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# Steroid-Binding Site of Human and Rabbit Sex Steroid Binding Protein of Plasma: Fluorescence Characterization with Equilenin†

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ABSTRACT: The interaction of the estrogen *d*-3-hydroxy-1,3,5(10),6,8-estrapentaen-17-one (equilenin) with the human and rabbit sex steroid binding proteins (hSBP and rSBP, respectively) has been investigated by using fluorescence and absorption spectroscopy. Equilenin competes for the binding of 5α-dihydrotestosterone. The calculated binding constant of equilenin for rSBP is $1.9 \times 10^7$ $M^{-1}$ at 4 °C, which can be compared with the binding constant of $5.7 \times 10^7$ $M^{-1}$ reported for hSBP [Ross, J. B. A., Torres, R., & Petra, P. H. (1982) *FEBS Lett. 149*, 240]. The results of fluorescence quenching experiments with the collisional quenchers KI and acrylamide indicate that the bound steroid has limited accessibility to the bulk solvent and that there are no anionic surface groups near the steroid-binding site. The fluorescence excitation spectra of SBP–equilenin complexes are similar to the absorption spectra of equilenin in low-dielectric solvents. The fluorescence emission of the SBP–equilenin complexes, however, exhibits wavelength shifts (red shifts) opposite to those of the steroid in low-dielectric solvents or complexed with β-cyclodextrin (blue shifts) but similar to the red shift produced by addition of the proton acceptor triethylamine to equilenin in cyclohexane. These data indicate that the steroid-binding site of hSBP and rSBP is a nonpolar cavity containing a proton acceptor that participates in a specific interaction, possibly a hydrogen bond, with the 3′-hydroxyl group of the bound steroid.

The sex steroid binding protein (SBP)[1] of vertebrate serum binds sex steroids with high affinity, especially 5α-dihydrotestosterone (DHT) and testosterone, with binding constants on the order of $10^9$ $M^{-1}$ at 4 °C (Petra, 1979). Functional and structural properties of SBPs from human, macaque, baboon, and rabbit have been reviewed and compared with each other in several recent papers (Petra et al., 1983, 1986a,b; Turner et al., 1984). There is suggestive evidence that SBP may be involved in the transport of sex steroids into target cells (Bordin & Petra, 1980). In addition, pure human or macaque SBPs have been shown to directly affect the metabolic clearance rate of testosterone in the macaque (Petra et al., 1985). The human (hSBP) and rabbit (rSBP) proteins also bind estrogens such as 17β-estradiol, estrone, and equilenin, but with less affinity than androgens (Mickelson & Petra, 1978; Lata et al., 1980; Ross et al., 1982). Compared with hSBP, rSBP generally exhibits a weaker affinity for estrogens, suggesting evolutionary differences in the steroid-binding site (Petra et al., 1983).

Equilibrium sedimentation studies show hSBP and rSBP to be dimers of about 85 000 and 86 000 daltons, respectively (Petra et al., 1986a). According to sequence analysis of the half-cystine peptides, hSBP is a homodimer (Petra et al., 1986b). In addition, metal-binding studies using the lanthanide terbium indicate that rSBP and hSBP each have four metal-binding sites (Ross et al., 1985). Steroid-binding studies indicate that 1 mol of steroid is bound per SBP dimer (Petra, 1979; Turner et al., 1984; Petra et al., 1986a). On the basis of steroid–SBP stoichiometry and preliminary sequence data, Petra et al. (1983) suggested the interface of the two identical SBP subunits as a candidate for the steroid-binding site.

The present investigation was first to determine if the steroid binds at the protein surface or is buried within the polypeptide structure and second to characterize the specific chemical interactions involved in the steroid binding. Relatively little is known about the detailed structure of the steroid-binding site of different SBPs or about the interactions that account for the specificity and high affinity of steroid binding. Previous

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[1] Abbreviations: SBP, sex steroid binding protein; hSBP, human sex steroid binding protein; rSBP, rabbit sex steroid binding protein; equilenin, *d*-3-hydroxy-1,3,5(10),6,8-estrapentaen-17-one; DHT, 5α-dihydrotestosterone; β-CD, β-cyclodextrin; TEA, triethylamine; PPO, 2,5-diphenyloxazole; HPLC, high-pressure liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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studies have indicated that the steroid-binding site is hydrophobic and nonionizing (Lata et al., 1980), with hydrogen bonds possibly involved in the binding process (Ross et al., 1982).

In the studies reported here, we have used equilenin, a naturally occurring fluorescent estrogen, as a probe to characterize the steroid–protein interaction and the steroid-binding sites of hSBP and rSBP. Equilenin has been shown to bind to hSBP with an association constant of $5.7 \times 10^7$ $M^{-1}$ at 4 °C (Ross et al., 1982). The steroid A ring, containing the 3′-hydroxyl group, and its adjacent B ring are fully aromatic. These fused rings resemble 2-naphthol, a well-characterized fluorescent molecule whose hydroxyl group becomes more acidic in the excited state. Consequently, the hydroxyl group undergoes excited-state dissociation, which is sensitive to the presence of proton donors and acceptors (Weller, 1961; Laws & Brand, 1979). Equilenin exhibits similar, related behavior as discussed by Davenport et al. (1986). Moreover, the absorption spectrum of equilenin permits its selective excitation independent of the tryptophan residues of SBP, thereby avoiding interference from tryptophan fluorescence.

The interaction of equilenin with SBP is also of interest for medical and biological reasons. This steroid, obtained from equine pregnancy urine, is a major component of *Premarin* (Ayerst), a mixed-steroid preparation used in estrogen replacement therapy in humans. Since hSBP has a high affinity for equilenin (Ross et al., 1982), SBP–equilenin interactions may prove to be important in mediating the physiological effects of equilenin and similar steroids in humans.

## Materials and Methods

*Chemicals.* Equilenin, 5α-dihydrotestosterone, and 2,5-diphenyloxazole were purchased from Sigma Chemical Co. 5α-[$^3$H]Dihydrotestosterone was purchased from New England Nuclear. HPLC-grade triethylamine was obtained from Fisher Scientific Co. Spectroscopic-grade cyclohexane was purchased from Kodak, and toluene was obtained from J. T. Baker Chemical Co. β-Cyclodextrin was obtained from Aldrich Chemical Co. and recrystallized twice from water. All other chemicals were reagent grade and used without further purification.

*Purification of SBP.* hSBP and rSBP were purified from human pregnancy serum and rabbit serum, respectively, as described earlier for hSBP (Petra & Lewis, 1980). Pure SBP was stored frozen at −20 °C in a Tris-HCl buffer [10 mM Tris, 0.1 M NaCl, 5 mM $CaCl_2$, and 10% (v/v) glycerol, pH 7.4] containing $2 \times 10^{-5}$ M DHT.

*Determination of the SBP–Equilenin Binding Constant.* The binding constant of equilenin to SBP was determined at 4 °C by a filter assay which measures the competitive binding in diluted serum between [$^3$H]DHT and the nonradiolabeled estrogen (Mickelson & Petra, 1974; Mickelson & Petra, 1978). The data were analyzed graphically (Figure 1) by the method of Scatchard (1949) using linear least squares. The association constant for the SBP–equilenin complex was calculated by using the relationship:

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

where $K_a$ and $K_i$ are the association constants for [$^3$H]DHT and equilenin, respectively, and $K_p$ is the apparent association constant of [$^3$H]DHT in the presence of a given concentration of equilenin, [S].

*Preparation of SBP–Equilenin Complexes.* SBP–equilenin complexes were prepared by dialysis at 4 °C as previously described (Ross et al., 1982), using the Tris-HCl buffer described previously except that 10% sucrose (w/v) replaced glycerol. The protein concentration in the dialysis bag was about $4 \times 10^{-6}$ M, determined by the 280-nm extinction coefficient of 114 000 $cm^{-1}$ $M^{-1}$ for hSBP and 127 000 $cm^{-1}$ $M^{-1}$ for rSBP (Petra et al., 1986a). The equilenin concentration was $5 \times 10^{-6}$ M. After exhaustive dialysis, the protein was further dialyzed in the absence of steroid to remove the free and nonspecifically bound equilenin. The intensity of equilenin fluorescence in the last dialysate was less than 1% of the fluorescence intensity of the complex, indicating negligible free equilenin. Formation of an SBP–equilenin complex was indicated by the red shift in the fluorescence emission spectrum of equilenin (Ross et al., 1982).

*Spectroscopy.* Single-wavelength absorption measurements were made on a Hitachi Perkin-Elmer 139 spectrophotometer, while absorption spectra were recorded with a Perkin-Elmer 557 double-beam spectrophotometer. Uncorrected fluorescence excitation and emission spectra were measured at 20 °C with an SLM 4800 spectrofluorometer. The excitation and emission band-passes were 8 nm, and the spectra were recorded under magic-angle conditions to avoid intensity artifacts due to rotation of macromolecules (Azumi & McGlynn, 1962; Kalantar, 1968; Paoletti & LePecq, 1969). Since the absorption of tryptophan is negligible at 330 nm (Wetlaufer, 1962), excitation at 330 nm was used to avoid interference from tryptophan fluorescence.

Fluorescence lifetimes were measured on the SLM by the phase method at a modulation frequency of 30 MHz (Spencer & Weber, 1969). Since the maximum solubility of equilenin in aqueous buffer is about $10^{-5}$ M, a steroid concentration of $5.0 \times 10^{-6}$ M was used. Although the fluorescence intensities were low under these conditions, phase lifetimes were obtained with good precision. Modulation lifetimes, however, were not reproducible; results for known lifetime standards indicated that higher emission intensities were required for accurate modulation lifetime values. Therefore, only the average phase lifetimes are reported. To minimize wavelength- and geometry-dependent photomultiplier effects, lifetimes were determined with reference to a single exponential decay standard. 2,5-Diphenyloxazole (PPO) was used as the standard since it can be excited at 330 nm, and its emission overlaps that of equilenin at neutral pH. PPO, in 95% ethanol, has a phase lifetime of 1.4 ns (Lakowicz et al., 1981).

*Fluorescence Quenching.* KI and acrylamide were used for fluorescence quenching experiments. The KI solutions contained $10^{-4}$ M $Na_2S_2O_3$ to inhibit formation of $I_3^-$ (Lehrer, 1971). The quenching data were plotted according to the Stern–Volmer equation (Stern & Volmer, 1919):

$$F_0/F = 1 + K_{SV}[Q] \quad (2)$$

where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of quencher, respectively, and [Q] is the concentration of quencher. The Stern–Volmer quenching constant, $K_{SV}$, is equal to the product of $k_q$, the bimolecular quenching constant, and $\tau_0$, the lifetime of the fluorophore in the absence of quencher.

## Results

*Binding of Equilenin to rSBP.* A Scatchard plot of the binding of [$^3$H]DHT alone and [$^3$H]DHT in the presence of equilenin to rSBP is shown in Figure 1. Within experimental error, the abscissa intercepts are the same. An association constant of $3.6 \times 10^9$ $M^{-1}$ at 4 °C was obtained for [$^3$H]DHT binding. This can be compared to the association constants of $1.2 \times 10^9$ and $1.7 \times 10^9$ $M^{-1}$ calculated from the data of Mickelson and Petra (1978) and Kotite and Musto (1982), respectively. The association constant of equilenin binding to

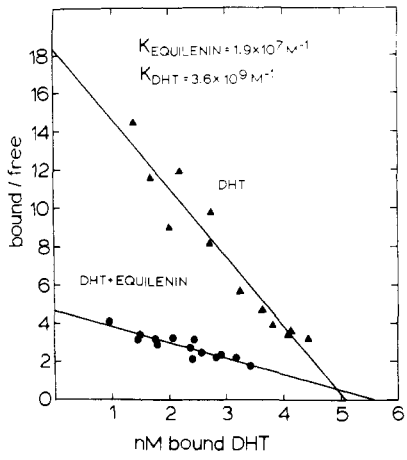


FIGURE 1: Determination of the association constants for the binding of [$^3$H]DHT and equilenin to rSBP at 4 °C by the competitive Scatchard method. The association constant of equilenin ($K_i$) was calculated from the apparent association constant ($K_p$) of [$^3$H]DHT in the presence of $1.7 \times 10^{-7}$ M equilenin (●) and the association constant ($K_a$) of [$^3$H]DHT alone (▲) as explained under Materials and Methods.

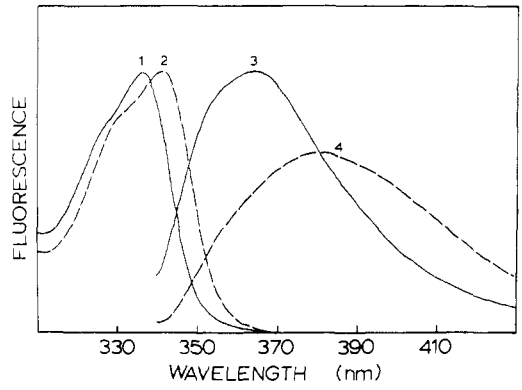


FIGURE 2: Fluorescence spectra of equilenin bound to rSBP (--) and equilenin liberated after addition of DHT to a final concentration of $2 \times 10^{-5}$ M to the same sample (—). Fluorescence emission spectra (3 and 4) give the relative emission intensities of free and bound equilenin, respectively. Excitation spectra (1 and 2), monitored at 385 nm, are peak-normalized. Emission spectra were recorded with excitation at 330 nm.

rSBP, calculated by using eq 1, is $1.9 \times 10^7\ M^{-1}$, which can be compared with the association constant of $5.7 \times 10^7\ M^{-1}$ for equilenin binding to hSBP (Ross et al., 1982).

*Fluorescence Spectra of SBP–Equilenin Complexes.* Figure 2 compares the uncorrected emission and peak-normalized excitation spectra of equilenin in neutral aqueous solution with those of equilenin bound to rSBP. The excitation maximum of free equilenin is at 336 nm and identical, within error, with the absorption maximum at 335 nm (Figure 3A). The excitation of the bound steroid is red-shifted about 5 nm. The fluorescence emission peak of free equilenin at neutral pH is near 363–364 nm, while that of equilenin bound to hSBP or rSBP is at 377 or 380 nm, respectively.

To measure the relative emission intensity of free and bound equilenin, DHT was added at a final concentration of $2 \times 10^{-5}$ M to solutions of hSBP and rSBP complexed with equilenin. Since DHT has about 2 orders of magnitude higher affinity for SBP, excess DHT should efficiently displace the bound equilenin. As shown in Figure 2, addition of DHT to rSBP–equilenin restores the emission and excitation spectra of free equilenin. Similar results are obtained for hSBP.

To estimate the stoichiometry of the SBP–equilenin complex, the concentration of equilenin liberated after addition

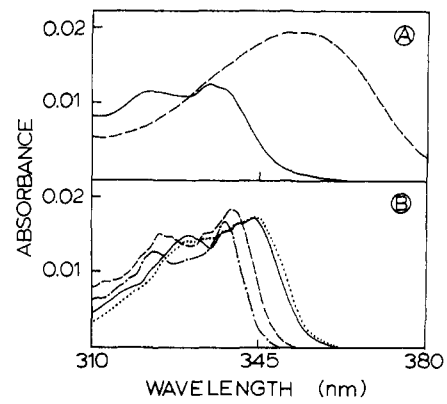


FIGURE 3: Absorption spectra of equilenin in different solvent systems. (A) In Tris-HCl buffer, pH 7.3 (—), and in 0.1 M NaOH, pH 13 (--). (B) In cyclohexane with (—) or without (-·-) 0.5 M TEA and in toluene with (···) or without (---) 0.5 M TEA. All spectra were recorded at room temperature. Equilenin concentration was $5.0 \times 10^{-6}$ M.

of DHT was compared with the concentration of SBP, assuming near-saturation of the steroid-binding site(s). The liberated equilenin concentration was determined by comparing its emission intensity with intensities of equilenin solutions of known concentrations. The SBP concentrations were calculated from the absorbances of solutions at 280 nm after correction for a small contribution from equilenin. The equilenin:SBP ratios were 1.1 and 0.8 for the human and rabbit complexes, respectively (single determinations). These values are in close agreement with the 1:1 stoichiometry observed for SBP–DHT complexes (Petra, 1979; Turner et al., 1984; Petra et al., 1986a).

*Spectroscopic Properties of Equilenin in Various Solvent Systems.* The absorption and emission of equilenin were examined in both aqueous and nonaqueous solvents to evaluate ground-state and excited-state interactions. The lowest energy ultraviolet (UV) absorption band of equilenin in water, at neutral pH (Figure 3A), has broad, but resolved, vibronic structure with a maximum at 335 nm. Near pH 13, where the 3′-hydroxyl group of the steroid is ionized, the lowest energy UV absorption band is shifted to 355 nm, increases in strength, and has no vibronic structure. The absorption spectra in cyclohexane (maximum at 338 nm) and toluene (maximum at 340 nm) are red-shifted and have better resolved vibronic bands than the spectrum in water at pH 7 (Figure 3B). When the proton acceptor triethylamine (TEA) is added (0.5 M final concentration) to equilenin in cyclohexane or in toluene, both absorption spectra shift to the red an additional 5 nm, and the vibronic bands are broadened.

The fluorescence emission spectra of equilenin in water at pH 7 and of ionized equilenin in 0.1 M NaOH are shown in Figure 4A. The fluorescence intensity and spectral bandwidth are constant between pH 3 and 7, with a maximum at 363–364 nm. At lower pH, there is a small amount of quenching as a result of the high proton concentration, similar to the fluorescence quenching of 2-naphthol below pH 2 (Laws & Brand, 1979). Ionized equilenin has a weaker, broader emission near 420 nm. In low dielectric constant solvents (Figure 4B), the emission spectrum of equilenin is blue-shifted relative to that in water and has resolved vibrational bands. For example, in cyclohexane there are two peaks at 347 and 360 nm. The blue shift is smaller in toluene. If TEA is added to equilenin in cyclohexane (or *n*-hexane), a red-shifted emission spectrum results with a single peak around 370 nm. With addition of TEA to equilenin in toluene, the red shift of the steroid emission is near 415 nm and closely resembles

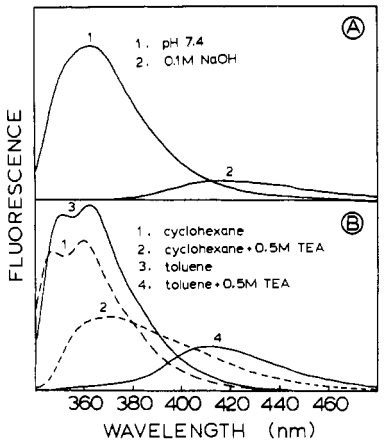


FIGURE 4: Fluorescence emission spectra of equilenin showing the relative emission intensities and peak positions in different solvent systems. (A) In Tris-HCl buffer, pH 7.4 (1), and in 0.1 M NaOH (2). (B) In cyclohexane with (2) or without (1) 0.5 M TEA and in toluene with (4) or without (3) 0.5 M TEA.

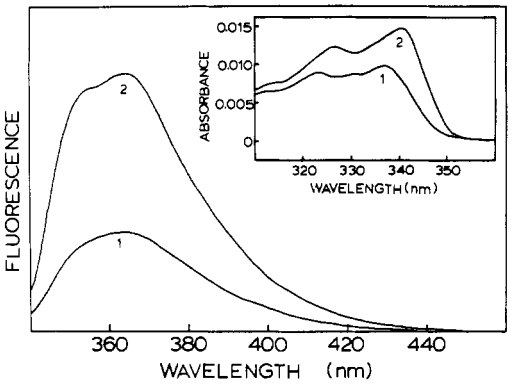


FIGURE 5: Fluorescence emission spectra showing the relative emission intensities of free equilenin (1) and equilenin in the presence of $8.8 \times 10^{-3}$ M β-CD (2) in Tris-HCl buffer, pH 7.4, at 20 °C. Excitation was at 330 nm. Inset: Absorption spectra of equilenin at room temperature in Tris-HCl buffer, pH 7.1 (1), and in the presence of $8.8 \times 10^{-3}$ M β-CD (2). Equilenin concentration was $5.0 \times 10^{-6}$ M.

that of ionized equilenin in high-pH aqueous solution (Figure 4B).

*Spectroscopic Properties of Equilenin Bound to β-Cyclodextrin.* As a comparison with SBP, we examined spectral characteristics of equilenin bound to β-cyclodextrin (β-CD). Cyclodextrins are doughnut-shaped cyclic polymers of glucose with hydrophobic cavities that enable them to form inclusion complexes with a wide range of compounds, including steroids and drugs (Saenger, 1980). β-CD contains seven D(+)-glucopyranose units attached by α-(1,4) linkages. The binding cavity of β-CD is about 8 Å high with an internal diameter of about 6.4 Å (Saenger, 1980).

In the presence of $8.8 \times 10^{-3}$ M β-CD, the emission band of equilenin is slightly blue-shifted compared to that in water, and there is more than a 2-fold increase in the intensity (Figure 5). In addition, between 300 and 350 nm, the absorption spectrum of the β-CD–equilenin complex (inset of Figure 5) is red-shifted about 4 nm compared with that of free equilenin, and the absorbance of the steroid increases. 2-Naphthol complexed with β-CD shows a similar red shift but a smaller increase in the absorbance (data not shown), in agreement with the results of Yorozu et al. (1982).

*Fluorescence Lifetimes of Equilenin in Different Environments.* The phase lifetimes of equilenin in various solvent systems are presented in Table I. In water between pH 1 and 7, there is no significant change in the fluorescence decay rate.

Table I: Fluorescence Phase Lifetimes of Equilenin in Different Solvent Systems[a,b]

| solvent | λ (nm) | $\tau_p$ (ns) |
|---|---|---|
| Tris-HCl, pH 7.3 | 350 | 6.0 ± 0.5 |
| | 365 | 5.8 ± 0.4 |
| | 380 | 5.9 ± 0.3 |
| 0.1 M HCl, pH 1.4 | 365 | 6.1 ± 0.2 |
| 0.1 M NaOH, pH 12.8 | 415 | 1.2 ± 0.3 |
| Tris-HCl + β-CD, pH 7.3[c] | 350 | 8.1 ± 0.8 |
| | 365 | 8.3 ± 1.1 |
| | 380 | 8.3 ± 0.7 |
| cyclohexane | 350 | 6.1 ± 0.4 |
| | 365 | 5.9 ± 0.3 |
| | 375 | 6.2 ± 0.5 |
| cyclohexane + TEA (0.05 M) | 350 | 3.3 ± 0.3 |
| | 365 | 3.5 ± 0.2 |
| | 375 | 3.5 ± 0.2 |
| cyclohexane + TEA (0.5 M) | 375 | 3.2 ± 0.2 |
| | 390 | 3.3 ± 0.3 |

[a] The lifetimes given were measured at 20 °C with modulation at 30 MHz and excitation at 330 nm. [b] Equilenin concentration was $5.0 \times 10^{-6}$ M. [c] β-CD concentration was $8.4 \times 10^{-3}$ M.

Table II: Fluorescence Phase Lifetimes of Equilenin Complexed with hSBP and rSBP[a]

| | $\tau_p$ (ns) | |
|---|---|---|
| λ (nm) | hSBP + equilenin | rSBP + equilenin |
| 355 | 5.2 ± 0.3 | 5.3 ± 0.6 |
| 360 | 5.2 ± 0.3 | 5.3 ± 0.3 |
| 380 | 5.2 ± 0.3 | 5.6 ± 0.4 |
| 390 | 5.3 ± 0.2 | 6.0 ± 0.3 |
| 400 | 5.4 ± 0.3 | 6.0 ± 0.3 |
| 410 | 5.6 ± 0.3 | 5.8 ± 0.4 |
| 420 | 5.5 ± 0.3 | 5.5 ± 0.6 |

[a] The lifetimes given were measured at 20 °C with modulation at 30 MHz and excitation at 330 nm.

The phase lifetimes also show no wavelength dependence between 350 and 380 nm. At pH 13, where the 3′-hydroxyl proton of equilenin is fully dissociated in the ground state, the phase lifetime is a factor of 5 shorter than that of the protonated form.

The lifetime of equilenin is not strongly affected by the dielectric constant of the solvent; the lifetimes in water and cyclohexane are essentially the same (Table I). The fluorescence lifetime of equilenin in cyclohexane decreases about a factor of 2 in the presence of TEA. There is no emission wavelength dependence of the lifetime, however, either in the presence or in the absence of TEA.

Equilenin bound to β-CD exhibits a 1.4-fold increase in the phase lifetime, compared with those of equilenin in water or cyclohexane (Table I); the lifetimes are independent of emission wavelength. The lifetimes reported for the SBP complexes (Table II) are slightly shorter than those of equilenin in water or cyclohexane. Within experimental error, the phase lifetimes of the SBP–equilenin complexes are emission wavelength independent.

*Fluorescence Quenching Experiments.* Experiments were performed with quenchers to obtain information about the electronic environment of SBP-bound equilenin and its accessibility to the solvent. Two types of quenchers were used: $I^-$, which is anionic, and acrylamide, which is polar but nonionic. The Stern–Volmer plots for the quenching of free equilenin and equilenin bound to rSBP are presented in Figure 6. The Stern–Volmer quenching constants, obtained from the slopes, were used along with the appropriate phase lifetimes to calculate bimolecular quenching constants. The results are given in Table III. Free equilenin is quenched by $I^-$ and by acrylamide with bimolecular quenching constants in the range

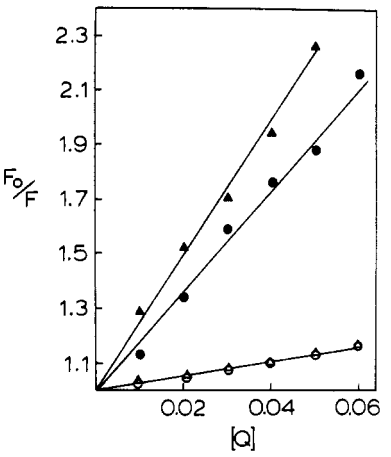


FIGURE 6: Stern–Volmer plots for the quenching of fluorescence of free (●, ▲) and rSBP-bound (○, △) equilenin by $I^-$ (○, ●) and acrylamide (△, ▲). Data were obtained at 20 °C in Tris-HCl buffer, pH 7.4. Excitation was at 330 nm, and the emission was monitored at 364 nm.

Table III: Fluorescence Quenching Data for Equilenin in Various Solvent Systems[a]

| sample | quencher | $K_{SV}$ ($M^{-1}$) | $k_q$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|
| buffer | $I^-$ | 18.0 | $3.0 \times 10^9$ |
| buffer | acrylamide | 25.0 | $4.2 \times 10^9$ |
| β-CD[b] | $I^-$ | 2.0 | $2.4 \times 10^8$ |
| β-CD | acrylamide | 1.8 | $2.2 \times 10^8$ |
| hSBP | $I^-$ | 2.9 | $5.5 \times 10^8$ |
| hSBP | acrylamide | 2.8 | $5.4 \times 10^8$ |
| rSBP | $I^-$ | 2.7 | $5.0 \times 10^8$ |
| rSBP | acrylamide | 2.9 | $5.2 \times 10^8$ |

[a] All measurements were at 20 °C in 0.01 M Tris-HCl buffer with 10% (w/v) sucrose, 1.0 M NaCl, and 0.05 M $CaCl_2$. Excitation was at 330 nm, and the emission was monitored at 364 nm. The bimolecular quenching constant, $k_q$, was calculated by using the phase lifetime measured at the detection wavelength. [b] β-CD concentration was approximately $8.8 \times 10^{-3}$ M, and 0.1 M NaCl was used in the buffer.

of diffusion-controlled rates. The bimolecular quenching constant is slightly larger for acrylamide, similar to the difference in quenching of indole fluorescence by $I^-$ and acrylamide (Eftink & Ghiron, 1981). The bimolecular quenching constants for $I^-$ and acrylamide are reduced about 10-fold when equilenin is bound to either hSBP or rSBP. No significant difference is observed in the quenching of the two protein complexes by either quencher. As indicated in Table III, the degree of quenching of $2 \times 10^{-6}$ M equilenin in the presence of $8.8 \times 10^{-3}$ M β-CD is less than that of the SBP–steroid complexes.

## Discussion

According to the SBP model proposed by Petra et al. (1983), the steroid-binding site is located at the interface of the two SBP monomers, with each face of the bound steroid in contact with one of the subunits.[2] According to this model, the steroid should be buried in the protein structure and largely shielded from the bulk solvent. Furthermore, specific protein–steroid interactions must exist to account for the specificity and high binding affinity of certain steroids. We have examined these aspects of the model by comparing the absorption and fluorescence properties of equilenin in SBP complexes with those of equilenin in the β-CD complex and in different solvents.

*Fluorescence Quenching.* Dynamic quenching with KI or acrylamide has been used as a method to characterize the relative solvent exposure of fluorophores associated with macromolecules or membranes (Eftink & Ghiron, 1981). $I^-$ preferentially quenches groups fully exposed to solvent. If there are local negative charges, the quenching is somewhat inhibited; conversely, local positive charges enhance the quenching (Lehrer, 1971; Lehrer & Leavis, 1978). Acrylamide, on the other hand, is a neutral, polar quencher. Therefore, its quenching is relatively insensitive to the local charge.

The fluorescence quenching of equilenin complexed with SBP was inefficient compared with that of free equilenin in solution, indicating that the bound steroid is in a shielded environment from which bulk water and anionic compounds are excluded. No difference is observed in the quenching of equilenin bound to either hSBP or rSBP by either iodide or acrylamide, indicating equivalent shielding of the bound steroid in both species of SBP. If surface-charged groups were in the immediate vicinity of the bound steroid, we would expect differences in the quenching efficiency between acrylamide and $I^-$.

Fluorescence quenching of the β-CD–equilenin complex by acrylamide and $I^-$ is less than that for the SBP complex (Table III). The low degree of quenching likely results from formation of a 2:1 complex in which a β-CD molecule binds to each end of an equilenin molecule. This type of complex between dye molecules and cyclodextrins has been reported in the literature (Kondo et al., 1976; Gerasimowicz & Wojcik, 1982). Our binding studies (not shown) indicate an association constant of about $10^4$ $M^{-1}$ for a 1:1 complex, and the stoichiometry is greater than 1:1 at β-CD concentrations higher than about $8 \times 10^{-4}$ M. In a 2:1 complex, both aromatic rings of equilenin would be largely inaccessible to solvent and therefore well protected from quenchers. Comparison of the bimolecular quenching constants of the β-CD–equilenin complex with both SBP–equilenin complexes indicates that the aromatic rings of equilenin are not as shielded from solvent when the steroid is bound to SBP.

*Chemical Environment of the Steroid-Binding Site.* Binding site interactions that affect the spectra of probe molecules can be deduced from model solvent studies. Solvent-dependent shifts in absorption and fluorescence emission have been classified in terms of general and specific solvent effects (Nagakura & Gouterman, 1957; Lakowicz, 1983). General effects are a result of the bulk characteristics of the solvent, such as its refractive index and dielectric constant. Specific solvent effects result from direct interactions between the fluorophore and solvent molecules, including complex formation and hydrogen bonding (Nagakura & Gouterman, 1957; Lakowicz, 1983). Both general and specific interactions may contribute to the absorption and fluorescence spectra as well as to the excited-state decay kinetics of equilenin bound to SBP.

The quenching data indicate that the bound equilenin is essentially buried in the protein. Since a protein's interior is thought to have a much lower dielectric constant than water (Brant & Flory, 1965; Scheraga, 1968), bound equilenin would be expected to have a red-shifted absorption and a blue-shifted emission, similar to its spectra in low-dielectric solvents (Figures 3 and 4). Such spectral shifts are characteristic of aromatic molecules bound to hydrophobic sites in proteins and

[2] Conformational asymmetry in the subunits is included in the model to account for the binding of 1 mol of steroid per dimeric protein. Asymmetric conformations in identical subunits have been observed in several other proteins. For example, yeast hexokinase is composed of two chemically identical subunits that associate asymmetrically to form a single binding site (Steitz et al., 1976).

reflect nonspecific interactions that are similar to those which produce general solvent effects. Comparing the red-shifted fluorescence excitation spectrum of the SBP–equilenin complex to the red-shifted absorption (or excitation) spectra of equilenin in cyclohexane, toluene, or the β-CD complex, we conclude that the SBP steroid-binding site is a low-dielectric environment.

The emission spectra give a different view of the SBP-binding site. When equilenin is complexed with β-CD, the emission spectrum is intermediate between that in water and cyclohexane. By analogy to solvent effects, this spectrum reflects nonspecific interactions. By contrast, the red-shifted emission spectrum of SBP-bound equilenin, which is intermediate between that of ionized and unionized equilenin, is evidence for specific interaction(s) between the steroid and some amino acid side chain(s) in the low-dielectric steroid-binding site.

To assess the effects of specific interactions upon the spectra of equilenin, we examined solvent systems in which specific interactions have been demonstrated for 2-naphthol. It has been shown that 2-naphthol and TEA form a hydrogen-bonded complex in nonpolar solvents such as *n*-hexane, *n*-heptane, benzene, and toluene. This complex is characterized by a red shift in the near-UV region of the 2-naphthol absorption spectrum (Nagakura & Gouterman, 1957), indicating a ground-state interaction. Furthermore, the formation of the complex also causes a red shift in the 2-naphthol fluorescence emission spectrum (Mataga & Kaifu, 1962; Matsuzaki et al., 1974). In benzene, for example, the observed red shift is almost as large as the shift due to dissociation of 2-naphthol in aqueous solution (Mataga & Kaifu, 1962).

Since equilenin, like 2-naphthol, becomes more acidic in the excited state (Davenport et al., 1986), it is possible that during the lifetime of the excited state either a hydrogen bond could be made or proton transfer could occur in the presence of a suitable acceptor. In either event, emission would be expected from both the non-hydrogen-bonded and hydrogen-bonded species or from both the protonated and unprotonated species. In either case, since there are two emitting species, the average fluorescence lifetime measured by the phase technique should exhibit emission wavelength dependence (Lakowicz & Balter, 1982a,b). Equilenin in aqueous solution, however, has not been shown to undergo excited-state proton transfer, except in the presence of a strong proton acceptor such as acetate (Davenport et al., 1986). Since the phase lifetime of equilenin bound to SBP is wavelength independent, as was also found for equilenin in water, low-dielectric solvents, and the β-CD complex, there is no evidence for either proton transfer or hydrogen-bond formation in the excited state.

By comparing the excitation and emission spectra of equilenin bound to SBP with the spectra of equilenin in different model systems, we can develop a physical picture of the SBP steroid-binding site. First, we note that the red shift observed in the near-ultraviolet absorption band of equilenin with TEA, in either cyclohexane or toluene (Figure 3B), is similar to the red shift in the absorption spectrum of the 2-naphthol–TEA complex. This red shift indicates formation of a ground-state hydrogen bond between the 3′-hydroxyl group of equilenin and TEA. In the absence of TEA, however, the maximum of the absorption spectrum of equilenin in toluene is the same as that of the red-shifted excitation spectrum of equilenin bound to SBP (Figure 3B). Thus, on the basis of absorption and excitation spectra alone, we would conclude that the steroid-binding site is a low-dielectric cavity, but it is difficult to determine from the magnitude of the red shift whether or not a hydrogen bond is present in the SBP–equilenin ground-state complex. Second, we note that the red-shifted *emission* spectrum of the SBP–equilenin complex closely resembles the emission spectrum of the equilenin–TEA complex in cyclohexane. The similarity between the two emission spectra suggests that a hydrogen bond exists in the SBP–equilenin excited-state complex and that the steroid-binding site is a low-dielectric cavity with one or more proton-accepting groups. Since the phase lifetime data do not provide evidence for an excited-state reaction, formation of a hydrogen bond must then take place in the ground state. Such hydrogen-bond formation would be consistent with the abundant thermodynamic data showing that hydrogen bonds are major factors contributing to the stability of most protein–ligand complexes (Ross & Subramanian, 1981). We should point out, however, that our spectroscopic data do not prove ground-state hydrogen-bond formation. Although the SBP emission data are consistent with a hydrogen bond between equilenin and amino acid side chains in a hydrophobic environment, the absorption and excitation data are less sensitive to environmental effects and thus neither confirm nor reject the notion of ground-state hydrogen-bond formation.

In conclusion, our results show that the steroid-binding site of human and rabbit SBP is a low-dielectric cavity which effectively isolates the aromatic rings of equilenin from the bulk solvent. In addition, there are one or more proton acceptor groups in the cavity, shielded from the bulk solvent, that interact with the 3′-hydroxyl group of equilenin. Since in the excited state there appears to be a hydrogen bond, the acceptor(s) appear(s) to be in close proximity to the 3′-hydroxyl group. Reasons that a complete proton transfer does not occur might include (1) distance, (2) strength of the acceptor, and (3) the tendency of a low-dielectric environment to suppress ionization. Experiments are currently under way to clarify the details of this steroid-binding site interaction.

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# Solubilization and Reconstitution of Chick Renal Mitochondrial 25-Hydroxyvitamin $D_3$ 24-Hydroxylase†

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ABSTRACT: Chick kidney mitochondrial 25-hydroxyvitamin $D_3$ 24-hydroxylase has been solubilized with sodium cholate and reconstituted with NADPH, beef adrenal ferredoxin, and beef adrenal ferredoxin reductase, each component being essential for maximal 24-hydroxylase activity. The product 24(*R*),25-dihydroxyvitamin $D_3$ was identified by cochromatography with synthetic compound on straight-phase and reversed-phase high-performance liquid chromatography and by periodate oxidation. The enzyme has an apparent $K_m$ for 25-hydroxyvitamin $D_3$ of 0.67 $\mu$M. At 1 $\mu$M 25-hydroxyvitamin $D_3$, 24,25-dihydroxyvitamin $D_3$ production is linear with time for up to 15 min and with protein concentrations of up to 2 mg/mL. The antioxidant diphenyl-*p*-phenylenediamine ($1.3 \times 10^{-4}$ M) has no effect on this reaction. Reconstituted 24-hydroxylase activity is enhanced by the addition of NaCl and KCl up to 100 mM, with higher concentrations having an inhibitory effect. 1$\alpha$-Hydroxylase is not present in this preparation from vitamin D replete chicks. The similarities of this reconstituted system to the 25-hydroxyvitamin $D_3$ 1$\alpha$-hydroxylase and the adrenal systems suggest that the 25-hydroxyvitamin $D_3$ 24-hydroxylase is also a cytochrome P-450 type mixed-function oxidase.

Vitamin D is converted in the liver to 25-hydroxyvitamin $D_3$ (25-OH-$D_3$) (Blunt et al., 1968), which is further hydroxylated in the kidney to either 24,25-dihydroxyvitamin $D_3$ [24,25-$(OH)_2D_3$] (Holick et al., 1972), a relatively inactive metabolite (DeLuca, 1984), or 1,25-dihydroxyvitamin $D_3$ [1,25-$(OH)_2D_3$], the hormonal form of the vitamin active in intestinal calcium transport and bone mineral mobilization (Fraser & Kodicek, 1970). Both 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ have been shown in vivo and in vitro to be further metabolized to 1,24,25-trihydroxyvitamin $D_3$ [1,24,25-$(OH)_3D_3$] (Holick et al., 1973; Kumar et al., 1978; Tanaka et al., 1977), a less active analogue of 1,25-$(OH)_2D_3$ (DeLuca, 1984). Therefore, 24-hydroxylation is presumably an inactivation route in the metabolism of vitamin $D_3$.

Whether renal hydroxylation of 25-hydroxyvitamin $D_3$ occurs at the C-1 or C-24 positions appears to be regulated in a reciprocal manner by serum levels of calcium (Boyle et al., 1971), phosphorus (Tanaka & DeLuca, 1973), and 1,25-

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