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# Conformational Changes in the Progesterone Binding Globulin–Progesterone Complex†

Stephen D. Stroupe and Ulrich Westphal*

ABSTRACT: An improved purification procedure for the progesterone-binding globulin (PBG) of the pregnant guinea pig has been developed utilizing sulfopropyl Sephadex, a strong cation exchanger, in the first step. The method exploits the low p*I* (2.8) and favorable acid stability of the glycoprotein. Subsequent chromatographies on DEAE-cellulose and Sephadex G-200 afford a highly purified PBG that exhibits the previously observed polydispersity (R. M. Burton et al. (1974), *Biochemistry 13,* 3554–3561). Circular dichroism, optical rotatory dispersion, and difference uv spectra all indicate the purified protein to undergo a conformational transition upon forming a complex with a steroid ligand. The CD and ORD spectra cannot be interpreted in terms of tertiary structure probably due to carbohydrate contributions. However, the difference spectra indicate strong perturbation of both a tryptophan residue and the steroid chromophore in the complex.

Progesterone-binding globulin (PBG)[1] isolated from the blood of pregnant guinea pigs is a glycoprotein of an unusually high carbohydrate content. It binds progestogens and androgens with high specificity whereas the affinity to corticoids is lower. The protein is found at relatively high levels during late pregnancy. The binding specificity and relative abundance make PBG well suitable for the study of high affinity steroid binders.

Several laboratories have reported the purification of PBG (Milgrom et al., 1973; Lea, 1973; Burton et al., 1974), but all reported methods are rather involved and none of them takes advantage of PBG's acid stability and extremely low isoelectric pH of 2.8 (Harding et al., 1974). The present communication reports on the use of a strong cation exchanger to separate the acidic PBG molecule from the bulk of plasma proteins in one step. A preliminary account of this method has been given (Stroupe and Westphal, 1974).

Many reports on the interaction of proteins with steroids have emphasized the influence of temperature, pH, ionic strength, specific salts, and steroid structure on the affinity of binding (Westphal, 1971). There appear to be almost no observations of changes in conformation in high affinity binders upon forming steroid complexes. To explore the conformational aspects of the PBG-steroid complex, measurements of circular dichroism (CD), optical rotatory dispersion (ORD), and uv difference spectra were performed with the PBG purified by the improved method.

## Materials and Methods

Pooled pregnant guinea pig serum was obtained from Grand Island Biologicals. Sulfopropyl (SP) Sephadex C-50

† From the Department of Biochemistry, University of Louisville School of Medicine, Health Sciences Center, Louisville, Kentucky 40201. *Received February 24, 1975.* This is communication No. 32 in the series Steroid-Protein Interactions; for the preceding paper see Stroupe et al. (1975). This work was supported by a grant from the National Institute of Arthritis, Metabolism, and Digestive Diseases (AM-06369) and a research career award (U.W.) from the Division of General Medical Sciences (GM-K6-14, 138) of the U.S. Public Health Service.

[1] Abbreviations used are: PBG, progesterone-binding globulin; CBG, corticosteroid-binding globulin; pregnenolone, 5-pregnen-3$\beta$-ol-20-one; SP-Sephadex, sulfopropyl Sephadex; PAS, periodic acid–Schiff base.

and Sephadex G-200 were purchased from Pharmacia Fine Chemicals; DEAE-cellulose was from Matheson Coleman and Bell. Progesterone was a commercial product and was recrystallized several times (Westphal, 1957) before use. Pregnenolone was from Mann Laboratories. All other chemicals were reagent grade, and water was glass redistilled and deionized.

*Chromatography.* The serum was prepared for chromatography by equilibrating with 20 m*M* sodium acetate containing 0.02% azide at pH 7.0–7.4. All operations were done at 4°. Radiolabeled steroids, if used, were added to the serum before dialysis. The dialyzed serum was titrated to pH 4.50 ± 0.02 with 50% acetic acid and the precipitate removed by centrifugation at 0–4°. The serum was then applied to a SP-Sephadex column equilibrated with 20 m*M* sodium acetate containing 0.02% azide at pH 4.50. For large preparations (50–100 ml of serum) a 4.5 × 40 cm bed of the resin was used. Empirically it was found that a ratio of bed volume to serum volume of at least 6:1 was necessary for optimal separation of PBG. Utilizing the 4.5 × 40 cm bed the serum was loaded at ~100 ml/hr and eluted with the pH 4.50 buffer at 150–200 ml/hr.

Following SP chromatography, the PBG was pooled, neutralized, and loaded directly to a DEAE-cellulose column at pH 7.4 in 10 m*M* Tris-HCl buffer. PBG was eluted with a linear NaCl gradient in the same buffer. The fractions containing PBG were pooled, concentrated with an Amicon Diaflo cell using a PM 30 membrane, and chromatographed on a Sephadex G-200 column. The PBG fractions were pooled or subfractionated as described (Burton et al., 1974) and stored frozen. Freeze-drying was avoided; the Amicon apparatus was used to concentrate dilute solutions when desired.

*Gel electrophoresis* was performed by a modification of the method of Ornstein (1964) and Davis (1964). The stacking gel was omitted and the sample was mixed with glycerol before applying to the gel. Coomassie Blue was used to stain for peptide, and a PAS stain (Zacharius et al., 1969) was used for carbohydrate; quantitation was performed on a Gilford 240 gel scanner.

*Spectra.* CD and ORD spectra were obtained at 27° with a Cary 60 spectropolarimeter equipped with a 6001 CD attachment. A single solution was used for all spectra in cells of 5.0-, 1.0-, and 0.1-cm path length. The solution was 8.6 × $10^{-7}$ *M* in progesterone binding sites, determined by fluorescence titration (Stroupe et al., 1975). Complex was formed by adding a concentrated alcoholic progesterone solution to the PBG solution (1 mol/mol of binding site); dilution was about 0.12% and was neglected. At the concentration used (8.6 × $10^{-7}$ *M*) progesterone yielded no observable rotation; therefore correction for progesterone was unnecessary in the spectra of the complex. The protein stock solution was 0.25 mg/ml based on dry weight; however, PBG is a glycoprotein and to compute the mean residue ellipticity $[\theta] = \theta_{obsd}$ MRW/10*dc* the sample was assumed to be 25% peptide (Burton et al., 1974). The mean residue weight (MRW) was taken as 115; the path length *d* was in cm and *c* was the peptide concentration of 6.3 × $10^{-5}$ g/ml.

Difference uv spectra were obtained manually with a Zeiss PMQ II spectrophotometer. Readings were taken every 0.5 nm in regions of large absorbance changes and 1.0 nm elsewhere. Tandem Yankeelov (1963) cuvets were used to obtain the difference spectrum between the progesterone complex and free PBG. Since pregnenolone is transparent to ca. 220 nm, no correction for steroid absorption was necessary and regular 1-cm cuvets were used for obtaining the difference spectrum between PBG and its pregnenolone complex. The spectral titration with progesterone utilized the Yankeelov cuvets. Small aliquots of progesterone were added to the PBG solution in the sample cell and to the buffer compartment in the reference cell with an equal volume of solvent added to the reference protein compartment. Distortion of the difference spectra due to stray light is thought to be minimal because the prism monochromator of the PMQ II spectrophotometer transmits very little stray light, and difference titrations as in Figure 4 are linear, indicating the validity of Beer's law under the conditions applied.

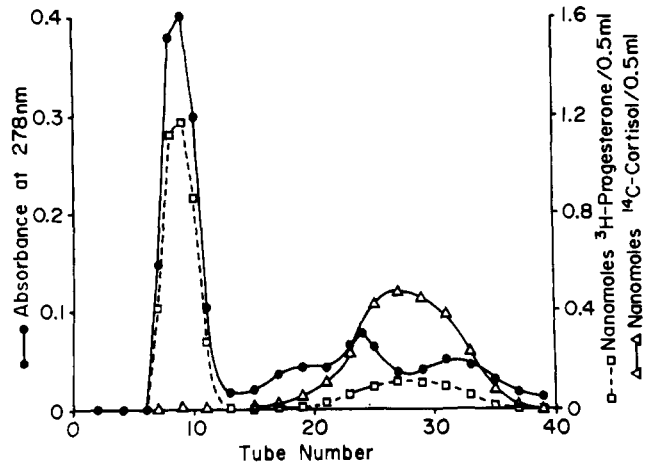


FIGURE 1: SP-Sephadex chromatography of pregnant guinea pig serum; 10 ml of serum was loaded with 179 ng of [$^3$H]progesterone and 206 ng of [$^{14}$C]cortisol corresponding to 5.7 × $10^{-8}$ *M* in each. Column dimensions were 2.2 × 30 cm and 5-ml fractions were collected.

*PBG binding activity* in chromatographic elution patterns was determined by counting radiolabeled progesterone tracer or by a fluorescence quenching method (Stroupe et al., 1975). In the quenching procedure, aliquots of the fractions to be analyzed were diluted to approximately the same $OD_{280}$ (typically 0.01–0.02). The fluorescence of 1.0 ml of the diluted solution was read before and after addition of 10 μl of $10^{-3}$ *M* progesterone.

*Steroid solutions* were prepared by weight, and the concentrations verified by uv absorption when applicable.

## Results

*Purification of PBG.* An SP-Sephadex chromatography of doubly labeled pregnant guinea pig serum is shown in Figure 1. The concentration of high affinity binding sites in pregnant guinea pig serum is 1.2 × $10^{-5}$ *M* for progesterone (Burton et al., 1974), and approximately 2 × $10^{-6}$ *M* for cortisol (Diamond et al., 1969). The progesterone binding activity is eluted in the void volume of the column with all cortisol label appearing in the internal volume of the column. Equilibrium dialysis indicated there was no cortisol binding activity associated with the cortisol peak, presumably due to acid inactivation (Seal and Doe, 1962). Inactive CBG was not found in subsequent analyses of the PBG peak, and is assumed to remain on the column together with about 98% of the serum proteins.

Use of SP-Sephadex with 100 ml of serum gave a broad peak of PBG beginning at the breakthrough volume. The active fractions were pooled and further purified on DEAE-cellulose. A single skewed peak was obtained. Progesterone-

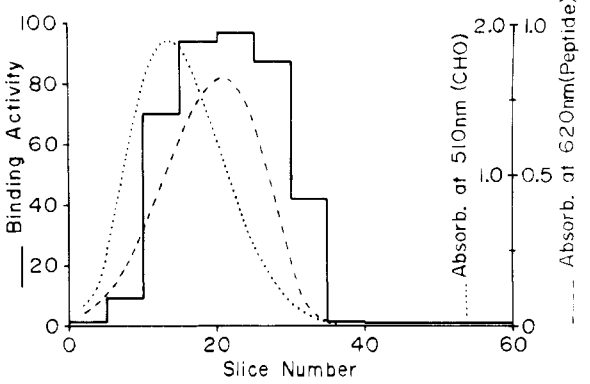


FIGURE 2: Progesterone binding activity in relation to protein and carbohydrate staining in polyacrylamide gel electrophoresis of PBG, applying 350 μg/gel. Coomassie Blue (- - -) was read at 620 nm, and PAS (• • •) at 510 nm. Binding activity (—) was determined by multiple equilibrium dialysis (Westphal, 1969) of 12 groups of 5 slices (1.4 mm) in 50 m*M* phosphate buffer (pH 7.4) and is reported as cpm inside/cpm outside. Anodic migration from left to right.

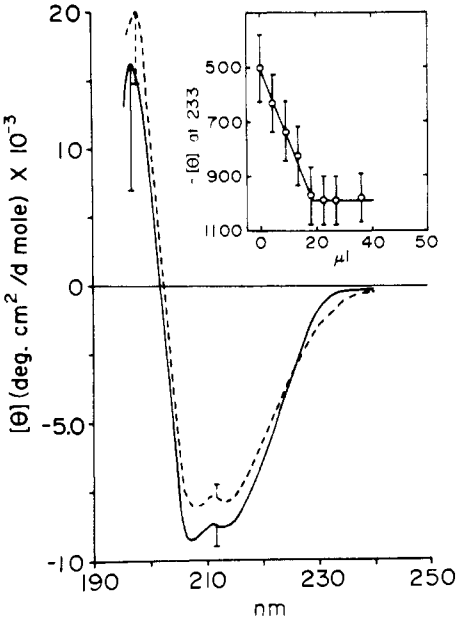


FIGURE 3: CD spectra of PBG (—) and PBG–progesterone complex (- - -). Path lengths 5.0 cm, 240–230 nm; 1.0 cm, 230–210 nm; 0.1 cm, 210–196 nm. Base lines were obtained using the Tris-Cl (10 m*M*)–NaCl (0.1 *M*) (pH 7.40) buffer. Progesterone binding site concentration for both spectra, $8.6 \times 10^{-7}$ *M*. Note change of scale of ordinate. Inset: CD titration of PBG by progesterone. Aliquots of a $7.30 \times 10^{-4}$ *M* progesterone solution in ethanol were added to 15.0 ml of PBG solution in a 5.0-cm cell. Each error bar corresponds to the sum of the base line and the scan noise. At the equivalence point (18.8 μl), 1.08 mol of progesterone has been added per binding site.

induced fluorescence quenching indicated the PBG to be concentrated in the leading edge of the peak; all fractions with more than 60% quench were pooled.

Sephadex G-200 chromatography of the pooled and concentrated peak from the DEAE-cellulose gave a single broad peak. Fractions with quenching above 65% were taken as purified PBG. The glycoprotein thus prepared can be further subfractionated as described previously (Burton et al., 1974), but this was not done for the studies in the present paper. The PBG thus obtained is free from contaminants by the criterion of disc gel electrophoresis at loads up to 400 μg. However, as seen in Figure 2 the material is polydisperse. Carbohydrate and peptide stains do not coincide; the binding activity is associated with the faster migrating peptide-rich fractions.

Overall yield from 100 ml of serum was 102 mg of PBG corresponding to $5.7 \times 10^{-7}$ mol of progesterone binding sites. Since the initial binding site concentration was $1.2 \times 10^{-5}$ *M*, the yield was 48%.

*Spectral Studies.* Conformational differences between free and liganded PBG were detected by difference uv, CD, and ORD spectroscopies. Figure 3 gives the CD spectra of PBG and its progesterone complex. The absolute value of the ellipticity in the aromatic amino acid region (240–300 nm) was less than 150 deg cm²/dmol, close to the limit of detection under the conditions employed; that region, therefore, is not shown. The PBG–progesterone complex has a more negative ellipticity from 240 to 227 nm where it becomes more positive. Both samples have minima at 213 and 207 nm, and exhibit zero ellipticity at 203 nm with maxima at 198 nm for the complex and 197 nm for PBG.

To demonstrate that the CD difference between PBG and liganded PBG is indeed due to complex formation, the titration of PBG with progesterone was monitored by the ellipticity at 233 nm where the percent difference is maximal. The CD titration in Figure 3, inset, gave a progesterone binding site concentration of $9.3 \times 10^{-7}$ *M*, in agreement with the value of $8.6 \times 10^{-7}$ *M* obtained by fluorescence quenching measurements. The CD signal changes linearly with the addition of progesterone until the binding sites are saturated.

The ORD spectra of PBG and its progesterone complex were recorded from 350 to 210 nm (figure not given). Both samples gave a minimum at 224 nm with a crossover to positive rotation at 210 nm. The mean residue rotation at 224 nm was −5400 ± 300 deg cm²/dmol for PBG and −4500 ± 300 deg cm²/dmol for the progesterone complex. The rotation of PBG was more negative than that of the complex over the entire range scanned.

Figure 4 gives the uv difference spectra of PBG against its progesterone and pregnenolone complexes. Both steroids induce a large positive signal with $\Delta\epsilon = 2500\ M^{-1}\ cm^{-1}$ at 294–295 nm. The pregnenolone complex has three smaller peaks at 289, 283, and 275 nm. The progesterone complex exhibits the same three peaks, but they are superimposed on a broad negative signal at 268–270 nm with $\Delta\epsilon = -4000\ M^{-1}\ cm^{-1}$. Both steroid complexes exhibit a large positive signal at shorter wavelengths, with the progesterone complex having $\Delta\epsilon = 19{,}000\ M^{-1}\ cm^{-1}$ at 233 nm, and the pregnenolone complex $\Delta\epsilon = 12{,}000\ M^{-1}\ cm^{-1}$ at 234 nm.

The signals at 294–295, 270, and 233–234 nm can all be used to quantitate the concentration of binding sites. Figure 4, inset, gives a titration of PBG with progesterone following the short wavelength signal; a binding site concentration of $1.03 \times 10^{-5}$ *M* was obtained. Titrations with both pregnenolone and progesterone following the signals at the different wavelengths all gave similar plots. The signal changes were linear with respect to added steroid until the binding sites were saturated; addition of steroid beyond saturation gave no further signal change. Therefore, the difference spectra given by the progesterone and pregnenolone complexes are the result of steroid binding to a specific site and not of a nonspecific solvent perturbation.

## Discussion

Utilization of SP-Sephadex as the first step in PBG purification has a significant advantage over hydroxylapatite (Burton et al., 1974) since the cation exchanger offers more

reproducible flow characteristics and higher retention of unwanted serum proteins. Avoiding lyophilization eliminates the aggregate peaks seen in Figure 2A and B of Burton et al. (1974). Relatively large quantities of PBG can be readily prepared by the improved method: 100 ml of serum yields approximately 100 mg of purified PBG within a week. Recovery of progesterone binding sites is typically 50%. A single SP-Sephadex chromatography gives a PBG preparation free of albumin and CBG which is suitable for many purposes not requiring complete purity. The physical properties of the purified material are similar to those reported previously from this laboratory (Burton et al., 1974). The purified PBG is polydisperse, as seen in Figure 2, in accordance with the previously reported results indicating varying amounts of carbohydrate attached to the same peptide core (Burton et al., 1974; Stroupe and Westphal, 1974).

There have been several previous observations on the induction of conformational changes in proteins upon steroid binding. Alfsen (1963) found bovine serum albumin to become less levorotatory and more acidic upon binding testosterone. Both human serum albumin and bovine serum albumin develop a difference spectrum in the aromatic absorption region when complexed with different steroids (Ryan, 1968; Ryan and Gibbs, 1970). Apparently there are only two reports concerning high affinity serum binders. Rat CBG polymerizes when its ligand is removed (Chader and Westphal, 1968), and addition of steroid to the polymerized protein reverses the aggregation. This offers indirect evidence that the conformation of rat CBG is ligand dependent. Similar observations have been made with rabbit CBG (Chader et al., 1972). In both of these cases, the aggregation is accompanied by reversible loss of steroid binding affinity.

No reports on CD spectra of high affinity steroid-binding proteins have appeared in the literature. The spectra given in Figure 3 are remarkable primarily for their low ellipticity and unusual double minima at 207 and 213 nm. Attempts to apply different methods of interpreting the spectrum of PBG in terms of $\alpha$ helix, $\beta$-pleated sheet, and random coil segments (Greenfield and Fasman, 1969; Chen et al., 1974) were unsuccessful. The reason may be the contribution to the observed ellipticity of the amide chromophores (Kabat et al., 1969) present in the *N*-acetylhexosamine and sialic acid residues of PBG. Similarly, the ORD spectrum of PBG cannot be interpreted in terms of peptide conformation.

Upon binding progesterone, the CD spectrum of PBG becomes more negative over the range 240–228 nm and more positive below 227 nm. Assuming that the steroid binding affinity resides in the peptide portion of the glycoprotein (see Figure 2), the steroid ligand should not influence the CD signal from the carbohydrate residues. The signal change must therefore reflect a conformational transition in the peptide portion of PBG.

The uv difference spectra given in Figure 4 confirm that PBG and its steroid complexes have different conformations. Both progesterone and pregnenolone induce a large positive signal at 294–295 nm with smaller peaks at 288 and 283 nm. The longest wavelength signal must be due to the perturbation of a tryptophan residue since tyrosine absorbs very little at this wavelength. The smaller peaks at 288 and 283 nm can be assigned to tyrosine and tryptophan, respectively (Herskovits, 1967). The peak at 294 nm and its relationship to the 283-nm peak suggest that the tryptophan difference spectrum might originate from a charge perturbation rather than the more familiar solvent perturbation as indicated by the ratio $\Delta\epsilon_{283}/\Delta\epsilon_{294}$ of 0.26 for the pregnenolone spectrum (Andrews and Forster, 1972). The progesterone spectrum is complicated by the large trough at 268–270 nm. However, both spectra are consistent with the removal of a positive charge from the immediate vicinity of a tryptophan residue upon binding a steroid.

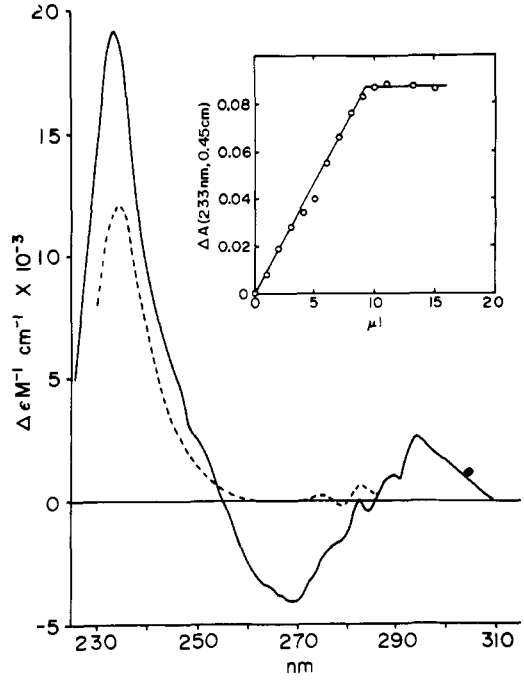


FIGURE 4: Uv difference spectra of PBG complexes with progesterone (—) and pregnenolone (- - -). Spectra were recorded as described under Materials and Methods. Between 285 and 310 nm the difference spectrum of the pregnenolone complex is identical with that of the progesterone complex. Inset: Spectral titration of PBG with progesterone at 233 nm. Aliquots of $1.12 \times 10^{-3}$ *M* progesterone solution in ethanol were added to 1.0 ml of PBG yielding a binding site concentration of $1.03 \times 10^{-5}$ *M*. At the equivalence point (9.2 $\mu$l), 1.01 mol of progesterone has been added per binding site.

The large negative signal at 268–270 nm present in the progesterone PBG spectrum and absent in the spectrum of the pregnenolone complex can be assigned to a solvent perturbation of the $\Delta^4$-3-keto chromophore in progesterone. This negative signal is the basis of a spectral method for measuring protein–steroid interactions (Westphal, 1957). A similar difference spectrum was reported (Ryan, 1968; Ryan and Gibbs, 1970) for albumin–$\Delta^4$-3-keto steroid complexes; however, these spectra have their minima at the shorter wavelength of 259–260 nm. The simplest interpretation of the minimum signal at 268–270 nm is a solvent-induced shift of the progesterone $\lambda_{max}$ when bound to PBG. Ryan and Gibbs (1970) concluded that testosterone bound to human serum albumin was in an alcohol-like environment. In order to induce a $\Delta\epsilon$ of $-4000$ $M^{-1}$ cm$^{-1}$ at 270 nm, progesterone would have to be in an environment sufficiently apolar to shift its $\lambda_{max}$ to about 238–240 nm when bound to PBG.

The positive peak at 233–234 nm in both difference spectra (Figure 4) is probably due to perturbation of the short wavelength transitions of aromatic amino acids (Herskovits, 1967). In addition to the positive contribution by the protein, the progesterone difference spectrum shows a contribution from the perturbed $\Delta^4$-3-keto group. This progesterone signal accounts almost entirely for the difference between the progesterone and pregnenolone spectra at the

short wavelength peak. If the difference spectrum of the pregnenolone complex is mathematically substracted from that of the progesterone complex, the remaining spectrum is a perturbed steroid difference spectrum similar to Figure 1 of Ryan (1968). This result indicates the conformational change in PBG upon binding progesterone and pregnenolone is the same.

The previously reported pregnenolone difference spectrum gave a $\Delta\epsilon_{232}$ of approximately 9000 $M^{-1}$ $cm^{-1}$ (Stroupe et al., 1975). The value of $\Delta\epsilon_{234}$ = 12,000 $M^{-1}$ $cm^{-1}$ is considered more accurate because the absorbance difference was approximately eight times greater in the current report as a result of using a higher PBG concentration.

The data of this report and the previous results of fluorescence studies (Stroupe et al., 1975) allow the proposal of a binding scheme. Fluorescence quenching measurements indicate that there is a tryptophan residue very close to the bound steroid in the PBG–progesterone complex. This conclusion is strengthened by the difference spectra in Figure 4 which indicate strong perturbation of both a tryptophan residue and the steroid chromophore. On the basis of our results we may conclude that the steroid as it associates with the protein enters a more hydrophobic environment resulting in perturbation of its absorption spectrum. In the binding site the steroid interacts with the tryptophan residue as seen by the quenching of the PBG fluorescence. Complex formation results in a conformational change of PBG accompanied by perturbation of a tryptophan residue.

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