# STEROID-PROTEIN INTERACTIONS. PURIFICATION OF PROGESTERONE-BINDING GLOBULIN BY AFFINITY CHROMATOGRAPHY*

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Received 28 February 1976

## 1. Introduction

The progesterone-binding globulin (PBG) found in the serum of pregnant guinea pigs [1] has been purified by various methods, based mainly on ion exchange and gel chromatographic techniques [2–4]. It has been recognized as a polydisperse glycoprotein with the unusually high carbohydrate content of about 70% [4]. Taking advantage of the low p*I* of 2.8 and favorable acid stability [5], the isolation procedure of PBG was improved [6] by utilizing chromatography on sulfopropyl (SP) Sephadex, a strong cation exchanger.

The purified PBG preparations obtained in our laboratory often had values of *n*, i.e., number of progesterone-binding sites per protein molecule, of significantly less than unity. Assuming that this discrepancy was caused by contamination with inactive material, we purified the PBG by affinity chromatography on modified Sepharose. The present paper describes these studies.

## 2. Materials and methods

### 2.1. *Materials*

The PBG preparations subjected to affinity chromatography were pre-purified on an SP-Sephadex column [6]. Sepharose 4B was from Sigma Chemical Company. 19-Nortestosterone, 5α-pregnane-3,20-dione, and desoxycorticosterone-21-hemisuccinate were purchased from Steraloids, Inc.; progesterone and 11-desoxycorticosterone were obtained from Mann Research Laboratories. All melting points were checked. The other chemicals used were reagent grade or of the highest purity available. Polygram Sil G/$UV_{254}$ plastic sheets for thin-layer chromatography were from Brinkman Instruments, Inc. Double-distilled deionized water was used throughout.

### 2.2. *Preparation of the affinity column*

The affinity gel was prepared according to published methods [7,8]. Sepharose 4B was activated with cyanogen bromide and coupled with diaminodipropylamine. The 17-hemisuccinate of 19-nortestosterone was prepared according to Benson et al. [8]; completion of the reaction was ascertained by thin layer chromatography in ethyl acetate. The nortestosterone ester was recrystallized from acetone–water or methanol–water, and condensed with the diaminodipropylamino derivatized agarose with the aid of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Different preparations of nortestosterone Sepharose bound approximately 1–2 nmol of PBG per ml gel. A similar affinity gel was prepared by coupling the hemisuccinate of desoxycorticosterone to the diaminodipropylamino derivative of Sepharose 4B.

### 2.3. *Affinity chromatography*

SP-Sephadex-purified PBG was applied to an affinity gel column equilibrated with 10 mM Tris chloride (pH 7.4) containing 0.1 M NaCl. Following sample application, the column was washed with the same buffer until the fluorescence of the eluate decreased to a constant value. The PBG was eluted

* This is Paper 35 in the series 'Steroid–Protein Interactions'; for the preceding paper see Kute, T. and Westphal, U. (1976) Biochim. Biophys. Acta 420, 195–213.



*North-Holland Publishing Company – Amsterdam*

by the same buffer containing 10 μM progesterone or 5α-pregnanedione and 5 to 10% ethanol. All affinity chromatography steps were performed at room temperature. The protein concentrations of the eluted fractions were measured by fluorescence [9]. The PBG was routinely freed of steroid by delipidation [10] after concentration of the affinity-purified protein to about 1 mg/ml utilizing ultrafiltration with an Amicon Diaflo cell.

2.4. *Characterization of the purified PBG*

Association constant and number of progesterone binding sites per molecule PBG were determined by fluorescence quenching titration [9] and by equilibrium dialysis [11]. The molecular weight was determined by sedimentation equilibrium ultracentrifugation [12], using a value for partial specific volume, $\bar{v}$, of 0.66 [4]. The pure PBG was subjected to Sephadex G-200 gel filtration and the symmetrical peak divided into two equal portions (A and B) as done before [4]. Polyacrylamide gel electrophoresis of each of the two portions and analysis for protein, carbohydrate, and progesterone binding were performed as described [4,6]. The carbohydrate content was measured by methods applied previously [4].

## 3. Results

3.1. *Isolation of PBG*

A substantial portion of the PBG applied to the nortestosterone affinity column was not adsorbed and appeared in the breakthrough volume (fig.1, peak I). Elution with buffer containing progesterone or 5α-pregnanedione gave a fraction (peak II) consisting of pure PBG. A similar fractionation of PBG was obtained using a desoxycorticosterone affinity column. The elution profile was not influenced by the flow rate which varied from approximately 10 to 70 ml/h.

The method of fluorescence quenching titration indicated lack of progesterone binding for peak I (fig.2). The failure to bind progesterone was not caused by saturation of binding sites, since attempted displacement with 5α-pregnanedione [9] did not result in increased fluorescence, and delipidation [10] did not produce an active protein.

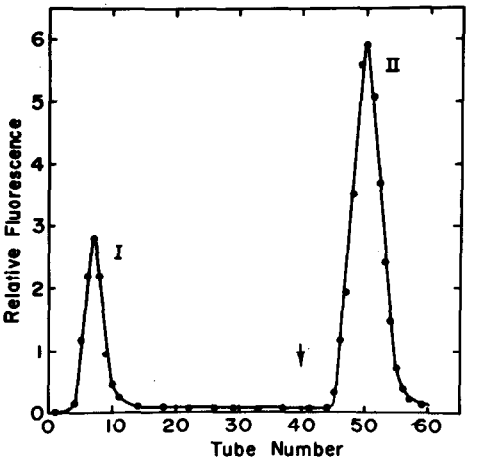


Fig.1. Affinity chromatography of SP-Sephadex-purified PBG. 12 mg of protein were applied to a 90 ml column of 19-nortestosterone affinity gel. Fractions of 100 drops were collected. At the arrow the column was eluted with 10 μM 5α-pregnane-3,20-dione.

3.2. *Characterization of affinity-purified PBG*

3.2.1. Fluorescence quenching titration

Fig.2 shows that formation of the progesterone–PBG complex quenches the fluorescence of PBG (peak II) by more than 80%. The affinity constant, $K_a$ at 23°C, was calculated to be $1.5 \times 10^9$ $M^{-1}$. The

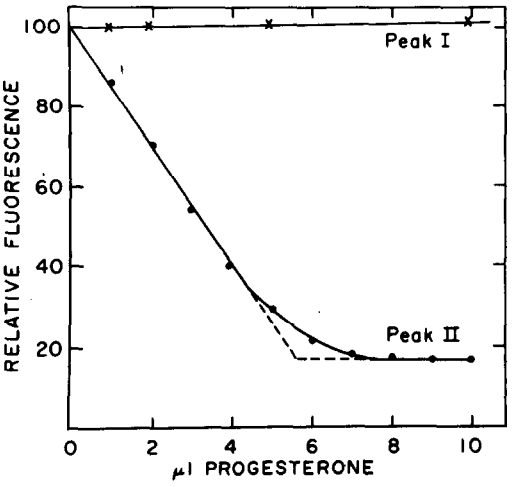


Fig.2. Fluorescence quenching titration of the delipidated peaks I and II (fig.1). One ml of the appropriate solution was placed in the cuvette and aliquots of 10.8 μM progesterone in methanol were added.

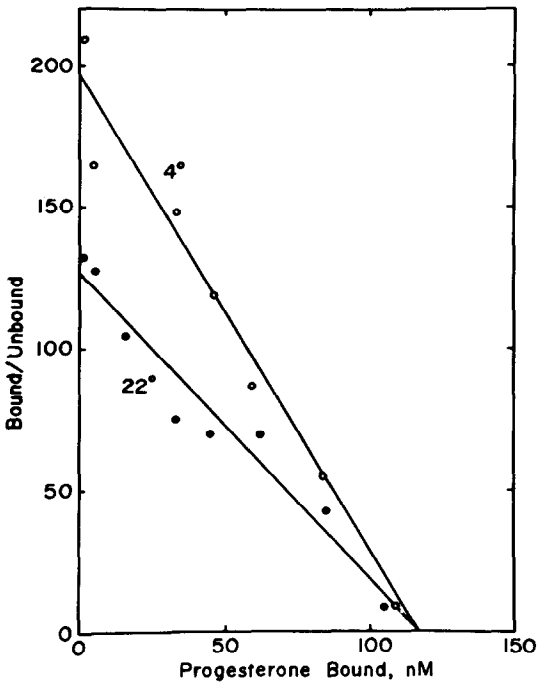


Fig.3. Equilibrium dialysis of affinity purified PBG at 4°C and 22°C. PBG concentration was 10 μg/ml and the samples were equilibrated for 48 h. The lines were calculated by a least squares computer program.

*n*-value was 0.98 ± 0.07 (SEM) as an average of ten determinations.

3.2.2. Equilibrium dialysis

An *n*-value of 1.03 was found as an average of 6 determinations at different temperatures. The association constants at 4°C and 22°C were $1.7 \times 10^9$ and $1.1 \times 10^9$ $M^{-1}$, respectively (fig.3).

3.2.3. Sedimentation equilibrium ultracentrifugation

The affinity-purified PBG gave a linear Yphantis plot [13]; the apparent weight-average molecular weight was 88 000.

3.2.4. Carbohydrate analysis

Table 1 shows the carbohydrate content of the inactive peak I and the purified PBG (peak II).

3.2.5. Polyacrylamide gel electrophoresis

Gel electrophoresis of fractions A and B obtained

Table 1
Carbohydrate content of peak I and peak II

| | percent | |
|---|---|---|
| Carbohydrate | I | II |
| Sialic acid | 11 | 17 |
| Hexose | 30 | 29 |
| Hexosamine | 34 | 23.5 |
| Fucose | 2.3 | 1.5 |

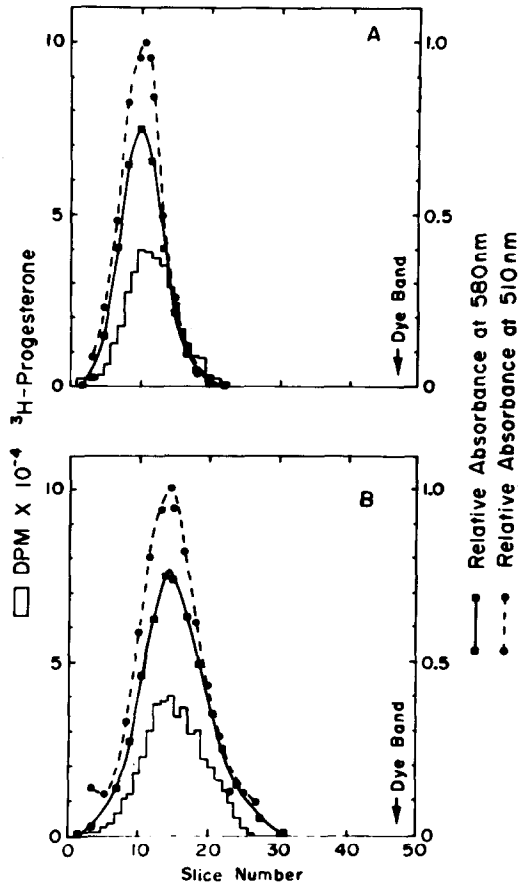


Fig.4. Polyacrylamide gel electrophoresis of fractions A and B of pure PBG, obtained by dividing the gel filtration peak (see section 2.4). Samples were run in triplicate. One gel was stained with Coomassie blue and scanned at 580 nm; another was stained by the periodic acid–Schiff technique and scanned at 510 nm; the third was loaded with PBG saturated with [$^3$H]progesterone and counted [4]. (A) fraction A; (B) fraction B.

from the Sephadex G-200 chromatography indicated different migration rates (fig.4). Protein and carbohydrate staining coincided with the binding activity in each fraction.

## 4. Discussion

In affinity chromatography it is advantageous to couple such ligands to the agarose that produce complexes of somewhat lower affinity than the displacing ligand which serves as eluent [14]. The binding affinity of 19-nortestosterone to PBG is lower than that of progesterone and 5α-pregnanedione [15]; a $K_a^{23°C}$ value of $0.9 \times 10^9$ $M^{-1}$ for 19-nortestosterone has been determined by fluorescence quenching titration*. A similar $K_a$ value was obtained for 11-desoxycorticosterone [9,16].

The elution profile in fig.1 illustrates the excellent resolution of PBG from the contaminants in peak I. Washing of the column with 1 M NaCl buffer before the steroid elution step yielded no appreciable protein peak thus demonstrating that nonspecific adsorption of proteins to the column is minimal. The fluorescence between peaks I and II (fig.1) does not return to baseline due to the continuous dissociation of PBG from the immobilized steroid. The relatively rapid dissociation of steroids from their PBG complex [17] is advantageous in affinity chromatography because the pure protein can be eluted by simply changing to a steroid-containing buffer without the incubation step needed with slower dissociating steroid-binding proteins [18,19]. Prepurification of a crude protein mixture before affinity chromatography has proven useful in protecting the affinity column [14]. In the present case the combination of the SP-Sephadex purification with affinity chromatography yields pure PBG in two simple steps.

Upon binding of progesterone, the fluorescence of the purified PBG (peak II) was quenched by more than 80%, a significantly greater quenching effect than the 60% seen with previous PBG preparations [9]. This increase in quenching is due to the elimination of the fluorescence originating from peak I which does not bind progesterone and therefore does not show the quenching effect.

* Unpublished results with A. T. Blanford.

As to be expected from a pure protein, fluorescence quenching titration and equilibrium dialysis consistently gave *n*-values of unity for the PBG–progesterone complex. The affinity constants do not differ significantly from those obtained previously. The mol. wt. of 88 000 is within the range of the polydisperse species described previously [4]. Gel electrophoresis confirmed the polydispersity: the two fractions (A and B in fig.4) had distinct mobilities indicating species of different size [4]. The coincidence of protein, carbohydrate and progesterone binding activity is in contrast to previous findings obtained with less pure material (fig.2 in ref. [6]). The different migration rates of fraction A and B (fig.4) show that the specific purification of PBG by affinity chromatography does not eliminate the polydisperse nature [4] of this glycoprotein. The carbohydrate content of over 70% is the same as found before and is again considerably higher than observed in other laboratories [4].

## Acknowledgements

This work was supported by a grant from the National Institute of Arthritis, Metabolism and Digestive Diseases (AM-06369) and a research career award (UW) from the Division of General Medical Sciences (GM-K6-14, 138) of the United States Public Health Service. The authors thank Mr George B. Harding for the equilibrium dialysis studies, Mrs Karen Acree for the hexosamine and fucose analyses, and Mr Benjamin Van Osdol for the ultracentrifugation experiment.

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