) average of 3 runs; (○ I) (at 0.4 μM progesterone) average of 4 runs ± SD.

Table 1
Rate of association of cortisol and progesterone with CBG at 20°C

Steroid	k_{on} ($M^{-1}\ s^{-1} \times 10^{-7}$)	k_{off}[a] (s^{-1})	k_{on}/k_{off} ($M^{-1} \times 10^{-8}$)	K_a[a] ($M^{-1} \times 10^{-8}$)
Cortisol	1.4	0.031	4.4	3.5
Progesterone	5.8	0.16	3.6	2.9

[a]From [12]; K_a determined by equilibrium dialysis

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