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Investigation of a 17β-Estradiol-monoclonal Antiestradiol Antibody Binding Mechanism Using Dilute Solutions of Organic Solvents

S. de Lauzon, K. M. Rajkowski* and N. Cittanova†

Département de Biochimie, Faculté de Médecine, 45 rue de saints-Pères, 75006, Paris, France

Quantitative understanding of steroid hormone transport and receptor-mediated action requires knowledge of the bonding forces involved in each steroid–protein complex and the effects of a biological environment on these forces. An approach to these problems using dilute solutions of water-miscible organic solvents, with a range of polarity, dielectric and hydrogen bonding properties, was tested on an estradiol–antiestradiol antibody binding system on the basis that comparing the effects of the solvents would both permit the importance of hydrophobic and hydrogen bonding to be differentiated and give information on the effects of the environment on the reaction. The results were compared with thermodynamic measurements. All the solvents reduced the Gibbs free energy of binding as a function of their concentration in the medium. The decreases were virtually a monotonic function of their dielectric constant, indicating reduced hydrogen bonding. Analysis of the decreases in terms of the solvents' hydrogen bonding and polarity properties supported this. Thermodynamic measurement showed the binding reaction was enthalpy-driven with, overall, a slightly unfavorable entropy contribution. This again showed the hydrophobic effect was not the main bonding force. The most deleterious solvent, *iso*-propanol, not only decreased the enthalpic contribution to binding but rendered the entropic contribution more favorable. This approach still does not allow the relative importance of hydrogen bonding and van der Waals contacts in the actual binding to be differentiated but it does give indications on how a biological environment may affect a steroid–protein binding reaction *in vivo*.

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INTRODUCTION

The protein binding of steroid hormones plays a major role both in their delivery to target tissues and in their mode of action. Despite a vast amount of available information on steroid–protein binding interactions (e.g. reviews [1, 2]) unanimity has still not been reached, for example, on a quantitative model explaining how steroid hormones, overwhelmingly protein-bound in the circulation, cross the capillary epithelium of their target tissues [3–6]. One obstacle to the resolution of these problems may well be the incongruity of inferring quantitative *in vivo* binding distributions using parameters obtained from *in vitro* experiments carried out with simple buffers. This is incongruous because the energetic contributions to binding of hydrogen bonding and the hydrophobic effect will be strongly influenced by the physical nature of the milieu [7–9]. To correct for this and define the effects of biological environment on a given steroid–protein binding reaction not only must the physical properties of the environment be known but also the relative importance of each of the various binding forces (ionic bonds, van der Waals contacts, hydrogen bonding and the hydrophobic effect).

Classical thermodynamic studies on ligand–protein interactions give the overall enthalpic and entropic contributions to binding and, providing the protein is stable over a sufficient temperature range, the importance of the hydrophobic effect can be found from heat capacity changes [10]. However the degree of hydrogen bonding and the contribution of van der Waals contacts cannot be calculated directly from these experiments.

More complete knowledge can be obtained from molecular dynamics simulations applied to high resol-

*Correspondence to K. M. Rajkowski at his present address. INSERM U33, Laboratoire d'Hormones, Hôpital de Bicêtre, 80 Av. Général Leclerc, 94276 Le Kremlin-Bicêtre, France.
†Deceased.
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ution X-ray crystallographic or NMR structural data, which also allows calculation of heat capacity changes from the water-accessible surface areas of the ligand and empty binding site [11]. However, these techniques are cumbersome and their use is restricted since either good quality crystals must be available or, for NMR spectroscopy, the protein's molecular weight should not exceed 30kDa. It thus seems important to devise other ways of attacking this problem in order to see how the physical particularities of a biological milieu may affect ligand binding.

Steroid hormones are composed of a hydrocarbon skeleton to which few (for most, two) polar functions are attached. Except in the case of the estrogens they do not have ionizable functions and, for the steroid used here, the pK of 17β-estradiol's phenol is 10.0–10.2 [12] and it is not ionized at a physiological pH. Ionic bonding and salt bridges do not, therefore, have to be taken into account. For hydrogen bonding, not only are there limited possibilities but also Chothia and Janin [13] have proposed that, even for protein–protein associations in which more such bonding possibilities exist, hydrogen bonding would not make a major contribution to binding because the bonds in a complex are equalled by corresponding bonds between the dissociated reagents and water. Similarly, they pointed out that van der Waals contacts should only make a small contribution since, again, contacts in a complex must be set against dissociated reagent–water contacts. From these considerations the main contribution to the energy for steroid binding would come from hydrophobic bonding. Nevertheless, evidence has been provided [14] that hydrogen bonds and van der Waals contacts have a more important role to play than allowed by Chothia and Janin. For example, hydrogen bonds to ligand in the site's low dielectric environment are of higher energy than those between free ligand and the high dielectric solvent. Further, estradiol has an aromatic ring, which may be involved not only in van der Waals stacking but also in hydrogen bonding [15, 16]. The question thus remains open as to which of these forces is likely to be most important in steroid–protein binding.

Although structural information is needed to determine the van der Waals contacts in a complex it was decided that information could be obtained about hydrogen and hydrophobic bonding by determining the effect of changes in the environment, caused by addition of small amounts of water-miscible organic solvents, on the ligand binding energy. Providing the solvent concentrations were too small to induce significant protein unfolding then they would be expected to have a 2-fold effect: they would disrupt the solvent shells around the free ligand and in the empty site as a function of their polarity, decreasing the energy contribution of hydrophobic bonding and their presence would also lower the dielectric constant of the medium and thus lessen the difference between the strengths of ligand-site hydrogen bonds and free ligand-water/empty site-water hydrogen bonds [9, 14]. The differential effects of the different solvents on the ligand binding energy could then be used to gain an insight into the binding forces involved. Although the effect on steroid–protein binding of modulating the hydrophobic environment with KCl and urea has been reported [17], the use of dilute organic solvents to this end has not, to out knowledge, previously been attempted.

The solvents chosen for the study were three aliphatic monoalcohols of different polarity (methanol, ethanol, *iso*-propanol), a di-alcohol (ethylene glycol) and, because all these solvents are amphiprotic, an aprotic solvent (acetone) was included. The binding of 17β-estradiol by a high affinity antiestradiol monoclonal antibody was studied, rather than using sex hormone binding globulin or the estradiol receptor, because the antibody was very stable and readily available in sufficient quantities.

MATERIALS AND METHODS

Estradiol was obtained from Roussel-Uclaf (Paris, France) and 2,4,6,7[^{3}H]estradiol (3.26 TBq/mmol) was from Amersham (Bucks., England). Organic solvents were of fluorescence spectroscopic grade.

The monoclonal antiestradiol antibody was raised in this laboratory [18] against estradiol-3-*O*-carboxymethyloxime–bovine serum albumin. Immunoglobulins were purified from ascites fluid by precipitation with ammonium sulfate at 50% saturation and ion-exchange chromatography on DEAE-Sephacel (Pharmacia, Uppsala, Sweden).

Protein concentrations were determined by the Lowry method [19] and the concentrations of stock estradiol solutions were verified spectroscopically.

Estradiol–protein binding parameters were determined by equilibrium microdialysis [20] in low ionic strength buffer (5 mmol/l phosphate buffer pH 7.2) containing 100 mg/l gelatin and 0 to 7% v/v of one of the organic solvents (methanol, ethanol, *iso*-propanol, ethylene glycol or acetone). Dialysis was between two 80 μl half-cells separated by a Visking dialysis membrane: the protein solution (1 nmol/l) was introduced into one half-cell and the estradiol solution (1.5 to 4.5 nmol/l), with a constant amount of tritiated steroid (10,000 dpm), was introduced into the other. Dialysis was continued at constant temperature (4°C, except for thermodynamic determinations) for 20 h with shaking. (Preliminary experiments showed 20 h was required for a close enough approximation to equilibrium.) Then a 50 μl aliquot was taken from each half-cell and the radioactivity counted. Preliminary experiments showed that radioactivity counting efficiency was not significantly altered by the presence of the organic solvents in the concentration ranges used here. A single experiment comprised five replicate determinations with a range of estradiol concentrations for each concentration of an organic solvent, including the pure buffer control. Each experiment was carried out three times.

Summation of the total radioactivity measured in

both half-cells allowed rejection of outliers differing from the mean, in that experiment, by two standard deviations. Binding parameters were calculated by weighted least squares minimisation [21] but non-specific binding was found to be insignificant and the model for a single class of binding sites was used. Weighting was the normalized reciprocal variance at each point of the (free + bound) ligand concentrations as calculated from the counting results.

RESULTS

Initial experiments were carried out on the binding of estradiol by Fab fragments of the antibody but a Scatchard representation of the binding curve was clearly convex with only 50% of the expected number of binding sites (not shown). Identical results were obtained using repurified tritiated estradiol and also the same binding curve was obtained using different concentrations of tritiated estradiol in the absence of unlabeled estradiol. This effect was not found for the binding of estradiol by purified, undigested immunoglobulin, which showed high affinity binding ($k_a = 14.1 \pm 1.6$ l/nmol at $n = 2.3 \pm 0.2$ equivalent sites) and insignificant non-specific binding. A Scatchard representation is shown in Fig. 1. The immunoglobulin preparation was used henceforth.

Low ionic strength buffer was used to minimize ion disruption of water structure [22] but it was still necessary to include an inert protein in the buffer in order to prevent steroid adsorption by glass- and plasticware. Gelatin was used for this since it is one of the proteins least interfering with steroid–antibody interactions [23] but, to avoid this protein affecting the properties of the buffer, a minimum concentration was sought. A concentration of 100 mg/l was the lowest at which no decrease in the number of binding sites (equivalent to protein loss) was detected.

The binding free energy for the estradiol–immunoglobulin interaction was found to decrease as a linear function of the mole fraction solvent for all five of the solvents tested (Fig. 2). In all these cases the number of binding sites per protein molecule was maintained except with the highest concentration (7% v/v) of *iso*-propanol ($n = 1.7$) at which the change in binding energy also deviated from linearity. This point was excluded from ensuing calculations. The weighted linear regression slopes of these plots are given in Table 1 as are those for the similarly linear plots as a function of the fraction of solvent by weight. These two measures—mole fraction and fraction by weight of solvent—are not directly proportional to each other, thus binding energies cannot be linearly related to both of them simultaneously. Nevertheless, given the virtually identical correlation coefficients (Table 1), it was not possible to establish of which of these measures the binding free energy was more a direct function.

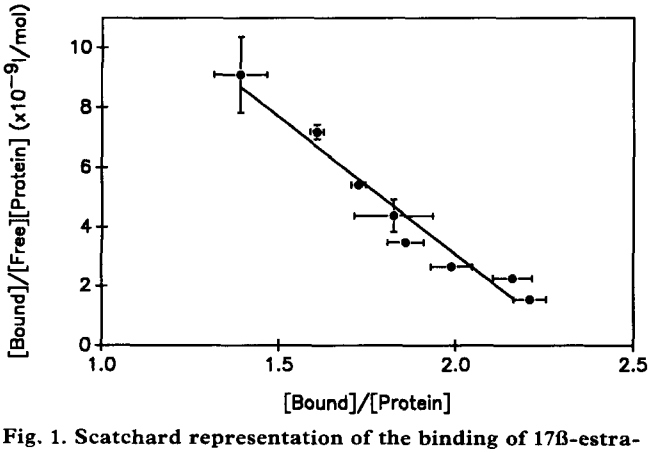

Fig. 1. Scatchard representation of the binding of 17ß-estradiol (1.5 to 5 nmol/l) by purified monoclonal antiestradiol immunoglobulin (1 nmol/l) in 5 nmol/l phosphate buffer pH 7.2 containing 100 g/l gelatin.

The presence of organic solvents in buffer changes its pH [24]. Although the pH changes found at the solvent concentrations used here (7.20 to 7.37) were small it was necessary to ensure that it was not this which was responsible for the decreased binding energies. Figure 3 shows the binding free energy as a function of pH, in the absence of organic solvent. It can be seen that there was no significant effect over the pH range tested.

An attempt was made to determine if the effect of the solvents could be interpreted in terms of an altered protein/solvent partition coefficient for estradiol due to increased estradiol solubility in the solvent. No significant changes in estradiol solubility could be found with the solvent mixtures used for the binding experiments; any changes were less than the experimental errors in the solubility determinations.

The slopes of the plots in Fig. 2 were compared with various physical properties of the solvents. Figure 4 shows plots of the slopes given in Table 1 as a function of the reciprocal of the dielectric constants. These two curves are virtually monotonic nonlinear functions of the dielectric constants. To analyze these results further in terms of solvent properties, the general empirical formula proposed by Taft and coworkers [25] was used:

$$XYZ = XYZ_0 + a\alpha + b\beta + s\pi^*$$

where XYZ is some free energy-related property and a, b, and s are variables representing measures of the susceptibility of XYZ to each of the solvent properties α, β and π^*. The latter are empirical constants for the solvent—obtained by a solvatochromic method, on an *ad hoc* zero to (approximately) unity scale—representing the hydrogen bonding acidity, basicity and polarity–polarizability of that solvent, respectively. The values of these constants for the solvents used here are given in Table 2. Since the dependent variable XYZ is a slope ($\Delta\Delta G^\circ$) the parameter XYZ_0, i.e. the change in binding free energy with no added organic solvent, is zero and the equation becomes:

$$\Delta\Delta G^\circ = a\alpha + b\beta + s\pi^*$$

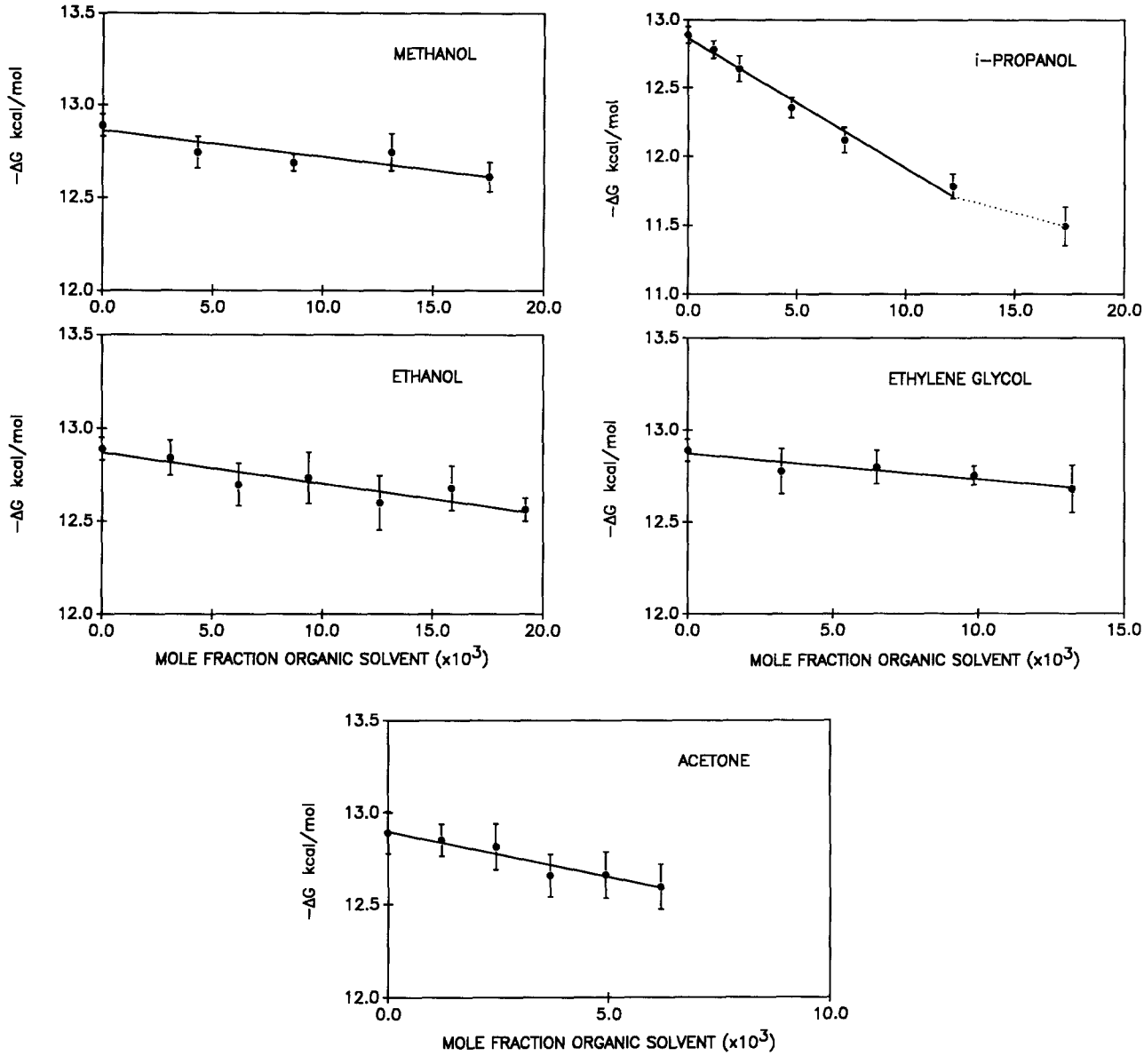

Fig. 2. The Gibbs free energy of 17β-estradiol binding by the monoclonal antiestradiol immunoglobulin as a function of the mole fraction of each organic solvent present in the buffer. The weighted linear regression lines for each plot are shown.

Resolving the over-determined system of simultaneous equations, one for each solvent, gave the values for the three variables representing the importance of each factor in the solvent effect on the binding energy for the reaction under consideration. Least squares minimization was used to solve these ill-conditioned equations and the values obtained for the constants were:

$\Delta\Delta G°$ as a function of mole fraction solvent:

$$a = -63.6 \quad b = 137 \quad s = -16.9 \quad (r = 0.76)$$

Table 1.

Solvent	Slope *f*(mole fraction) kcal/mol	r^2	Slope *f*(weight fraction) kcal/mol	r^2
Methanol	11.47 ± 4.10	0.85	6.38 ± 2.25	0.83
Ethanol	16.75 ± 2.09	0.96	6.75 ± 0.83	0.96
iso-Propanol	95.07 ± 5.60	0.99	29.24 ± 1.60	0.99
Ethylene glycol	14.04 ± 2.76	0.98	4.18 ± 0.82	0.98
Acetone	49.45 ± 5.32	0.95	14.46 ± 1.67	0.95

Weighted linear regression slopes of Gibbs free binding energy as a function of the fraction of organic solvent in the buffer, expressed as a mole fraction and as a fraction by weight. The corresponding correlation coefficients for each plot are shown.

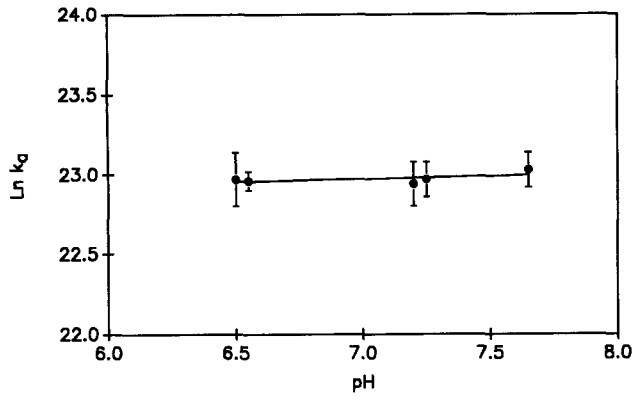

Fig. 3. The effect of buffer pH (6.5 to 7.6) on the Gibbs free energy of 17β-estradiol binding by the antiestradiol immunoglobulin in the absence of added organic solvent.

$\Delta\Delta G°$as a function of weight fraction of solvent:

$$a = -16.4 \quad b = 41.6 \quad s = -6.8 \quad (r = 0.77)$$

where r is the coefficient of multiple correlation.

The values in either set, which can be compared because all the variables in a given set are approximately on the same scale, indicate that the hydrogen bonding basicity of the solvent is the factor mainly responsible for the loss of binding affinity. (The negative values for s corresponds to a variable representing polarity, and not nonpolarity.) According to these results, increased hydrogen bonding acidity of the solvent relative to water would lead to an increase in binding energy.

A van t'Hoff plot of binding energies over a 4 to 20°C temperature range in the absence of organic solvent (Fig. 5) showed that the binding reaction was enthalpy-driven ($\Delta H° = -14.7 \pm 1.0$ kcal/mol), with a low, unfavorable entropy contribution ($-T\Delta S° = +1.8 \pm 1.0$ kcal/mol at 4°C). This supports the previous findings that the hydrophobic effect is not the most important bonding force here. Over the temperature range used the plot was linear and there was no evidence for an entropy compensation effect.

Finally, the effect of temperature on the binding reaction in the presence of the most deleterious solvent, *iso*-propanol, was determined. In order to avoid problems of solvent evaporation and of protein denaturation the experiments were carried out over only small temperature and solvent concentration ranges. The resulting van t'Hoff plots are shown in Fig. 6 and the changes in the enthalpic and entropic contributions to binding as a function of the mole fraction of *iso*-propanol are shown in Fig. 7. This figure shows a compensating effect, with a decreasingly unfavorable entropy contribution partially offsetting a decreasingly favorable enthalpy contribution to the binding free energy.

Table 2.

Solvent	α	β	π*
Water	1.017	0.14	1.09
Methanol	0.99	0.62	0.586
Ethanol	0.85	0.77	0.54
iso-Propanol	0.687	0.92	0.505
Ethylene glycol	0.792	0.51	0.932
Acetone	0	0.507	0.683
Ref.	26	27	25

Taft's empirical solvatochromatic constants representing hydrogen bonding acidity (α), hydrogen bonding basicity (β) and polarity–polarizability (π*).

DISCUSSION

Equilibrium microdialysis as used here, while laborious, is a very sensitive method for studying steroid–protein binding and there are few interfering factors to be controlled. However, even with high specific activity radiolabeled estradiol, the antibody binding affinity was so high that, to have statistically significant radioactivity counting rates, it was necessary to work with estradiol concentrations at the upper end of the desirable concentration range ($0.1\text{–}10 \times K_d$). This contributed to the errors associated with the binding parameters, though these were of the order of 10% and thus well within the limits normally accepted [28]. The

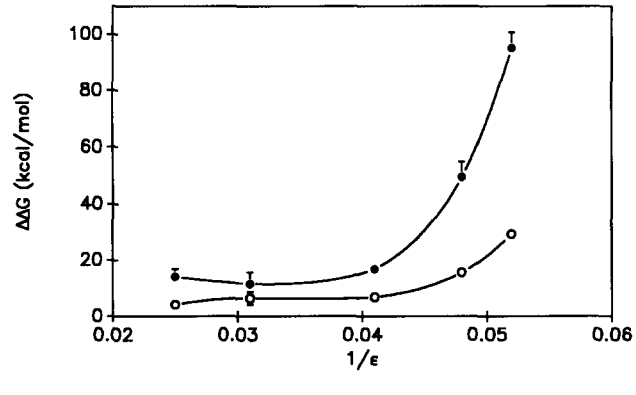

Fig. 4. The rates of decrease in the Gibbs free binding energy per mole fraction (●—●) and per weight fraction (○—○) of an organic solvent plotted as a function of the reciprocal of that solvent's dielectric constant.

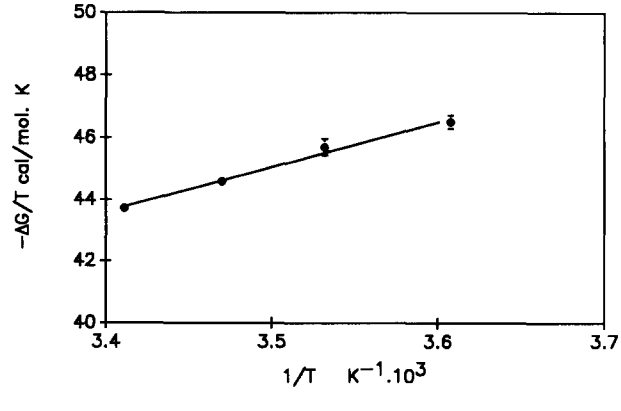

Fig. 5. Van t'Hoff plot for the binding of 17β-estradiol by the antiestradiol immunoglobulin over a 4 to 20°C temperature range. The weighted linear regression is shown and yields: $\Delta H° = -14.7 \pm 1.0$ kcal/mol, $\Delta S° = -6.6 \pm 3.6$ cal/mol K.

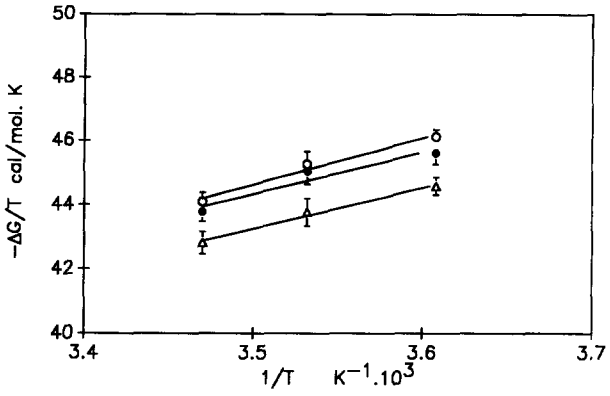

Fig. 6. Van t'Hoff plots for the binding of 17β-estradiol by the antiestradiol immunoglobulin over a 4 to 15°C temperature range in the presence of 0.5% (○—○), 1.0% (●—●) and 2.0% (Δ—Δ) *iso*-propanol.

use of long equilibration times also meant that thermodynamic measurements could only be made over a restricted temperature range to prevent evaporation of the medium, particularly when an organic solvent is present. Calculation of binding parameters by least squares minimization may appear unjustified when there is no (error-free) independent variable, the total ligand concentration in the protein-containing half-cell being calculated from the measured free and bound ligand concentration. Nevertheless, for a simple single site class binding model it has been shown that weighted least squares minimization gives the same results as a more rigorous maximum likelihood estimation [29].

The binding system examined here was chosen on the basis of the availability, affinity and stability of the steroid-binding protein. However it was not possible to simplify the system by working with antibody Fab fragments because of their over-complex binding curves; these were found not to be caused by impure radioactive ligand nor by differences in protein affinity for the cold and radioactive ligand. Similar convex Scatchard plots have been found with single-site steroid receptors and ascribed to protein dimerization [30]

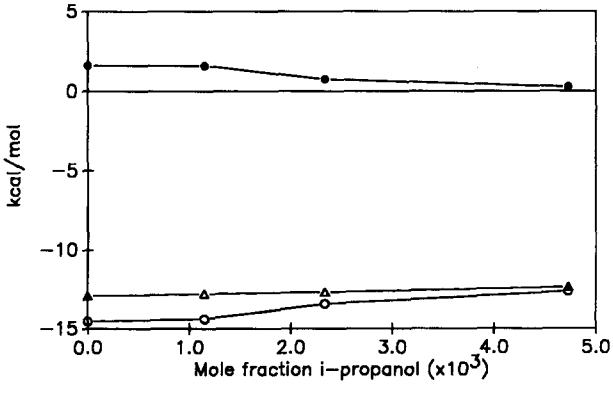

Fig. 7. Changes in the Gibbs free energy (Δ—Δ), enthalpy (○—○) and entropy (●—●) of 17β-estradiol binding by antiestradiol immunoglobulin as a function of the proportion of *iso*-propanol present in the buffer.

and to single site cooperativity [31]. Nevertheless it is difficult to see why cooperativity, stemming from protein conformational changes upon steroid binding, should occur with Fab fragments but not whole immunoglobulin molecules. A third explanation for the convex Scatchard plot is that, unlike the immunoglobulin, the Fab fragments are unstable in a low ionic strength medium but are stabilized by the presence of their ligand. Since undigested immunoglobulin did not give such convex curves, this was used for the experiments.

As would be expected, whatever the binding mechanism, the Gibbs free energy for estradiol binding by the antibody decreased when organic solvents were included in the buffer. Such a decrease has already been reported for estrogen receptor binding in the presence of 10% dimethylformamide [32]. The decreases found here appeared to be linear functions of the fraction of solvent present, at least until protein denaturation began to occur (with 7% *iso*-propanol). The small changes in binding energies, relative to their associated errors, within the small range of solvent concentrations used did not allow a differentiation to be made between whether linearity was better as a function of the mole fraction or the fraction by weight of solvent. The former would be the better measure for chaotropic activity (changes in hydrophobic bonding energy), which would depend on the relative numbers of molecules of water and solvent, while the latter would be a better measure for solvent dielectric properties (changes in hydrogen bonding energy). The finding that the slopes of the plots as a function of mole fraction solvent were different for each solvent does show that the effect depended on solvent properties and not simply on (small) changes in water concentration; as has recently been reported for the activity of the hydrolytic enzyme adenosine deaminase [33].

While the slopes of these plots were virtually monotonic nonlinear functions of the solvent dielectric constants no transformation, e.g. the form in the Clausius-Mossotti relation or that in the Kirkwood theory of dielectrics $[9\epsilon/(\epsilon + 2)(2\epsilon + 1)]$, could be found which linearized either of them. The free energy decreases did not appear to be a function of solvent dipole moment (plot not shown): acetone has a large dipole moment but also causes a large loss of binding energy.

The virtually monotonic curves as a function of dielectric constant suggested that the solvents were acting on hydrogen bonding energies rather than by solvent shell disruption (decreased hydrophobic bonding). This was further supported by setting the rates of decrease in binding energy into the empirical Taft equation relating free energy related processes to the hydrogen bonding and polarity properties of the solvents in which they occur [25–27]. The use of this equation, involving the properties of pure organic solvents, is justified here because the binding energy in solvent-free buffer is not taken into account in the calculation and the comparison is only between the

effects of the different solvents on binding energy changes. Nevertheless the caveat must be added that for a least squares minimization solution to the simultaneous equations with 3 unknowns it would have been preferable to use 15 sets of variables rather than 5. It is perhaps worthy of note that, by algebraic artifice, the slopes of the plots in Fig. 3 also correspond to (fictitious) binding free energies in pure solvent under the condition that the effects occurring in the 1–5% solvent concentration range, and only those effects, are extrapolated as acting in the same way up to 100% solvent. The results, giving solvent hydrogen bonding basicity a 7–8 times more important role than polarity, on the scale used, also indicated that solvent hydrogen bonding acidity would serve to increase binding energy. Unfortunately it is not possible to test this with solvents since water is already the most protic solvent. While hydrogen bonding acid solutes exist and could be tested as additives in the buffer, the problem remains that compounds with an aromatic ring might be partially "recognized" by the binding site and thus act as competitors for estradiol binding.

Classical thermodynamic experiments appeared to confirm, to the extent they could, the results obtained with the solvent studies, that is they showed that the hydrophobic effect was not the main contributor to the binding energy. (Including the cratic contribution to entropy of -7.98 cal/mol. K gives unitary energies of binding at 4°C of: $\Delta G°_u = -15.2$ kcal/mol, $\Delta H° = -14.7$ kcal/mol and $-T\Delta S°_u = -0.4$ kcal/mol). The absence of evidence for an entropy compensation effect here is understandable: the reaction is not entropy-driven, so any entropy compensation effect could only be small and only a relatively small temperature range was covered here. Wolff *et al.* [34] reported such an effect between 0 and 16°C for corticosterone binding by the glucocorticoid receptor but this had not previously been found by Schaumberg and Bojesen [35] and Spolar *et al.* [11] have noted that heat capacity changes, causing curvatures of van t'Hoff plots, usually only become significant at temperatures much higher than 20°C (e.g. Herron *et al.* [36]). At such temperatures, in the presence of organic solvents, protein denaturation would become the major effect [37].

The unfavorable entropy contribution to binding does not mean there is no hydrophobic bonding. The loss of rotational and translational degrees of freedom upon ligand binding corresponds to an entropy loss of approx. 50 cal/mol K, though approximately half is regained through new normal modes appearing [28]. If the overall entropy loss is approx. 25 cal/mol K (7 kcal/mol at 4°C) then there must be a favorable hydrophobic contribution to binding of about 5 kcal/mol at 4° to yield the final entropy loss of 2 kcal/mol ($T\Delta S$) given by the van t'Hoff plot. The free energy change to be expected from hydrophobic bonding of estradiol can be calculated approximately. The water accessible surface of estradiol is of the order of 500 Å^2 (a value of 568 Å^2 has been reported for cortisol [38]) and if, as has previously been found for antibody–hapten binding, 80–95% of the hapten surface is engulfed in the binding site [39] then some 800 Å^2 steroid and binding site surface would be rendered water-inaccessible. Using the heat capacity increment per square angström of *nonpolar* surface removed from water and the hydrophobic bonding free energy per unit heat capacity change, given by Spolar *et al.* [11], one obtains values of $\Delta Cp \approx 220$ cal/mol K and $\Delta G°hyd \approx -17$ kcal/mol at 4°C for this binding reaction. The latter value is close to that of -16 kcal/mol calculated by Wolff *et al.* [34], using Bondi surface areas, for the engulfment of progesterone in a protein binding site. This estimated hydrophobic bonding free energy is evidently out of proportion with the calculated 5 kcal/mol entropy change, underlining the lack of hydrophobic bonding here and even suggesting that much of the binding site surface is of a polar nature.

The finding that this steroid–antibody reaction did not follow the classical scheme of hydrophobic bonding reinforced by short-range contacts [14], as found for corticosteroid binding by hepatoma cell glucocorticoid receptors [34] and estradiol binding by uterine estrogen receptors [40], was unexpected. Enthalpy-driven steroid binding has, however, previously been found for cortisol and progesterone binding by human corticosteroid binding globulin [41] and progesterone is significantly more hydrophobic than estradiol. More recently, the integration of hydrophobic molecules into a phospholipid membrane has been shown to be enthalpy-driven [42], with van der Waals contacts providing the binding energy.

Of the enthalpic bonding possibilities here, hydrogen bonding to the C-17 hydroxyl group of estradiol is a candidate and the aromatic A ring could be a hydrogen bond acceptor. Hydrogen bonding to estradiol's phenolic hydroxyl group is less evident because the antibody was raised against estradiol coupled to albumin via its C-3 position so the steroid's C-3, and the phenolic group, is unlikely to be contained in the binding site. If it is, residues hydrogen bonding with the C-3 oxygen may exist, since a proton acceptor (nitrogen) also existed in this position in the steroid-albumin immunogen, but any bonding to the phenolic hydrogen would be fortuitous. With respect to van der Waals contacts it is relevant that a tyrosine residue has been shown to be involved in estradiol binding by the uterine receptor [43], presumably through stacking.

The reduction in hydrogen bonding by the organic solvents and the enthalpy-driven nature of the binding reaction do not in themselves mean that these hydrogen bonds are between the ligand and the site. They could be intra-protein bonds maintaining the structural integrity of the binding site. However, the thermodynamic results with increasing amounts of *iso*-propanol are evidence against this, all the more so that the increasingly favorable entropy contribution may even be underestimated, since rising *iso*-propanol concentrations would increasingly disrupt water structure and reduce such hydrophobic bonding as there is. If the solvent were breaking intra-protein hydrogen bonds

and loosening binding site structure then steroid binding would be accompanied either by a more unfavorable or no change in the entropy contribution compared to that in the absence of solvent, according to whether the steroid's presence rigidified the site or not. The more favorable entropy contribution rather indicates breaking of steroid–protein bonds so that bond rotations etc., otherwise forbidden to a tightly bound ligand, become possible.

Solvents can decrease hydrogen bonding indirectly or directly. Indirectly, reducing the milieu's dielectric reduces the energy difference between protein–steroid hydrogen bonds in the complex and dissociated reagent-water hydrogen bonds in the solution [14]. Directly, the solvents may act via their own hydrogen bonding properties as suggested here by the overriding importance of solvent hydrogen bonding basicity and not acidity. Solvent molecules replacing water in the binding site could reduce steroid binding in two ways: being larger than water they have a hindering effect on binding and having a lower hydrogen bonding valency than water they are less likely to act as hydrogen bonding bridges between the protein and ligand. (The importance of intercalated water molecules mediating protein–ligand bonding has been shown by Quiocho *et al.* [44].)

Overall these results show the dangers of inferring *in vivo* binding properties from *in vitro* results. Minton [45] has pointed out some of the problems, such as the presence of inert, space-filling macromolecules and structures meaning that reagent concentrations will be higher than expected and that most of a dissolved reagent will be in an environment contiguous to, and under the influence of, solid structures. (In this respect, the use of aqueous two-phase partitioning to determine steroid binding parameters [46] goes some way to imitating biological conditions since the environment contains 10–20% polymers and salts.) The results obtained here show that the effects of all small molecules, considered inert to a binding reaction, in a biological milieu must also be taken into account because of their effect on its polarity and dielectric. It is noteworthy that a value of 8.0–8.5, less than half the dielectric constant of pure *iso*-propanol, has been reported for the dielectric constant of whole blood [47]. Obviously no quantitive conclusions can be derived from this because the dielectric constant of such a heterogenous substance as blood is at best a weighted average over all the physical environments that coexist, compartmentalized, in it. Moreover, the conditions dictated by the aims of the present study—hypotonic buffer and low inert carrier protein concentration—are even further from those *in vivo* than are usual in binding studies, in particular the dielectric constant of an isotonic buffer would be smaller than that of buffer used here. Only qualitative inferences about how steroid binding reactions would be affected by *in vivo* conditions can be drawn from the present results both since Taft solvatochromic constants for biological fluids have not, to our knowledge, been determined and since effects of the milieu on steroid binding systems will depend on the relative importance of van der Waals, hydrogen and hydrophobic bonding for each particular system. Nevertheless these results reinforce the suggestion that binding reactions may be of very much lower affinity *in vivo* than *in vitro*—the presence of 5% *iso*-propanol leading to a 10-fold decrease in binding affinity here—and show that, while one would expect the solvent's polarity and structure to influence hydrophobic bonding, its composition also significantly influences hydrogen bonding. Therefore only those reactions in which the binding energy is largely provided by van der Waals forces may be relatively insensitive to the medium. (To reassure the more Dionysian reader, the maximum permitted blood ethanol level for drivers of 0.8 g/l would, in buffer, only correspond to a loss of 5 cal/mol in binding energy for the system examined here.)

While these results do show the potential utility of studying the effect of additives such as organic solvents for ligand binding studies more cases need to be studied, especially that of an entropy-driven binding system, in order to refine the method. The fluorescein–antifluorescein binding system studied by Kranz *et al.* [48] and Herron *et al.* [36] is one such system and the results would be all the more enlightening that the structure of that binding complex is known to high resolution. Determination of the effect on ligand binding of a biological environment's physical characteristics would, however, be problematic both because indicator molecules for determining those characteristics would be bound by endogenous proteins, such as albumin in serum, and because one is dealing with continua rather than discrete environments.

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