Summers, M. F., South, T. L., Kim, B., & Hare, D. R. (1990) *Biochemistry 29*, 329-340.
Tam, J. P., Heath, W. F., & Merrifield, R. B. (1983) *J. Am. Chem. Soc. 105*, 6442-6455.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley, New York.
Wüthrich, K., Billeter, M., & Braun, W. (1983) *J. Mol. Biol. 169*, 949-961.

Fluorescence Investigation of the Sex Steroid Binding Protein of Rabbit Serum: Steroid Binding and Subunit Dissociation†

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ABSTRACT: The relationship between steroid binding and protein subunit interactions of rabbit sex steroid binding protein (rSBP) has been studied by steady-state and time-resolved fluorescence spectroscopy. The high-affinity ($K_a \sim 10^8$ M^{-1} at 4 °C), fluorescent estrogen *d*-1,3,5(10),6,8-estrapentaene-3,17β-diol [dihydroequilenin (DHE)] was used as a fluorescent probe of the steroid-binding site. Perturbation of the binding site with guanidinium chloride (Gdm·Cl) was monitored by changes in the steady-state fluorescence anisotropy of DHE as well as by changes in fluorescence quenching of DHE with acrylamide. The results of acrylamide quenching at 11 °C show that, while between 0 and 1 M Gdm·Cl the steroid-binding site is completely shielded from bulk solvent, there is decreased DHE binding. To study the subunit–subunit interactions, rSBP was covalently labeled with dansyl chloride in the presence of saturating 5α-dihydrotestosterone (DHT), which yielded a dansyl-conjugated protein that retained full steroid-binding activity. The protein subunit perturbation was monitored by changes in the steady-state fluorescence anisotropy of the dansyl group. At 11 °C, the dansyl anisotropy perturbation, reflecting changes in global and segmental motions of the dimer protein, occurs at concentrations of Gdm·Cl above 1 M. The Gdm·Cl titration in the presence of steroids with equilibrium association constants less than 10^8 M^{-1} shows a plateau near 3 M Gdm·Cl at 11 °C; at this Gdm·Cl concentration, no DHE is bound. No plateau is observed at 21 °C. At higher Gdm·Cl concentrations, the dansyl fluorescence anisotropy decreases further and shows no steroid dependence. Recovery of steroid-binding activity (assayed by saturation binding with [^{3}H]DHT), under renaturation conditions, is dependent on both steroid concentration and affinity. Both unlabeled and dansyl-labeled protein recovery the same amount of activity, and according to fluorescence anisotropy, dansyl-labeled rSBP re-forms a dimer upon dilution below 1 M or removal of Gdm·Cl. From the steroid requirement for recovery of steroid-binding activity, it appears that a conformational template is required for the dimeric protein to re-form a steroid-binding site with native-like properties.

The blood of most vertebrates contains a high-affinity sex steroid binding protein, generally referred to as SBP[1] (also called sex hormone binding globulin, abbreviated SHBG), which binds both estrogens and androgens [for reviews, see Westphal (1986) and Moore and Bulbrook (1988)]. The relative affinities for estrogens and androgens vary among different species; androgens are generally bound with higher affinity. For example, compared with primate SBPs, rabbit SBP has a significantly weaker affinity for 17β-estradiol, but these species have similar affinities for testosterone and 5α-dihydrotestosterone (DHT) (Mickelson & Petra, 1978; Kotite & Muto, 1982). It has been proposed that SBP is a blood carrier for the sex steroids and that its biological function is to maintain the proper balance in the free steroid concentrations. This hypothesis is consistent with the observation that increased SBP concentrations reduce the clearance rate of specific steroids in the blood (Vermeulen et al., 1969; Petra et al., 1985; Plymate et al., 1990). This hypothesis is also consistent with the view held by many endocrinologists that steroids enter target cells by free diffusion. Recent data from several laboratories, however, have provided suggestive evidence for the existence of specific SBP receptors on the membranes of target cells (Sakiyama et al., 1988; Hryb et al., 1985). These data could mean that the biological function of SBP involves more than control of the free steroid concentration in plasma and that an additional function of SBP is to target the bound steroid to specific cells.

Our laboratories have been investigating the mechanism of steroid binding by rabbit and human SBP in an effort to understand the basis for their differences in specificity and affinity for the different sex steroids (Örstan et al., 1986).

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[1] Abbreviations: SBP, sex steroid binding protein; rSBP, rabbit SBP; hSBP, human SBP; DHT, 5α-dihydrotestosterone; DHE, 17β-dihydroequilenin; DNS, dansyl or 5-(dimethylamino)naphthalene-1-sulfonyl; Gdm·Cl, guanidinium chloride.

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Recently, the chemical composition and polypeptide sequences of human and rabbit SBP have been determined (Petra et al., 1986a), indicating that 79% of their residues occupy identical loci (Griffin et al., 1989). Both proteins and homodimeric glycoproteins with molecular weights of about 90000 (Petra et al., 1986b), which are stabilized against heat denaturation by calcium (Rosner et al., 1974; Ross et al., 1985). Steroid-binding studies show that only one steroid is bound per dimer (Petra et al., 1986b). One possible explanation for this stoichiometry is that the steroid-free protein has two equivalent steroid sites, but steroid binding proceeds in a negatively cooperative manner, with the association microconstant for filling the second site being orders of magnitude less than that for the first site. Alternatively, the protein subunits might associate in an asymmetric fashion, generating a single high-affinity steroid site involving the dimer interface.

At the present time, very little is known about how SBP monomers interact to form the native dimeric structure and how different sex steroids affect the formation and stability of the active dimers. In this paper, we report the results of guanidinium chloride (Gdm·Cl) solvent perturbation on the steroid and subunit interaction of rabbit SBP (rSBP). We made use of the naturally fluorescent equine estrogen 17β-dihydroequilenin (DHE) to measure the Gdm·Cl perturbation of steroid binding. Since fluorescence anisotropy has been used extensively as a tool to study dissociation of oligomeric proteins (Weber, 1953; Mejillano & Himes, 1989), we also measured the fluorescence anisotropy of the 5-(dimethylamino)-naphthalene-1-sulfonyl-labeled protein–steroid complex (DNS-rSBP) to investigate the subunit interaction. Finally, to assess the role of steriods in stabilizing the dimeric structure, we first compared the Gdm·Cl denaturation of pure dansyl-labeled and unlabeled rSBP in the presence of different, structurally related steroids, which have different affinities for rSBP in dilute serum. We then compared the recovery of anisotropy (DNS-rSBP) and steroid-binding activity ([^{3}H]-DHT binding by pure labeled and unlabeled rSBP) following renaturation in the presence of different concentrations of these steroids.

Experimental Procedures

Chemicals. 17β-Estradiol, equilenin, testosterone, and 5α-dihydrotestosterone (DHT) were from Sigma Chemical Co., [1,2-^{3}H]DHT was from New England Nuclear, and 17β-dihydroequilenin (DHE) was from Steraloids. Guanidinium chloride (Gdm·Cl) (UV grade) was purchased from U.S. Biochemical Corp. and used without further purification. Fresh Gdm·Cl solutions were prepared daily; the Gdm·Cl concentrations were determined from density measurements (Kawahara & Tanford, 1966). Acrylamide, used for fluorescence quenching, was recrystallized once from ethyl acetate. All other chemicals were reagent grade.

Preparation of Protein. rSBP was purified by affinity chromatography followed by preparative acrylamide gel electrophoresis (Petra & Lewis, 1980; Griffin et al., 1989) and stored at −70 °C in a 10 mM Tris (pH 7.4 at 5 °C), 0.1 M NaCl, 5 mM $CaCl_2$, and glycerol (10% v/v) buffer containing 20 μM DHT. In the buffer used in the experiments, sucrose (10% w/v) was substituted for glycerol because it had a lower fluorescence background than glycerol.

Preparation of Steroid Complexes. The replacement of DHT by other steroids was accomplished by competitive dialysis of 1 mL of samples against 100 mL of buffer containing 2×10^{-5} M steroid (protein concentration was 1–10 μM). The dialysis buffer was changed every 12 h for a total of five changes. To reduce the free steroid concentration, a final dialysis was carried out against buffer either without steroid or with steroid equimolar in concentration to the protein sample. In the case of DHE, formation of the complex was evident from the red shift in the fluorescence emission spectrum of the equine estrogen, shown in Figure 1. The shift of the DHE emission spectrum is similar to that which was previously observed for the SBP complex with equilenin (Ross et al., 1982; Örstan et al., 1986). As in the case of equilenin, addition of excess DHT, which has about a 100-fold higher equilibrium association constant, results in an emission spectrum identical with that of DHE free in buffer. This shows that DHT readily displaces bound DHE and that the red shift in the fluorescence is due to specific binding; in a filter assay with [^{3}H]DHT (Mickelson & Petra, 1974), DHE acts as a simple competitive inhibitor. To eliminate errors due to possible sample dilution or loss during dialysis, protein and DHE concentrations were determined at the conclusion of dialysis by absorption spectra, extinction coefficients of 1.27×10^5 M^{-1} cm^{-1} at 280 nm for rSBP (Petra et al., 1986b) and 2.5×10^3 M^{-1} at 340 nm for DHE being used, where the estrogen absorption is due only to its 2-naphthol chromophore (Hurwitz & Liu, 1977; Ross et al., 1982). At equimolar concentrations of protein and steroid, the absorption contribution of DHE to the 280-nm extinction of the complex is about 1% that of the protein alone. Therefore, the protein concentration was determined at 280 nm, the 1% contribution from DHE being ignored. Assuming that the change in the peak extinction coefficient of the lowest energy absorption band of DHE upon binding to rSBP is small, the bound steroid concentration was then determined by the absorption difference between the sample and the dialyzate at 340 nm, where there is no absorption due to the protein. This method of calculation of steroid binding was checked by fluorescence, first by measuring the total DHE in the sample. This was accomplished by addition of excess DHT (20 μM) to the sample and then by subtraction of the fluorescence spectrum of the dialyzate (the free steroid concentration) from that of the sample (the total steroid concentration) to yield that fluorescence due to DHE specifically competed by DHT. Comparing this difference with a standard curve of free DHE fluorescence as a function of concentration gave the bound steroid concentration. The fluorescence and absorption estimates of the bound steroid concentration typically agreed within 10%.

Steroid Binding Activity. The total steroid binding activity of pure rSBP was determined with a modified filter assay based on the procedure for diluted serum described by Mickelson and Petra (1974). It was difficult to obtain reproducible values of binding activity for the pure protein. Addition of serum albumin as a carrier protein helped reduce the problem. Further studies showed that ovalbumin worked more efficiently, and it was used in the present studies. The undiluted pure rSBP concentration was determined by absorption

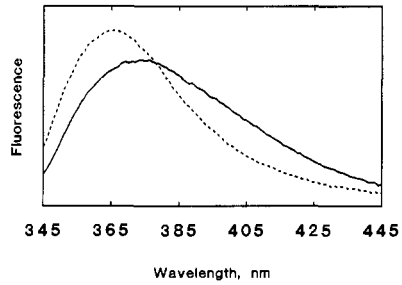

FIGURE 1: Comparison of the emission spectra, at 11 °C and pH 7.5, of DHE complexed with rSBP (—) (1 μM) and after addition of 20 μM DHT (– –). Excitation was at 330 nm, and the excitation and emission band-passes were 5 and 10 nm, respectively.

spectra. Pure rSBP was diluted into a 10 mM Tris buffer (pH 7.4 at 5 °C) containing ovalbumin. The final assay concentration of diluted rSBP was 3 nM, and the final carrier protein concentration was 0.04% (w/v); the [^{3}H]DHT concentration was 30 nM to assure complete saturation of the protein. To measure nonspecific binding, a second sample included a 100-fold excess of radioinert DHT. Assuming binding of 1 mol of steroid/mol of dimer protein, different preparations of rSBP had binding capacities between 50 and 110%; most preparations were about 90% active protein by these criteria. Only the latter preparations were used in the experiments reported here.

Preparation of Dansyl-Labeled rSBP. The DHT–protein complex was covalently labeled at 4 °C with 2 mM 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl) chloride in a 10 mM phosphate buffer (pH 8). Excess DHT (20 μM) was used to assure protection of the steroid-binding site. The reaction was quenched after 15 min by addition of Tris base (final concentration 0.1 M). The labeled protein was separated from reagents by desalting on a Sephadex G-25 column, eluted with 10 mM Tris, 1 mM $CaCl_2$, 0.1 M NaCl, and 1 μM DHT (pH 7.4 at 5 °C). The fractions containing dansyl-conjugated protein were detected by their intense yellow fluorescence when excited by a mercury lamp (365-nm line). The fractions with yellow fluorescence, characteristic of dansyl–protein conjugates, were pooled, and sucrose and calcium were added to final concentrations of 10% (w/v) and 5 mM, respectively, to stabilize the protein [see Ross et al. (1985)].

The extent of labeling was calculated by assuming that the absorption of the dansyl label covalently bound to rSBP is approximately the same as that of the model compound ϵ-dansyllysine in water at pH 7; we obtained extinction coefficients at 340 and 280 nm of 3.94×10^3 and 1.58×10^3 M^{-1} cm^{-1}, respectively, for this model compound. The concentration of dansyl groups was calculated from the rSBP conjugate absorption at 340 nm. This concentration was then used to calculate the expected absorption contribution due to dansyl at 280 nm. Using an extinction coefficient at 280 nm of 1.27×10^5 M^{-1} cm^{-1} for native rSBP (Petra et al., 1986b) and assuming additivity of the dansyl and protein extinction coefficients, we obtained labeling ratios of 1–2 mol of dansyl/mol of protein monomer. Dansyl-rSBP (DNS-rSBP) in the modified filter assay showed no detectable alteration in the total binding of [^{3}H]DHT.

Renaturation of rSBP and DNS-rSBP. A stock solution of either pure native rSBP or dansyl-labeled rSBP (ca. 5 μM) was diluted in 2.5 or 5.2 M Gdm·Cl (final concentration) to a final protein concentration of 1 μM and then maintained at 5 °C for 6–12 h. After this time there was no further change in fluorescence properties. Renaturation was accomplished by four to five changes of dialysis buffer containing either no steroid or different concentrations of steroid (either DHE, 17β-estradiol, testosterone, or DHT), including excess steroid (20 μM).

Spectroscopy. Absorption measurements were carried out with an IBM 9430 UV–visible spectrophotometer. Fluorescence excitation and emission spectra were obtained either with an SLM 4800, converted by us to a single-photon counting instrument, or with an SLM-Aminco SPF-500C spectrofluorometer. Both fluoresence instruments were equipped with a thermostated cuvette holder, and the measurements were made with polarizers set at the magic angle to avoid artifacts due to molecular rotation during the lifetime of the excited state (Kalantar, 1968; Badea & Brand, 1979).

The steady-state anisotropy $\langle r \rangle$, defined by the relationship

$$\langle r \rangle = I_{vv}(G - I_{hv})/I_{vv}(G + 2I_{hv}) \quad (1)$$

was obtained by measuring the vertical and horizontal components of the fluorescence emission with excitation vertical (I_{vv} and I_{vh}, respectively) and horizontal (I_{hv} and I_{hh}, respectively) to the emission axis. The G factor ($G = I_{hh}/I_{vh}$) corrects for the transmissivity bias of the excitation monochromator (Azumi & McGlynn, 1962; Paoletti & LePecq, 1969).

The average rotational correlation time, $\langle \phi \rangle$, of the labeled protein was calculated from the Perrin equation [see Lakowicz (1983)]:

$$\langle \phi \rangle = \langle \tau \rangle \langle r \rangle / (r_o - \langle r \rangle) \quad (2)$$

where $r_o = 0.308$ is the limiting anisotropy of the dansyl group in the absence of rotation (Weber, 1953). $\langle \tau \rangle$ is the mean fluorescence lifetime:

$$\langle \tau \rangle = \sum_{j=1}^{n} \alpha_j \tau_j^2 / \sum_{j=1}^{n} \alpha_j \tau_j \quad (3)$$

τ_j is the lifetime and α_j the fractional population of the jth emitting species, and assumes that the fluorescence decay is represented by a sum of exponentials:

$$I(t) = \sum_{j=1}^{n} \alpha_j e^{-t/\tau_j} \quad (4)$$

The value of $\langle \phi \rangle$ is a weighted harmonic mean:

$$\langle \phi \rangle = \sum_{i=1}^{m} \beta_i [\sum_{i=1}^{m} \beta_i / \phi_i]^{-1} \quad (5)$$

where $\sum \beta_i$ is the limiting anisotropy, r_o. Equation 2 assumes that all emission lifetime components, τ_j, are short compared to the rotational correlation time components, ϕ_i.

Fluorescence Lifetimes. A time-correlated single-photon counting instrument assembled in this laboratory was used to measure the fluorescence lifetimes. Vertically polarized excitation was provided by a Spectra Physics YAG-pumped-dye laser, cavity dumped at 4 MHz. The fluorescence intensity decay was measured with a detection polarizer set at the magic angle, and the photons were detected with a R2809U-06 Hammamatsu microchannel plate photomultiplier. The intensity decay data were analyzed by iterative reconvolution (Knight & Selinger, 1971; Grinvald & Steinberg, 1974), a solution of ludox being used for the instrument response. The reduced χ^2 value and the mean-weighted residuals with their autocorrelation were used as statistical benchmarks for determining a satisfactory fit to the data. The statistical limits for meaningful fits to the data were determined by the best statistics obtained for reference compounds of known decay behavior (Ross et al., 1981).

Fluorescence Quenching. Acrylamide was used to differentially quench the fluorescence of free and bound DHE. Samples were prepared by dilution of concentrated rSBP–DHE ($\sim$10 μM) complex to a final protein concentration of about 1 μM. For steroid-binding site perturbation experiments, a concentrated solution of Gdm·Cl (5 M) was added to the solution containing the steroid–protein complex to obtain the final desired concentration of Gdm·Cl ($\leq$1 M). After incubation in ice for 1 h or longer, 0.8 mL of solution was transferred to a cuvette. Quenching titrations were then carried out with acrylamide from a 4 M stock solution containing the same concentration of Gdm·Cl as the protein sample. Control quenching titrations were carried out with solutions of free DHE at the different concentrations of Gdm·Cl.

The quenching data of free DHE, in the presence and absence of Gdm·Cl, were plotted according to the Stern–Volmer

equation (Stern & Volmer, 1919), as modified by Eftink and Ghiron (1981):

$$F_o/F = (1 + K_{sv}[Q])e^{V[Q]} \quad (6)$$

where F_o is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity in presence of quencher, [Q] is the quencher concentration, and V represents a volume immediately surrounding the excited fluorophore within which instantaneous quenching occurs. Quenching within this volume is called static quenching and depends upon the random distribution of quenchers throughout the solution; static quenching can also arise from equilibrium association between the quencher and the fluorophore, as discussed below. The Stern–Volmer constant, K_{sv}, describes the dynamic quenching process and is the product of the mean fluorescence lifetime in the absence of quencher, $\langle\tau_o\rangle$, and the bimolecular collisional rate constant, k_q. The K_{sv} and V values of free DHE were obtained, the experimental data being fitted according to eq 6, by use of a nonlinear least-squares procedure (Bevington, 1969). The Stern–Volmer constant was confirmed from the time-resolved measurements by the decrease in the mean lifetime, $\langle\tau\rangle$, in the presence of quencher:

$$\langle\tau_o\rangle/\langle\tau\rangle = 1 + K_{sv}[Q] \quad (7)$$

To analyze the quenching data for the rSBP–DHE complexes, where both free and bound DHE species are present (designated by the subscripts f and b, respectively; i.e., $K_{sv,f}$ and $K_{sv,b}$ or V_f and V_b), requires fitting five independent variables for the quenching parameters. These are $K_{sv,f}$, $K_{sv,b}$, V_f, V_b, and either the fractional fluorescence intensity of the free, f_f, or the fractional fluorescence intensity of the bound (i.e., $f_b = 1 - f_f$). Fitting all these parameters is difficult: we do not know, a priori, the difference between $K_{sv,f}$ and $K_{sv,b}$ or between V_f and V_b, and moreover, the quenching parameters, $K_{sv,i}$ and V_i, of each species, i, are correlated. Therefore, we simplified the problem by assuming that the parameters for free DHE were the same in the presence or absence of rSBP. Consequently, the values of $K_{sv,f}$ and V_f, obtained from independent experiments, described above, were constants in the analyses. In that way, the nonlinear least-squares fitting for quenching of free and bound DHE was limited to only the parameters associated with the bound steroid: $K_{sv,b}$, V_b, and f_b.

As discussed below, the physical interpretation of $K_{sv,b}$ can be described by three limiting cases, and the quenching data for the rSBP–DHE complexes were analyzed according to each of these models. For each case, we were able to use the following equation for heterogeneous systems:

$$\frac{F_o}{F} = \left[\sum_{i=1}^{n}\left[\frac{f_i}{(1 + K_{sv,i}[Q])e^{V_i[Q]}}\right]\right]^{-1} \quad (8)$$

where the species i represents the free or bound steroid and the quenching parameters for the bound steroid, $K_{sv,b}$ and V_b, are model dependent.

The first case assumed that a difference in quenching of bound DHE would include both dynamic and static interactions, similar to free DHE. The ratio of the static constant to the dynamic constant of the bound steroid was assumed to be the same as that of free DHE.[2] Thus, the data were fit for two independent invariables ($K_{sv,b}$ and the fractional intensity of the bound, f_b) and one dependent variable (V_b of the bound). On the basis of the fluoresence decay of the complex, however (see Results and Table I), there was no evidence for dynamic quenching of the bound steroid. Consequently, the second and third cases only considered the two limiting static quenching models. Accordingly, in the second case, by making $K_{sv,b}$ a constant equal to 0, the static quenching was fit according to the random distribution model:

$$F_{o,b}/F_b = e^{V_b[Q]} \quad (9)$$

This equation predicts upward curvature for $F_{o,b}/F_b$ as a function of quencher concentration. Finally, in the third case, we considered the possibility of weak, ground-state equilibrium binding of acrylamide to rSBP in the immediate vicinity of the bound steroid. This could be considered as nonspecific binding of acrylamide by the protein. In this situation, the steady-state quenching of bound DHE was obtained by making V_b a constant equal to 0 and iterating for $K_{sv,b}$, where

$$K_{sv,b} = [\text{rSBP–DHE–Q}]/[\text{rSBP–DHE}][Q] \quad (10)$$

In contrast to the random distribution model of static quenching, the equilibrium binding model predicts that $F_{o,b}/F_b$ will be a linear function of quencher concentration.

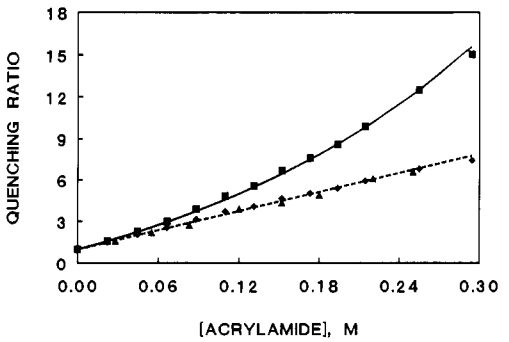

FIGURE 2: Quenching of 3 μM DHE by acrylamide in pH 7.5 Tris buffer with 10% (w/v) sucrose at 11 °C. The fluorescence quenching as a function of acrylamide concentration is shown for the steady-state emission by the ratio F_o/F (■) and for the mean lifetime by the ratio τ_o/τ (▲). Correction for static quenching of the steady-state fluorescence is shown by the ratio $F_o/(Fe^{V[Q]})$ (◆). The steady-state quenching was measured with excitation at 330 nm with a band-pass of 5 nm. The time-resolved quenching was measured with laser excitation at 325 nm. Detection was at 365 nm with a 10-nm band-pass.

RESULTS

Solvent Accessibility of the Steroid-Binding Site Probed by Quenching of 17β-Dihydroequilenin Fluorescence. After exhaustive competitive dialysis to replace DHT with DHE, some samples were further dialyzed in the absence of added estrogen to reduce the concentration of free steroid and nonspecific binding. At the end of dialysis, the molar ratio of bound steroid to dimer was slightly less than 1:1; when the total concentration of steroid was in significant excess of the protein concentration, the molar ratio of *bound* steroid to total protein did not exceed 1:1, indicating the presence of only one high-affinity steroid binding site per dimer.

The solvent exposure of the fluorescent estrogen DHE was measured by fluorescence quenching with acrylamide. Figure 2 compares the steady-state and time-resolved fluorescence quenching of free DHE by acrylamide. The upward curvature of the steady-state data indicates both static and dynamic quenching of the free steroid. Analyzing these data by eq 6, we found collisional and static quenching constants of K_{sv} = 23.1 M^{-1} ($=K_{sv,f}$) and V = 2.4 M^{-1} ($=V_f$), respectively, reported in Table I. A value for K_{sv} was obtained independently from time-resolved measurements, and was 22.5 M^{-1}, as reported in Table I. Within error, this is the same value as that obtained

[2] This assumption is based on the observation by Eftink and Ghiron (1981) that there is an approximately constant relationship between V and K_{sv} for tryptophan residues in different proteins. In these cases, when acrylamide is the quencher, V is about 10% of K_{sv}.

Table I: Acrylamide Quenching of DHE Free and Bound to rSBP[a]

	steady-state fluorescence			time-resolved fluorescence	
	K_{sv} (M^{-1})	V (M^{-1})	f_b	K_{sv} (M^{-1})	$\langle\tau_o\rangle$ (ns)
Free DHE: Dynamic/Static					
0 M Gdm·Cl	23.1	2.4	0	22.5	8.4
1 M Gdm·Cl	23.0	2.0	0	21.3	8.4
Bound DHE: Dynamic/Static					
0 M Gdm·Cl	2.1	0.2	0.96		
1 M Gdm·Cl	3.1	0.3	0.46		
Bound DHE: Static, Random Distribution					
0 M Gdm·Cl		1.6	0.88	0	6.2
1 M Gdm·Cl		2.3	0.42	0	6.2
Bound DHE: Static, Equilibrium Binding					
0 M Gdm·Cl	2.7		0.99	0	6.2
1 M Gdm·Cl	3.9		0.48	0	6.2

[a] Steady-state excitation was at the isosbestic wavelength of 330 nm for both free and bound DHE. Detection of free DHE was at 365 nm, near its emission peak, and detection of bound DHE was at 380 nm, near the isoemissive point for the free and bound species. The respective intensity decays of free and bound DHE were measured at 365 and 390 nm in both the presence and absence of acrylamide. The steady-state quenching data for the complex were analyzed in three ways (see Experimental Procedures, eq 8–10): first, according to a dynamic model in which K_{sv} is the product of the bimolecular quenching constant and the mean fluorescence lifetime, including an additional static quenching term based upon a statistical interaction volume, $e^{V[Q]}$; second, according to a pure static model with a statistical interaction volume, $e^{V[Q]}$; third, according to a pure static model in which K_{sv} describes the equilibrium association constant for a ground-state complex.

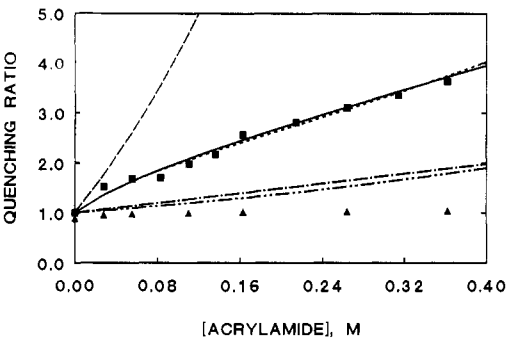

FIGURE 3: Comparison of quenching at 11 °C of DHE free in solution and DHE bound to rSBP. The steady-state and time-resolved quenching of the steroid–protein complex is represented by F_0/F (■) and τ_0/τ (▲), respectively. The theoretical curve (– –) is for the steady-state fluorescence quenching of free DHE. The theoretical curves (—) and (- - -) are for static quenching of the steady-state fluorescence of bound DHE either by equilibrium binding or by random distribution of quencher, respectively, in a mixture of free and bound DHE. The theoretical curves (–·–) and (–··–) are for static quenching of the steady-state fluorescence of bound DHE alone either by equilibrium binding or by random distribution of quencher, respectively. The steady-state quenching was measured with excitation at 330 nm with a band-pass of 5 nm, and the time-resolved quenching was measured with laser excitation at 325 nm. Detection was at 380 nm, with a band-pass of 10 nm, for steady-state measurements, and at 390 nm for time-resolved measurements, to decrease the contribution from residual free DHE fluorescence. In this particular experiment free and bound DHE were 54 and 46%, respectively.

from the steady-state data. The value of V from the steady-state data was about 10% that of K_{sv}.

The fluorescence decay of free DHE at 365 nm, and at pH 7.5, is a double exponential with time constants of 8.5 ± 0.1 and 1.7 ± 0.05 ns with average amplitudes of 0.89 and 0.11, respectively, yielding a mean lifetime of 8.3_5 ns. Similar results have been obtained by Davenport et al. (1986). By use of the mean lifetime, a bimolecular rate constant of 2.7×10^9 M^{-1} s^{-1} was calculated for acrylamide quenching in the 10% sucrose buffer at 10 °C. As indicated in Table I, 1 M Gdm·Cl had essentially no effect on the mean lifetime or on the dynamic and static quenching constants for free DHE.

Stern–Volmer plots of acrylamide quenching of the steady-state and time-resolved fluorescence of an rSBP–DHE complex in the presence of excess DHE (ca. 45% bound) are compared in Figure 3. Between 0.1 and 0.5 M acrylamide (data shown to 0.4 M acrylamide), the mean fluorescence lifetime was about 6.2 ns, and the intensity decay had a dominant 6.8–7.1-ns time constant, which was unaffected by acrylamide. From the quenching parameters of free DHE, indicated in Figure 3 (see Figure 2), 75 and 97% of free DHE is quenched at 0.1 and 0.5 M acrylamide, respectively. At 0.5 M acrylamide, the intensity decay kinetics of the DHE complex were best fit by a triple exponential with decay constants of 0.5, 2.5, and 6.8 ns, with respective fractional fluorescence intensities (see also eqs 3 and 4)

$$f_j = \alpha_j\tau_j / \sum_{j=1}^{3} \alpha_j\tau_j \qquad (11)$$

of 3, 9, and 88%. Since, at 0.5 M acrylamide, the dominant 8.6-ns decay component of free DHE is quenched to about 0.7 ns, free DHE may account for part or all of the 0.5-ns lifetime observed in the triple-exponential fit of the intensity decay of the complex. If there is neither static nor dynamic quenching of DHE bound to rSBP, at 0.5 M acrylamide we would expect about a 1% intensity contribution from free DHE at 390 nm. At 0.1 M acrylamide, where the main lifetime component of the free DHE is about 2.6 ns, we would expect about a 10% intensity contribution from free DHE at 390 nm. Thus, from 0.1 to 0.5 M acrylamide the essentially constant mean lifetime of the complex is largely due to the highly efficient collisional quenching of the excess free DHE. Since, above 0.1 M acrylamide, the main decay component with the 6.8–7.1-ns lifetime is unaffected and the mean lifetime remains essentially constant, there is no evidence for dynamic quenching of bound DHE. The decrease in the steady-state fluorescence of DHE bound to rSBP must then be the result of static quenching.

Static quenching can be considered as resulting from a random distribution of quenchers with an effective sphere for instantaneous quenching. Another possible, but very different, static model is the formation of an equilibrium complex between the quencher and the fluorophore. This model would suggest binding of acrylamide to rSBP in close vicinity to the bound steroid. The fits of the steady-state quenching data predicted by these two static models for the bound steroid are compared in Figure 3.

Solvent Accessibility of Bound 17β-Dihydroequilenin during Guanidinium Chloride Perturbation. Although increased steady-state fluorescence quenching of the DHE complex is observed for increasing Gdm·Cl up to 1 M, as shown in Figure 4, Gdm·Cl perturbation does not affect the mean fluorescence lifetime of bound DHE. Apparently, under these apparently mild perturbing conditions, the bound steroid remains inaccessible to acrylamide quenching. Regardless of which quenching model was used to fit the data, the fraction of steroid bound to the protein decreased with increasing Gdm·Cl concentration. The results obtained in 0 and 1 M Gdm·Cl, fitting the mixed dynamic and static and the two pure static fluorescence quenching models, are summarized in Table I. A comparison of the models is made under Discussion.

Guanidinium Chloride Induced Subunit Dissociation and Denaturation of rSBP Probed by Fluorescence Anisotropy of a Covalently Bound Dansyl Group. The mean fluorescence lifetimes of dansyl-labeled rSBP at increasing concentrations of Gdm·Cl, at 10 °C, are given in Table II. The mean lifetime

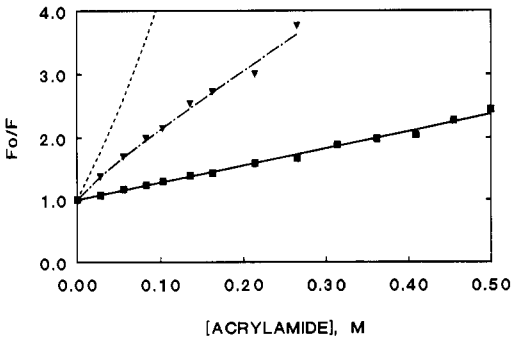

FIGURE 4: Steady-state fluorescence quenching curves of DHE free in solution (- - -) and DHE free and bound to rSBP at 1 M Gdm·Cl (-·-) and at 0 M Gdm·Cl (—). DHE in this complex was 95% bound. Other conditions are as described in Figure 3. Fitting of the bound DHE assumed static quenching by equilibrium binding of acrylamide.

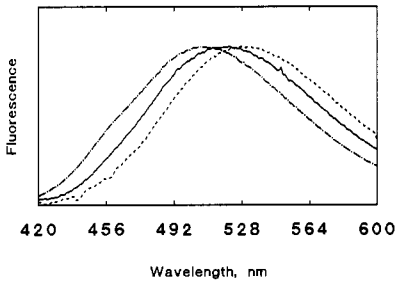

FIGURE 5: Comparison of the fluorescence emission spectra of dansyl-labeled rSBP complexed with DHT at 0 M Gdm·Cl (—) and at 5.2 M Gdm·Cl (- - -) (incubation time 6–12 h) and after renaturation by dialysis of the 5.2 M Gdm·Cl sample against buffer containing 20 μM DHT (-·-). Excitation was at 340 nm with a band-pass of 5 nm, and the emission spectrum was recorded with a band-pass of 10 nm.

Table II: Fluorescence Parameters of DNS-rSBP[a]

Gdm·Cl (M)	⟨r⟩	⟨τ⟩ (ns)	⟨φ⟩ (ns)
0	0.196	13.8	24.2
1.0	0.193	13.9	23.5
3.0	0.138	12.3	9.9
5.2	0.101	10.5	5.1
renatured	0.20	16	29

[a] Excitation and emission wavelengths were at 340 and 520 nm, respectively, for the equilibrium steady-state anisotropy in the presence of 17β-estradiol and at 318 and 500 nm, respectively, for fluorescence lifetime measurements (pH 7.5 Tris-HCl buffer, 11 °C). The fluorescence decay of the dansyl probe was multiexponential, and the mean lifetime is reported here (eq 3). The fluorescence intensity decays were also measured at 520 nm, and no significant differences were observed between the 500- and 520-nm data sets.

decreases by about 10% at 3 M Gdm·Cl and by about 20% at 5.2 M Gdm·Cl. The emission spectra at 0 and 5.2 M Gdm·Cl and after renaturation are compared in Figure 5. No shift occurred between 0 and 3 M Gdm·Cl. At 5.2 M Gdm·Cl, however, all preparations showed an emission shift to lower energy ($\Delta\lambda_{max}$ = 10 nm), consistent with increased solvation of the dansyl chromophore. After renaturation, the dansyl emission spectrum was shifted to higher energy, indicating decreased solvation.

Figure 6 compares the change in the steady-state fluorescence anisotropy of dansyl-labeled rSBP in the presence of 17β-estradiol at 11 and 21 °C, between 0 and 5.2 M Gdm·Cl. The profile of the Gdm·Cl titration at 11 °C is distinctly biphasic. The anisotropy of the steroid complex remains at a constant value up to about 1.5 M Gdm·Cl. Above this concentration, the anisotropy starts to decrease and reaches a plateau at about 2.5 M Gdm·Cl. Above 3.5 M Gdm·Cl, the anisotropy again decreases. By contrast, at 21 °C the profile

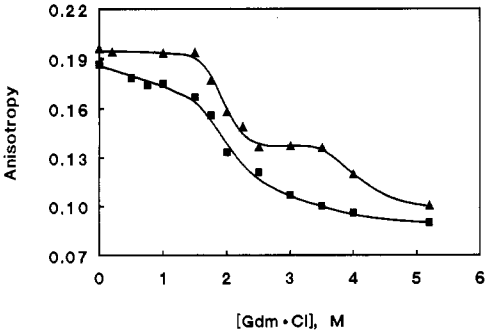

FIGURE 6: Change in the steady-state fluorescence anisotropy of the dansyl-labeled rSBP complexed with 17β-estradiol at 11 (▲) and 21 °C (■) as a function of Gdm·Cl concentration. Measurements were made as described under Experimental Procedures. The concentration of the labeled protein was ca. 1 μM, and the total steroid concentration was ca. 2 μM. Gdm·Cl titration up to 4 M was made by adding aliquotes of 7 M Gdm·Cl (10 mM Tris-HCl, pH 7.5, at each temperature) directly to the cuvette. The sample was incubated at least 1 h at each temperature for each point. The last point at 5.2 M Gdm·Cl was obtained by direct addition of crystalline Gdm·Cl. Excitation was at 340 nm with a band-pass of 5 nm, and emission was at 520 nm with a band-pass of 10 nm.

Table III: Effect of Steroids with Different Binding Affinity on Recovery of [^{3}H]DHT Binding by rSBP and DNS-rSBP after Renaturation from Gdm·Cl[a]

		% [^{3}H]DHT binding recovered	
20 μM steroid	K_a (M^{-1})[b]	2.5 M Gdm·Cl	5.2 M Gdm·Cl[c]
no steroid		7 (±6)	5 (±3)
17β-estradiol	1.2×10^7		10
DHE	4.8×10^7	18	14 (±3)
testosterone	3×10^8		20
DHT	1.2×10^9	45 (±4)	23 (±8)

[a] Renaturation was by dialysis in 10 mM Tris·HCl, 10% sucrose (w/v), 0.1 M NaCl, and 5 mM $CaCl_2$, pH 7.5 at 5 °C, in either the presence or absence of steroid; the [^{3}H]DHT binding activities of rSBP and DNS-rSBP were the same before denaturation (92 ± 10%), and their recovery of activity was identical. Concentration of steroids less than 20 μM yielded lower values of recovered binding activity. [b] The equilibrium association constants for 17β-estradiol, testosterone, and DHT (4 °C) in diluted rabbit serum are from Mickelson and Petra (1978). The equilibrium association constant of DHE in diluted rabbit serum, based on its competitive binding with [^{3}H]DHT at 4 °C, was 4 times that of 17β-estradiol; the value for DHE binding reported here is relative to the binding affinity of 17β-estradiol reported by Mickelson and Petra (1978). For comparison, the DHE equilibrium association constant was measured by spectroscopic assay (fluorescence anisotropy and acrylamide quenching) of the pure protein. Taking into account the total protein and total ligand concentrations and fraction of ligand bound, the value of k_a at 11 °C was 10^8 (×/÷2) M^{-1}. [c] The deviation, where indicated, is based on the average of three experiments. The other values are single observations.

suggests a single, broad transition. The anisotropy decreases in an essentially linear fashion up to about 1.5 M Gdm·Cl and then decreases in a smooth titration, reaching a limiting value between 5 and 6 M Gdm·Cl.

The same experiment was carried out in the presence of DHT, a higher affinity steroid, instead of 17β-estradiol. The results are summarized in Table IV. The same trends were observed in the presence of DHT. An important difference is that the decrease in anisotropy in the presence of 17β-estradiol reaches equilibrium in less than 1 h for each point in the titration; however, in the presence of DHT, the approach to equilibrium in the first part of the titration between 1.7 and 3.5 M Gdm·Cl takes many hours. By contrast, above 3.5 M Gdm·Cl, in the range up to 5.2 M Gdm·Cl, the kinetics of the approach to equilibrium of the anisotropy values in the presence of DHT and 17β-estradiol are essentially the same; the anisotropy change is complete within a few minutes, and

Table IV: Effect of Steroids on the Dimer Dissociation of rSBP by Gdm·Cl[a]

	% anisotropy of DNS-rSBP	
guanidinium chloride (M)	17β-estradiol	5α-dihydrosterosterone
0	100	100
1.5	100	100
1.75	62	100
2.0	31	63
2.5	12	33
3.0	0	13
3.5	0	5

[a] The anisotropy values between 0 and 3.5 M Gdm·Cl (11 °C) are assumed to reflect the entire dimer dissociation curve (see Figure 6 for conditions). The percent anisotropy represents the fractional change for the titration curve over this concentration range of Gdm·Cl. Each value has an estimated error of ±8%. The rSBP concentration was 1 μM, and the steroid concentration was 2 μM for each titration.

there is no evidence of a steroid-dependent kinetic effect.

The kinetics in the dansyl anisotropy at concentrations less than 3.5 M Gdm·Cl appear to be related to the different rates of dissociation of the two steroids. To investigate the effect of Gdm·Cl on equilibrium steroid binding, parallel 11 °C experiments were conducted, the change in anisotropy of the fluorescent steroid DHE, bound to unlabeled rSBP, being monitored as a function of time and Gdm·Cl concentration. At 4 °C, the equilibrium binding of DHE in dilute serum is about 4-fold stronger than that of 17β-estradiol and about 10-fold weaker than that of DHT (see Table III). Assuming that the differences in steroid affinity are dominated by the rate of steroid dissociation, which is the case for 17β-estradiol and DHT (Mickelson & Petra, 1978), the effects of Gdm·Cl on DHE binding to rSBP should be intermediate between those on 17β-estradiol and DHT binding. The fraction of bound DHE was measured by exciting at the isosbestic point and detecting the anisotropy of the fluorescence at the isoemissive point. Under these conditions, the anisotropy changes are directly related to changes in equilibrium binding (Lakowicz, 1983). Perturbation of the DHE fluorescence anisotropy was evident at lower concentrations of Gdm·Cl than that required to perturb the fluorescence anisotropy of the DNS-labeled protein in the presence of 17β–estradiol or DHT. For example, unlabeled rSBP with about 90% of the total DHE bound in the absence of Gdm·Cl binds about 70% of the steroid at 0.5 M Gdm·Cl, and equilibrium was reached within a few minutes. By contrast, at 1.75 M Gdm·Cl (where in the presence of 17β-estradiol the DNS-labeled protein anisotropy starts to decrease) about 15% of DHE is bound, and the time for the steroid anisotropy to reach equilibrium was about 1 h. At higher concentrations of Gdm·Cl, this equilibrium is reached in substantially shorter time. For example, at 2 M Gdm·Cl less than 5% of DHE is bound, and equilibrium of the DHE fluorescence anisotropy is reached in less than 1 min; at 3 M Gdm·Cl (the plateau region in the DNS-rSBP fluorescence anisotropy at 11 °C—see Figure 6 and Table IV) there is no evidence of DHE binding.

Renaturation of Native and Dansyl-Labeled rSBP. The proteins were denatured in either 2.5 or 5.2 M Gdm·Cl at 11 °C. The samples were then dialyzed against buffer containing either no steroids or else varying concentrations of different steroids. These results are summarized in Table III. It is noteworthy that essentially no binding activity was recovered if renaturation was carried out in the absence of steroid. If steroids were present, however, there was partial recovery of [^{3}H]DHT binding activity. Although, as indicated in Table III, full binding activity was never recovered after renaturation, there was a correlation between the percent activity recovered in the presence of different steroids and their respective binding activities. rSBP exposed to only 2.5 M Gdm·Cl and dialyzed in the presence of saturating DHT regained the greatest binding activity.

The fluorescence anisotropy of renatured dansyl-labeled rSBP was equal to or slightly greater than that measured prior to denaturation (Table II), and the dansyl fluorescence emission spectrum was shifted about 10 nm to higher energy (Figure 5). In addition, as indicated in Table II, the fluorescence lifetime increased by about 2 ns. This leads to a higher apparent value for the calculated rotational correlation time. A greater rotational correlation time could result from aggregation to higher oligomers, or as explained under Discussion, other factors could be important. The shift in the emission spectrum and the change in the fluorescence lifetime of the dansyl probe were essentially independent of renaturation condition; there was no correlation between the alteration in the fluorescence properties of the dansyl group and the recovery of the steroid-binding activity. Thus, while steroids are essential for recovery of activity, they do not appear to be essential for dimer formation.

Discussion

On the basis of the results presented here, we propose that the steroid-binding interaction in SBP is linked to the protein subunit–subunit interaction. This model accounts for the observed stoichiometry of steroid binding. It also predicts that the dimeric form of SBP should be stabilized by specifically bound steroids and that steroids of decreasing affinity should be less effective in providing a stabilizing influence. We have examined this model by studying the influence of different affinity steroids on Gdm·Cl perturbation of rSBP. To monitor the steroid-binding interaction, we used DHE, a naturally fluorescent estrogen which binds with moderately high affinity to rSBP. The changes observed in the fluorescence anisotropy and acrylamide quenching of this chromophore would be related to structural perturbations occurring at the rSBP steroid-binding site as well as to decreased binding affinity. To monitor perturbation of the protein subunits, we covalently labeled the protein with the dansyl group. The change in the fluorescence anisotropy was used as an indicator of dimer dissociation and denaturation. The fluorescence anisotropy and spectral properties of the dansyl probe were used in conjunction with [^{3}H]DHT binding to both labeled and unlabeled rSBP to characterize renaturation.

Nature of the Steroid-Binding Site. In our initial studies comparing the steroid-binding sites of human and rabbit SBP, with the estrogen equilenin as a fluorescent probe, we observed that the emission of the bound steroid was shifted to lower energy (Ross et al., 1982; Örstan et al., 1986). We observe the same fluorescence shift with DHE bound to rSBP (Figure 1). On the basis of model studies, the emission shift of the bound steroid was ascribed to the 3′-hydroxyl group forming a hydrogen bond with a proton acceptor in the protein (Örstan et al., 1986). In addition, steady-state acrylamide quenching of the equilenin complex with SBP was interpreted as indicating limited accessibility of the bound steroid to the bulk solvent. The present acrylamide quenching results for the DHE complex with rSBP, combining observations from both time-resolved and steady-state experiments, show that the quenching of the bound steroid is due to static, not dynamic, interactions. Therefore, we conclude that the aromatic rings of the bound steroid (the A and B rings) are completely shielded from bulk solvent.

Two possible models were examined for the pure static binding interaction (no dynamic quenching of bound steroid;

see Figure 3). The first model was a random distribution of quenchers with a certain probability of being within a reaction volume that allows instantaneous, nondiffusional fluorescence quenching of the steroid. Because the random distribution model obeys Poisson statistics ($e^{V[Q]}$), it is expected that the $F_{o,b}/F_b$ contribution for the bound species alone should exhibit upward curvature as a function of linearly increasing quencher concentration. The second model was equilibrium association of the fluorophore with the quencher; binding of quencher at (or near) the same site where the probe is bound is an equivalent description. Because, this model obeys the law of mass action, the $F_{o,b}/F_b$ contribution for the bound species alone should increase linearly as a function of linearly increasing quencher concentration. As shown in Figure 3, however, both pure static models fit the steady-state quenching data equally well. In addition, it should be pointed out that the steady-state quenching data are also fit equally well by the mixed dynamic and static model. The latter model, however, can be rejected because, above 1 M acrylamide, the mean fluorescence lifetime and the dominant 6.8–7.1-ns component of DHE bound to the protein are essentially independent of acrylamide concentration. Since $K_{sv,b}$, obtained from the equilibrium binding model, and V_b, obtained from the random distribution model, are small, their differential effect on the steady-state quenching is difficult to distinguish. This is seen by examination of the theoretical curves in Figure 3 for the two static quenching models, by comparison of either the curves through the data, which include the effect on the total fluorescence intensity, or the curves extracted for the bound steroid, which include the effect on the bound steroid fluorescence alone. The upward curvature predicted by random distribution is clearly evident by the curves through the data; at low concentration of acrylamide this curve falls below the curve predicted by the equilibrium binding model, and at higher concentrations of acrylamide both curve cross. If one accepts the equilibrium binding model, the magnitude of K_{sv} is what would be expected for weak, nonspecific binding. Nonetheless, regardless of which static model is used to fit the steady-state quenching data, it is clear that the bound steroid is well buried in the protein structure and inaccessible to dynamic quenching.

Steroid Binding and Subunit Dissociation of rSBP in Guanidinium Chloride. Low concentrations of Gdm·Cl that do not result in subunit dissociation (less than 1 M) do result in perturbation of steroid binding. This is evident from the increased steady-state fluorescence quenching of DHE in the perturbed complex, shown in Figure 4. The quenching and fluorescence lifetime parameters are summarized in Table I. No change is observed in the mean fluorescence lifetime of the bound steroid. Consequently, the increased steady-state fluorescence quenching of bound DHE observed in the presence of up to 1 M Gdm·Cl is not related to an increase in the accessibility of the steroid bound to the quencher. Rather, there is decreased affinity of the steroid with a concomitant increase in total quenching due to a larger free fraction; the fractions of free and bound based on quenching are consistent with those observed from fluorescence anisotropy of DHE. In addition, the observed changes in the values of the acrylamide quenching constants of the bound steroid, fitting the different quenching models, are not significant, and the two static quenching models cannot be distinguished (Table I). At these low concentrations of Gdm·Cl, moreover, there is no change in either the fluorescence anisotropy or fluorescence lifetime of the dansyl label on the DNS-rSBP steroid complexes; the anisotropy is that of the native, dimer protein. *Thus, the reduction of steroid-binding efficiency due to Gdm·Cl perturbation results from a reduction in the free energy of steroid binding without subunit dissociation.* This could be caused by minor disruption of subunit–subunit and/or intrapolypeptide interactions. The perturbation, however, is insufficient to change the accessibility of the steroid-binding site to acrylamide, yet there is a substantial decrease in steroid affinity.

Between 0 and 1.5 M Gdm·Cl the fluorescence anisotropy of DNS-rSBP is stable. Between 1.5 and 2.0 M Gdm·Cl, where there is marked decrease in steroid binding, the anisotropy begins to decrease (Figure 6). To assess the effect of steroid binding upon subunit dissociation and denaturation, we compared 17β-estradiol and DHT (Table IV); 17β-estradiol has about a 100-fold lower affinity than DHT for rSBP (Mickelson & Petra, 1978). The anisotropy of the 17β-estradiol complex starts to decrease at lower Gdm·Cl concentrations than that of the DHT complex. Both complexes reach the same limiting anisotropy at about 3.5 M Gdm·Cl. Thus, it is evident that the strength of steroid interaction contributes to the stability of the dimeric protein structure.

The subunit dissociation/denaturation of the 17β-estradiol complex, monitored by the decrease in steady-state anisotropy of DNS-rSBP, can be resolved into at least two phases at 11 °C (Figure 6). In the first phase (1.5–3 M Gdm·Cl), the mean rotational correlation time, calculated from the steady-state anisotropy, decreases by more than a factor of 2 from about 24 to about 10 ns (Table II). The molecular volume of the unperturbed protein, V, estimated from the Stokes–Einstein equation (Einstein, 1906)

$$V = kT\phi/\eta \qquad (12)$$

where k is Bolzmann's constant, T is the absolute temperature, and η is the viscosity, predicts a hydrodynamic raidus of only 22 Å. By comparison, horse liver alcohol dehydrogenase, a dimer protein of molecular weight comparable to that of rSBP, has molecular dimensions of 45 × 55 × 110 Å (Creighton, 1984), and the equivalent volume sphere yields a radius of about 40 Å. The low estimate for the hydrodynamic radius of DNS-rSBP requires considerable segmental motion of the dansyl probe, in both the native and the Gdm·Cl-perturbed protein. As indicated in eq 5, the calculated rotational correlation time is a harmonic mean of all the probe motions including global rotation (whole molecule rotation) and segmental motions. Consequently, we cannot entirely eliminate the possibility that, in addition to dimer dissociation after loss of steroid binding, there is some unfolding of dimers and monomers in the first phase of the Gdm·Cl perturbation. It is clear, however, that the Gdm·Cl perturbation of DNS-rSBP at 11 °C is at least a three-state process. The stable plateau is indicative of a stable intermediate, probably the rSBP monomer. If steroid binding is energetically linked to the protein subunit–subunit interaction, we might expect the initial phase of the perturbation to be dominated by dimer–monomer dissociation. This would mean that the time required for complete equilibration of the SBP monomer–dimer concentrations would in turn affect the time for complete equilibration of steroid binding. Indeed, we observed an increase in the time required to reach equilibrium steroid binding when the Gdm·Cl concentration was increased to the range where protein subunit dissociation takes place. We would also expect that higher affinity steroids should stabilize the protein subunit interactions and make the protein less susceptible to the effects of Gdm·Cl. And this is what we observed when we compared the perturbation in the presence of 17β-estradiol and with that in the presence of DHT (see Table IV). If the monomers bind

steroids weakly, or not at all, subsequent denaturation of the monomers should be steroid independent. In fact, above 3 M Gdm·Cl the dansyl anisotropy perturbation is steroid independent. This is consistent with the observation that at 2 M Gdm·Cl less than 5% of the estrogen DHE is bound to unlabeled SBP. *These results indicate that the monomers formed in the presence of Gdm·Cl have essentially no affinity for steroids and that the anisotropy change above 3 M Gdm·Cl is dominated by unfolding of the monomers.* However, since Gdm·Cl clearly affects steroid binding, we cannot exclude the possibility that monomers in plasma, if they exist, could be competent to bind steroids.

It is evident that significant denaturation occurs by 2.5 M Gdm·Cl because, after dialysis to remove Gdm·Cl, the maximum recovery of [^{3}H]DHT binding is about 50%. Consequently, dissociation and denaturation/unfolding by Gdm·Cl cannot be easily resolved. The same has been observed for other oligomeric proteins, suggesting that native-like intermediates of association exist at intermediate denaturant concentration close to the oligomer–monomer transition (Jaenicke et al., 1987). The presence of such intermediates may partly account for the kinetic behavior observed in the case of rSBP perturbation below 3 M Gdm·Cl. If there is substantial overlap in the dependence on Gdm·Cl concentration of destabilization of the dimers and denaturation of the monomers, it is unlikely that two distinct phases would have been observed in the change in fluorescence anisotropy of the dansyl probe. The Gdm·Cl titration at 21 °C, seen in Figure 6, has this behavior.

The recovery in steroid-binding activity of unmodified rSBP and DNS-rSBP under different renaturation conditions indicated that proper refolding of the protein is dependent on the prsence of steroids which are specific for native rSBP; higher affinity steroids produced the higher regain in binding activity, and the absence of steroid resulted in essentially inactive protein. Whether or not steroids were present, the fluorescence anisotropy and mean lifetime of the dansyl group of the renatured protein were the same. The change in the calculated rotational correlation time of renatured rSBP could result from aggregation, decreased segmental flexibility, or alteration of the orientation of the probe with respect to the molecular axes of the protein. The last possibility would require a nonspherical structure for rSBP. Since the dansyl modification was carried out in the presence of saturating concentrations of DHT, to protect the integrity of the steroid-binding site, and since we obtained an extrinsically labeled protein with unaltered steroid-binding activity, it is not likely that the dansyl group is reporting on structural dynamics at the steroid-binding site. This conclusion is supported by the lack of correlation between the dansyl spectral properties of the renatured protein and the conditions (plus or minus steroids) used for renaturation. The observation that after renaturation there is a blue shift in the emission spectrum and an increase in the mean fluorescence lifetime of the dansyl group could mean that the protein conformation after renaturation is not exactly the same as that of the native protein but rather that of an intermediate or related form. If so, then depending upon the renaturation conditions, this intermediate can partially regain steroid-binding activity. Alternatively, depending on the renaturation conditions, there could be different distributions of fully active, partially active, and inactive dimers. The current data cannot distinguish among these possibilities.

In summary, our results indicate that with Gdm·Cl perturbation, subtle but significant changes occur in the steroid-binding site in dimeric SBP before subunit dissociation takes place. It is evident that the two events are linked processes because the subunit dissociation is steroid dependent. From the steroid dependence of renaturation, it appears that the complete information for the native, biologically active structure of SBP is not present in the mature form of the active, secreted protein, which circulates in blood. One possible explanation is that some structural information, which determines the correct configuration of the steroid-binding site, is lost or altered during posttranslational processing. Alternatively, it is possible that a specific steroid, such as DHT or testosterone, is present during protein folding in vivo. Whatever the case, it is clear from our results that if the appropriate steroid or steroids are present during in vitro refolding, a conformational template is provided that aids in re-formation of an SBP steroid-binding site with native-like properties.

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Registry No. DHE, 1423-97-8; DHT, 521-18-6; 17β-estradiol, 50-28-2; testosterone, 58-22-0.

References

Azumi, T., & McGlynn, S. P. (1962) *J. Chem. Phys. 37*, 2413–2420.

Badea, M. G., & Brand, L. (1979) *Methods Enzymol. 61*, 378–425.

Bevington, P. R. (1969) in *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Creighton, T. E. (1984) in *PROTEINS Structure and Molecular Properties*, p 268, Freeman, New York.

Davenport, L., Knutson, J. R., & Brand, L. (1986) *Biochemistry 25*, 1186–1195.

Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.

Einstein, A. (1906) *Ann. Phys. (Leipzig) 19*, 371–381; as referenced in Einstein, A. (1956) *Investigations on the Theory of the Brownian Movement*, Dover Publications, New York.

Griffin, P. R., Kumar, S., Shabanowitz, J., Charbonneau, H., Namkung, P. C., Walsh, K. A., Hunt, D. F., & Petra, P. H. (1989) *J. Biol. Chem. 264*, 19066–19075.

Grinvald, A., & Steinberg, I. (1974) *Anal. Biochem. 59*, 583–598.

Hryb, D. J., Khan, M. S., & Rosner, W. (1985) *Biochem. Biophys. Res. Commun. 128*, 432–440.

Hurwitz, A. R., & Liu, S. T. (1977) *J. Pharm. Sci. 66*, 624–627.

Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117–237.

Kalantar, A. H. (1968) *J. Chem. Phys. 48*, 4992–4996.

Kawahara, K., & Tanford, C. (1966) *J. Biol. Chem. 241*, 3228–3232.

Kotite, N. J., & Musto, N. A. (1982) *J. Biol. Chem. 257*, 5118–5124.

Knight, A. E. W., & Selinger, B. K. (1971) *Chem. Phys. Lett. 10*, 43–48.

Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.

Mejillano, M. R., & Himes, R. H. (1989) *Biochemistry 28*, 6518–6524.

Mickelson, K. E., & Petra, P. H. (1974) *FEBS Lett. 44*, 34–38.

Mickelson, K. E., & Petra, P. H. (1978) *J. Biol. Chem. 253*, 5293-5298.
Moore, J. W., & Bulbrook, R. D. (1988) *Oxford Rev. Reprod. Biol. 10*, 181-236.
Örstan, A., Lulka, M. F., Eide, B., Petra, P. H., & Ross, J. B. A. (1986) *Biochemistry 25*, 2686-2692.
Paoletti, J., LePecq, J. B. (1969) *Anal. Biochem. 31*, 33-41.
Petra, P. H., & Lewis, J. (1980) *Anal. Biochem. 105*, 165-169.
Petra, P. H., Stanczyk, F. Z., Namkung, P. C., Fritz, M. A., & Novy, M. J. (1985) *J. Steroid Biochem. 22*, 739-746.
Petra, P. H., Kumar, S., Hayes, R., Ericsson, L. H., & Titani, K. (1986a) *J. Steroid Biochem. 24*, 45-49.
Petra, P. H., Namkung, P. C., Senear, D. F., McCrae, D. A., Rousslang, K. W., Teller, D. C., & Ross, J. B. A. (1986b) *J. Steroid Biochem. 25*, 191-200.
Plymate, S. R., Namkung, P. C., Matej, L. A., & Petra, P. H. (1990) *J. Steroid Biochem. 36*, 311-317.
Rosner, W., Toppel, S., & Smith, R. N. (1974) *Biochim. Biophys. Acta 351*, 92-98.
Ross, J. B. A., Rousslang, K. W., & Brand, L. (1981) *Biochemistry 20*, 4361-4369.
Ross, J. B. A., Torres, R., & Petra, P. H. (1982) *FEBS Lett. 149*, 240-244.
Ross, J. B. A., Contino, P. B., Lulka, M. F., & Petra, P. H. (1985) *J. Protein Chem. 4*, 299-304.
Sakiyama, R., Pardridge, V. M., & Musto, N. A. (1988) *J. Clin. Endocrinol. Metab. 67*, 98-103.
Stern, O., & Volmer, M. (1919) *Phys. Z. 20*, 183-188.
Vermeulen, A. L., Verdonck, L., Van der Straeten, M., & Orie, M. (1969) *J. Clin. Endocrinol. Metab. 29*, 1470-1480.
Weber, G. (1952) *Biochem. J. 51*, 155-167.
Weber, G. (1953) *Adv. Protein Chem. 8*, 514-549.
Westphal, U. (1986) *Steroid-Protein Interactions II, Monogr. Endocrinol. 27*.

Estimating the Contribution of Engineered Surface Electrostatic Interactions to Protein Stability by Using Double-Mutant Cycles†

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ABSTRACT: Coulombic interactions between charges on the surface of proteins contribute to stability. It is difficult, however, to estimate their importance by protein engineering methods because mutation of one residue in an ion pair alters the energetics of many interactions in addition to the coulombic energy between the two components. We have estimated the interaction energy between two charged residues, Asp-12 and Arg-16, in an α-helix on the surface of a barnase mutant by invoking a double-mutant cycle involving wild-type enzyme (Asp-12,Thr-16), the single mutants Thr → Arg-16 and Asp → Ala-12, and the double mutant Asp → Ala-12,Thr → Arg-16. The changes in free energy of unfolding of the single mutants are not additive because of the coulombic interaction energy. Additivity is restored at high concentrations of salt that shield electrostatic interactions. The geometry of the ion pair in the mutant was assumed to be the same as that in the highly homologous ribonuclease from *Bacillus intermedius*, binase, which has Asp-12 and Arg-16 in the native enzyme. The ion pair does not form a hydrogen-bonded salt bridge, but the charges are separated by 5-6 Å. The mutant barnase containing the ion pair Asp-12/Arg-16 is more stable than wild type by 0.5 kcal/mol, but only a part of the increased stability is attributable to the electrostatic interaction. We present a formal analysis of how double-mutant cycles can be used to measure the energetics of pairwise interactions.

Electrostatic interactions are of fundamental and ubiquitous importance in binding, catalysis, protein folding, and the assembly of macromolecules (Perutz, 1978; Warshel & Russell, 1984). Special attention has been paid to the role of electrostatic interactions in stabilizing α-helices (Hol, 1985; Sali et al., 1988; Serrano & Fersht, 1989; Nicholson et al., 1988; Marqusee et al., 1987). Two types of electrostatic interactions in particular have been analyzed: charged residues with the helix dipole (Sali et al., 1988; Serrano & Fersht, 1989; Nicholson et al., 1988) and the interaction of charged residues within the helix (Nicholson et al., 1988; Marqusee et al., 1987). While several analyses indicate a value of 1.5-2.0 kcal/mol for the charge–helix dipole interaction, no quantitative values have been found for exposed charge–charge interactions on the surface of a helix, although qualitative evidence has been presented by Baldwin and co-workers (Nicholson et al., 1988; Marqusee et al., 1987). The small ribonuclease from *Bacillus amyloliquefaciens*, barnase (Hartley, 1989), has proved to be an excellent system for determining with high precision the energetics of interactions responsible for protein stability (Sali et al., 1988; Serrano & Fersht, 1989; Kellis et al., 1988, 1989). The enzyme of M_r 12 382 consists of a single polypeptide chain with no disulfide cross-links (Mauguen et al., 1982). It undergoes reversible unfolding induced either thermally or by solvents. Measurements of the energetics of unfolding of wild-type and mutant proteins may be used to quantify energies of interaction between side chains (Kellis et al., 1988, 1989) or long-range electrostatic interactions (Sali et al., 1988; Serrano & Fersht, 1989). Identical results are found from

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