# Equilenin: a specific fluorescent probe for steroid–protein interactions in sex steroid-binding protein

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Equilenin, a naturally fluorescent steroid, has high binding affinity for human sex steroid-binding protein (SBP). At 4°C the equilibrium association constant is $\sim 6 \times 10^7 M^{-1}$. The fluorescence excitation and emission spectra of the steroid–protein complex indicate that both hydrophobic interactions and hydrogen bonding of the 3′-hydroxyl group of the estrogen are important in its binding to the protein. Equilenin has a substantially different 3-dimensional spatial configuration compared with the normally bound androgens, and yet exhibits very tight binding to SBP. This suggests that SBP undergoes a conformational change to accomodate equilenin.

*Equilenin* *Sex steroid-binding protein* *Fluorescence* *Steroid–protein interaction*

## 1. INTRODUCTION

The sex steroid-binding protein (SBP) specifically binds sex-steroids in the plasma. The human and non-human primate proteins have greatest affinity for 5α-dihydrotestosterone and testosterone, but also bind, with less affinity 17β-estradiol [1–3]. The rabbit protein does not bind 17β-estradiol significantly [1]. Methods for obtaining pure SBP have been difficult to establish, but early efforts yielded enough material to begin physico-chemical characterization [4–7]. Improvements in the design of a steroid–agarose adsorbent have greatly increased the yield of pure protein, and it is now possible to isolate 10–15 mg/preparation, thereby greatly accelerating physico-chemical characterization [8]. The research developments on SBP and on other extra-cellular androgen-binding proteins have been reviewed [9]. The information on SBP has been updated with new findings about the molecular organization of SBP and the role of the protein in plasma [10].

We are interested in developing fluorescent probes for examining the nature of the steroid–protein interaction in pure SBP. One possible way is by covalently linking dyes to dihydrotestosterone analogues. Another way is to utilize fluorescent steroids which exhibit high affinity. Since the ground and excited states of aromatic molecules are very sensitive to the local environment, fluorescence studies have the potential of providing considerable information about the nature of the steroid binding site in SBP.

The competition of a variety of steroids with [$^3$H]testosterone for binding to partially-purified preparations of human SBP was compared in [11]. Equilenin, an estrogen isolated from horse urine, was among these steroids. This steroid was reported to have 33% binding to human SBP relative to testosterone, suggesting a binding constant somewhat $> 10^7 M^{-1}$.

Equilenin is of special interest because rings A and B are aromatic, containing a 3′-hydroxyl group. The aromatic structure is that of 2-naph-

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thol, a strongly fluorescent molecule with well-characterized excited state properties. 2-Naphthol undergoes a two-state exicted-state reaction, namely loss of the hydroxylic proton, which is pH-dependent and affected by various proton acceptors [12]. Furthermore, the absorption spectrum of equilenin permits excitation of the steroid independently of the protein tryptophan residues. These spectral properties make equilenin useful as a fluorescent binding site probe for SBP.

Here, we have determined the equilibrium association constant of equilenin to human SBP, and report the steady state fluorescence excitation and emission spectra of equilenin in the SBP complex. This should establish the steroid as a fluorescent probe to study the steroid–protein interaction.

## 2. EXPERIMENTAL

### 2.1. *Chemicals*

Equilenin was purchased from Sigma. All other chemicals were reagent grade and used without further purification. Other materials used were as in [5,6,8].

### 2.2. *Methods*

Human SBP, from fresh pregnancy serum, was isolated as in [8]. The pure protein was stored frozen at −20°C in a 10 mM Tris buffer (pH 7.4), containing 0.1 M NaCl, 5 mM $CaCl_2$, 20 $\mu$M dihydrotestosterone (DHT) and 10% glycerol (v/v).

The binding affinity of equilenin to SBP was determined by a filter assay [13]. This method measures the competitive binding between [$^3$H]DHT and non-radiolabeled steroid.

The equilenin complex with purified SBP was prepared by dialysis against the Tris buffer described above, except that 2 $\mu$M equilenin replaced DHT and 10% sucrose (w/v) replaced glycerol. No steroid was used in the final three changes of buffer. In this way most of the weakly bound and free steroid was removed.

Uncorrected fluorescence spectra of the SBP–equilenin complex were carried out using a Perking Elmer MPF 44A spectrofluorometer equipped with a thermostated cuvette holder. The typical excitation and emission bandpass was 5 nm. Conditions for the spectral measurements are given in the figures.

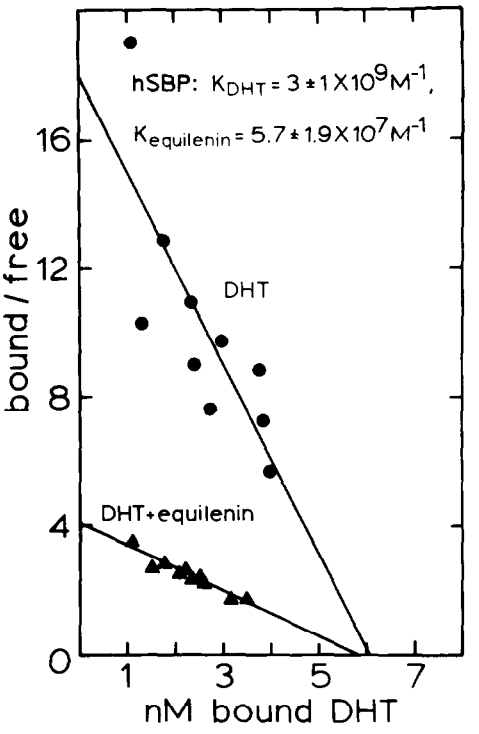


Fig. 1. Determination of the equilibrium association constants of DHT and equilenin binding to SBP in human serum at 4°C, as in [12]. The association constant of equilenin ($K_i$) was calculated from the apparent association constant ($K_p$) of DHT in the presence of $5.8 \times 10^{-8}$ M equilenin (▲) and the association constant ($K_a$) of DHT alone (●), as explained in the text.

## 3. RESULTS

### 3.1. *Equilenin binding to SBP in human serum*

Fig. 1 shows a competitive Scatchard plot from which the equilibrium association, at 4°C, of [$^3$H]DHT to SBP is obtained. The equilibrium association constant ($K_i$) for equilenin, the competing steroid, is calculated from the relationship:

$$K_a = K_p\,(1 + K_i[S])$$

where:

$K_a$ = the association constant for DHT;
$K_p$ = the apparent association constant reduced by a given concentration of the competing steroid, S.

Fitting the data by linear least squares yields a $K_a$ value of $3 \pm 1 \times 10^9$ $M^{-1}$ for DHT, in good

agreement with the reported value of $2.8 \pm 0.6 \times 10^9\ M^{-1}$ [13]. In the presence of $5.8 \times 10^{-8}$ M equilenin, a $K_p$ value of $7.04 \pm 0.04 \times 10^8\ M^{-1}$ is obtained. Accordingly, the calculated binding constant for equilenin ($K_i$) is $5.7 \pm 1.9 \times 10^7 M^{-1}$, about a factor of 50 than for DHT.

### 3.2. *Equilenin complex with pure human SBP*

Formation of the equilenin–SBP complex was carried out by dialysis. Inside the dialysis bag SBP was $5 \times 10^{-6}$ M, determined by the 280 nm extinction coefficient of $\sim 90000\ cm^{-1}.\ M^{-1}$ [1]. After further dialysis in the absence of steroid, the 280 nm absorbance of the protein solution was slightly higher than before dialysis in the presence of equilenin, indicating essentially no loss of SBP and that, in addition, equilenin was apparently bound. The protein solution had a new absorbance peak at 340 nm, not present prior to dialysis. The precise ratio of equilenin to SBP was difficult to determine since the absorbance of the 340 nm peak was small (~0.01). However, using the 340 nm extinction coefficient determined for equilenin in ethanol ($2500\ cm^{-1}.M^{-1}$), we estimate that 1 mol equilenin/mol SBP is bound, as is the case with DHT binding [13]. Free equilenin in the dialysate was not detectable by absorbance measurement. However, a weak equilenin fluorescence, <0.5% that of the sample, was observed.

### 3.3. *Fluorescence emission and excitation spectra*

The fluorescence emission spectra of free equilenin and that bound to SBP are compared in fig. 2. Excitation was at 330 nm to avoid contribution from tryptophan; SBP has a tryptophan absorbance with a red 'tail' extending to ~310–315 nm. The 280 nm excited tryptophan emission spectrum of the protein, prior to dialysis with equilenin, is also in fig. 2.

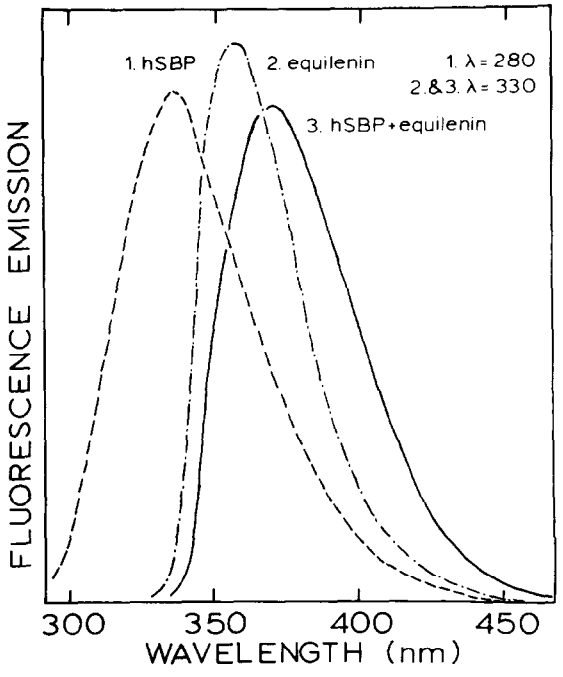


Fig. 2. Comparison of fluorescence emission spectra of equilenin bound to SBP and free in buffer, excited at 330 nm. The emission spectrum of SBP saturated with DHT, excited at 280 nm, shows the spectral distribution of the tryptophan fluorescence. All of the data were obtained at 20°C, as described in the text.

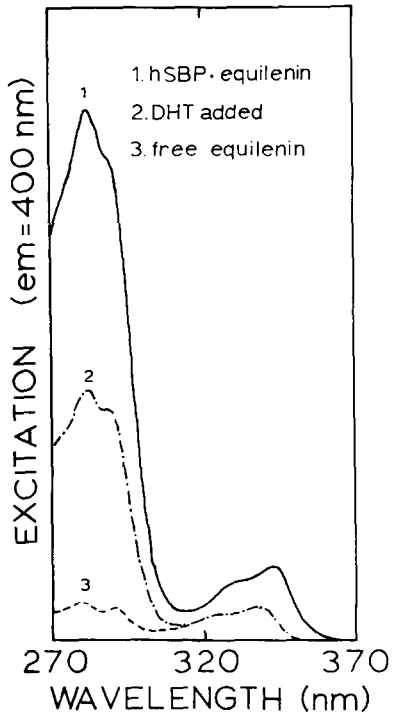


Fig. 3. Comparison of the excitation spectra, monitored at 400 nm, of equilenin bound to SBP, equilenin free in solution, and the equilenin–SBP complex after addition of saturating DHT (20 $\mu$M). The experimental conditions are as in fig. 2.

The fluorescence excitation spectrum of the complex, shown in fig. 3, is essentially due to the absorption of equilenin and of tryptophan in SBP. Addition of DHT to the equilenin–SBP complex dramatically alters the fluorescence excitation spectrum. Although tryptophan excitation, monitored at 400 nm, is still evident after addition of equilenin, the intensity of the entire excitation spectrum decreases. The spectral envelope above 300 nm is blue-shifted and superimposes on the free equilenin spectrum. Moreover, the fluorescence emission excited at 330 nm is identical with that of free equilenin. These results show that DHT effectively displaces equilenin, and that the remaining contribution of tryptophan below 300 nm is largely due to the SBP–DHT complex.

## 4. DISCUSSION

These results show that equilenin can serve as a potentially useful probe for studying the steroid –protein interaction. The equilibrium association constant of $\sim 6 \times 10^7\ M^{-1}$ is sufficiently strong to displace the natural steroid, DHT, from the binding site. The value for the association constant obtained by the competitive filter assay [13] agrees well with the binding measured at one concentration of the inhibitor [11]. Such a strong interaction between equilenin and SBP was somewhat unexpected because of the significant difference in the 3-dimensional spatial structure between equilenin and DHT. This means that aromatic residues may be important in the binding interaction by providing planar hydrophobic surfaces which can induce conformational changes in the structure of the protein. The equilenin absorption at 340 nm has good overlap with the tryptophan emission at 337 nm, ideal for singlet–singlet energy-transfer measurements which can provide insight into the role of tryptophan and tryrosine residues near or in the steroid binding site.

The direction of the fluorescence wavelength shifts and the intensity changes of bound *vs* free equilenin reveals significant information about the SBP steroid binding site. The excitation spectrum above 300 nm of the bound species is similar to that of equilenin in neutral ethanol, and is red-shifted with respect to the spectrum in more polar, aqueous buffer. Since the high pH excitation spectrum of free equilenin is red-shifted due to ground state ionization of the 3′-hydroxyl group, the red-shift in the complex could be due either to a hydrophobic binding site or to hydrogen bonding of the A ring hydroxyl group with an amino acid residue. We will discuss both possibilities.

The fluorescence emission of planar aromatic molecules bound to many proteins is blue-shifted compared to the emission in water. The similarity of the bound fluorophore's spectra to its spectra in less polar solvents is generally ascribed to a hydrophobic environment in the binding site. From the excitation spectrum of the complex we have argued that the steroid binding site may be hydrophobic. However, the emission spectrum of the complex is red-shifted by $>10$ nm, suggestive of hydrogen bonding or excited state ionization. The emission spectrum of free equilenin becomes broadened and progressively red-shifted at pH $>8$ as the excited state equilibrium is shifted more towards the ionized form of the steroid. Equilenate is less fluorescent than equilenin and has its maximum near 420 nm. Since the emission of the equilenin–SBP complex is similar to the pH 9 spectrum of the free steroid, it appears that the steroid is hydrogen-bonded in its binding site, and that the fluorescence emission is due to a mixture of emitting species depending upon the degree of ionization in the excited state. But since the emission of the free steroid in basic ethanol is also red-shifted, this argument does not preclude the role of hydrophobic interactions in the steroid binding site. The overall spectral data indicate that both kinds of interactions are present and important for the unexpectedly tight binding of equilenin by human SBP. The differences in the 3-dimensional structure of equilenin compared with either DHT or testosterone suggests that the tight binding of the estrogen induces a conformational change in SBP.

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