Ligand-Induced Self-Association of Human Luteinizing Hormone. Negative Cooperativity in the Binding of 8-Anilino-1-naphthalenesulfonate†

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ABSTRACT: The self-association of human luteinizing hormone (hLH) is enhanced in the presence of 8-anilino-1-naphthalenesulfonate (ANS). Sedimentation equilibrium measurements indicate that the hormone exists primarily as a dimer in the presence of excess ANS. It is shown that, for a self-associating protein system in which monomer and dimer have different affinities and/or capacities for ligand, both the shape and the position of the binding curve depend on protein concentration. Gel filtration and fluorescence measurements indicate that the hLH dimer has a single high affinity site ($K \gg 10^6\ M^{-1}$) for ANS while binding to the monomer is too weak to be observed. This leads to negative cooperativity in the binding and to a shift of the binding curve to lower free ligand concentration with increasing concentration of the hormone.

Cooperativity in the binding of ligands to multisubunit proteins is a subject of intense current interest because of the implications for biological control (see reviews by Koshland, 1969, 1970; Frieden, 1971; Phillips, 1974; Hammes and Wu, 1974; Dunne and Wood, 1975). The cooperative effects often result from subunit interactions in which the binding of the first ligand alters the apparent affinity of the remaining sites toward the same or other ligands. The binding of oxygen to hemoglobin is perhaps the most thoroughly studied example of this type of cooperativity (Antonini and Brunori, 1971; Saroff and Yap, 1972). However, cooperative effects can also arise from protein association if the different protein species (monomer, dimer, tetramer, etc.) have different affinities and/or capacities for ligand (Nichol et al., 1967; Frieden, 1971; Frieden and Colman, 1967). The latter type of cooperativity can be explained simply on the basis of mass action effects due to the differences in ligand binding to the various oligomers. In such cases, the extent of saturation and the degree of cooperativity will depend on the protein concentration as well as on other factors such as ionic strength and temperature which might perturb the self-association of the protein.

Human luteinizing hormone (hLH)[1] is a glycoprotein comprised of two nonidentical subunits held together by noncovalent bonds (Ward et al., 1973; Hartree et al., 1971). The subunits can be dissociated in acid or in concentrated solutions of urea and can be recombined by incubating at neutral pH. The fluorescence probe, ANS, has been used to measure the rates of subunit dissociation and recombination (Ingham et al., 1973). The method is based on the fact that ANS fluorescence is strongly enhanced by the native hormone whereas the subunits cause little or no enhancement.

It was previously reported that ANS binds to hLH with an apparent association constant of about $10^6\ M^{-1}$ (Aloj et al., 1973). However, subsequent studies have revealed that the hormone self-associates to form dimers in the presence of ANS and that the enhanced ANS fluorescence is due to its binding to the dimer. Because of the usefulness of ANS for investigating the kinetics of subunit interactions, and in order to characterize the ligand-induced self-association, we have examined the ANS binding in greater detail. In this paper, it is shown that the binding of ANS to hLH provides an interesting example of negative cooperativity arising from protein association. In the following paper (Ingham et al., 1975) we report the results of a similar study with hCG, which, although functionally and structurally similar to hLH, exhibits marked differences in its ANS binding properties.

Materials and Methods

Highly purified hLH (LER-1705) obtained from Dr. L. Reichert was used for all experiments. This material had a biological activity of 5060 IU/mg by the rat ventral prostate weight assay (Van Hall et al., 1971). Concentrations of hLH were determined from the absorbance at 276 nm using a molar extinction coefficient of 1.6×10^4 l. per mol per cm. The latter was calculated assuming seven tyrosine residues, one tryptophan residue, and 11 disulfide bonds per mole (Ward et al., 1973; Sairam et al., 1972; Closset et al., 1973; Shome and Parlow, 1973). Extinction coefficients of 5400, 1500, and 145 l. per mol per cm were used for tryptophan, tyrosine, and cystine, respectively (Brandts and Kaplan, 1973; Edelhoch, 1967). Concentrations determined in this manner were within 10% of those determined from the weight of lyophilized hormone assuming a molecular weight of 28,800 (Bishop and Ryan, 1973).

The buffer, in all experiments, was 0.01 M potassium phosphate, prepared in doubly distilled H_2O at pH 7.0. The magnesium salt of ANS was obtained from Eastman and used without further purification. Concentrations of ANS were determined using ϵ_{350} 6240 l. per mol per cm (Ferguson et al., 1975). All chemicals were reagent grade.

Fluorescence measurements were made on a Hitachi Perkin-Elmer MPF-3 spectrofluorometer equipped with a thermostated cell holder and polarizing filters for measuring fluorescence polarization. A solution of quinine sulfate in 0.1 N H_2SO_4 was used to monitor the stability of the instrument. ANS fluorescence titrations of dilute solutions of hLH were performed in the following manner. Equal ali-

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[1] Abbreviations used are: hLH, human luteinizing hormone; hCG, human chorionic gonadotropin; ANS, 8-anilino-1-naphthalenesulfonate; TNS, 2,6-*p*-toluidinonaphthalenesulfonate.

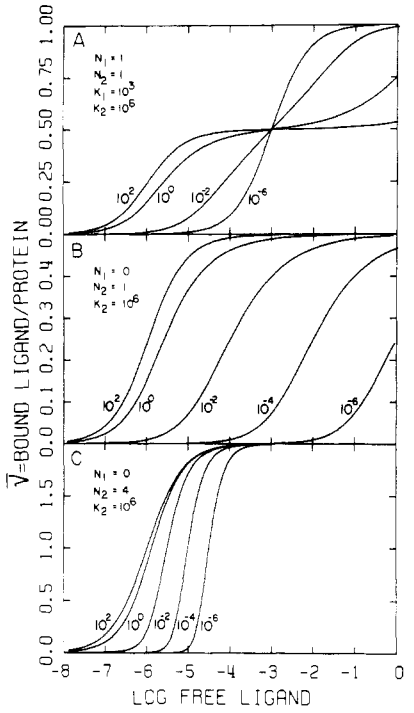

FIGURE 1: Theoretical saturation curves for a monomer–dimer model in which the monomer has N_1 equivalent and independent sites with affinity K_1, and the dimer has N_2 such sites with affinity K_2. The numbers next to the curves give the log of the product, K_dP_0. For a given value of the dimerization constant, K_d, the curves illustrate the effect of protein concentration on the shape and position of the saturation curve. Panels B and C correspond to cases in which the binding to monomer is sufficiently weak to be neglected ($K_1 \ll K_2$). This was formally accomplished by setting $N_1 = 0$. All numbers are based on concentration units of moles/liter.

quots of a concentrated solution of hLH were added to a series of tubes containing equal volumes of ANS at different concentrations. The solutions were incubated at room temperature for at least 1 hr before measuring the fluorescence at 25°C. It was found that ANS fluorescence signals in the presence of hLH were time dependent but that equilibrium values were always obtained within 1 hr (see below). In addition, some problems with adsorption of hLH to glass were encountered which accounts for some of the scatter in the titration curves at low hLH concentration.

ANS fluorescence polarization was measured at 480 nm using vertically polarized excitation at 360 nm. The polarization was calculated from the relation $P = (I_v - GI_H)/(I_v + GI_H)$ where I_v and I_H are the fluorescence intensities measured at an angle of 90° to the incident beam with the emission polarizer in the vertical and horizontal positions, respectively, and G is the ratio I_v/I_H measured with the excitation polarizer in the horizontal position (Chen et al., 1969). The fluoresence lifetime of ANS (10.0 μM) in the presence of hLH (1.0 μM) was measured at 25°C on a TRW, Inc. nanosecond apparatus as described by Chen et al. (1967).

Gel filtration experiments utilized a 1 × 50 cm Sephadex G-100 column whose void volume (V_0) was taken as the peak of the elution profile of 19S porcine thyroglobulin (mol wt 660,000 (Edelhoch, 1960)). Fractions were analyzed for hLH by measuring the enhancement of ANS fluorescence. When the columns were equilibrated with ANS, the fluorescence could be measured directly on the collection tubes. Otherwise, 50-μl aliquots were removed from each fraction and placed in 0.5-ml volumes of 100 μM ANS, and the fluorescence was measured at 480 nm with excitation at 360 nm.

Sedimentation equilibrium measurements employed a Spinco Model E ultracentrifuge and the meniscus depletion method of Yphantis (1964). The hormone was dissolved in 80 μM ANS and dialyzed overnight against the same ANS solution at room temperature. The final dialysate was used as the reference solvent in the double sector centrifugation cell. Interference optics were employed to calculate the hormone concentration at intervals along the cell radius. The variation in ANS concentration is too small to contribute to the interference pattern and only the protein concentration is recorded under these conditions. The base line was established by a blank run in which both cell compartments contained the dialysate. The sample was centrifuged at 22,000 rpm for 2 days at 22°C before photographing and the measurements were repeated 12 hr later to ensure that equilibrium had been established. A partial specific volume of $\bar{v}$ = 0.702 was used (Bishop and Ryan, 1973) to analyze the sedimentation data in a manner similar to that described in the accompanying paper (Ingham et al., 1975).

Fluorescence and sedimentation data were fit to the theoretical model (see below) using a nonlinear curve-fitting program based on the conjugate gradient method (Beckman, 1960). The uniqueness of the parameter values obtained from the fluorescence data was investigated by a parameter dependency study using an approach similar to that described by Endrenyi and Kwong (1973). Briefly, this consists of fixing the test parameter at various values above and below the "best" value and repeating the calculation, allowing the remaining parameters to vary. By observing the change in the standard deviation of the fit, one can assess the uniqueness with which the test parameter is determined.

Theoretical Section

Consider the binding of ligand (L) to a protein (P) which is involved in a monomer–dimer equilibrium as follows:

$$P + P \rightleftarrows P_2 \qquad K_d = [P_2]/[P]^2$$

$$\begin{aligned} &P + L \rightleftarrows PL \\ &PL + L \rightleftarrows PL_2 \qquad K_1 \\ &PL_{N_1-1} + L \rightleftarrows PL_{N_1} \end{aligned}$$

$$\begin{aligned} &P_2 + L \rightleftarrows P_2L \\ &P_2L + L \rightleftarrows P_2L_2 \qquad K_2 \\ &P_2L_{N_2-1} + L \rightleftarrows P_2L_{N_2} \end{aligned}$$

It is assumed that the monomer has N_1 equivalent and independent ligand binding sites, all having the same intrinsic association constant, K_1, modified by statistical factors (Klotz et al., 1946; Nichol et al., 1967). Similarly, the dimer has N_2 such sites with intrinsic association constant K_2. The binding is then described by eq 1 and 2, where L is the molar concentration of free ligand, P_0 is the total molar concentration of protein in units of monomer, and $\bar{\nu} = L_b/P_0$, where L_b is the bound ligand concentration. Equation 1, which was derived independently, can be easily obtained from the work of Nichol et al. (1967). In the above form, it conveniently expresses the binding in terms of statistical[2]

[2] By "statistical" we mean to infer the simplest kind of binding such as that described by the Langmuir (1918) isotherm or by the Henderson–Hasselbach equation (Mahler and Cordes, 1966). Such binding is characterized by Hill coefficients of unity (Brown and Hill, 1923) and gives linear Scatchard type plots (Scatchard, 1949). The analogous expression in the context of enzyme kinetics is the equation of Michaelis and Menten (1913).

type expressions for the monomer (1st brackets) and dimer (2nd brackets) whose relative contributions are regulated by the quantity R. Note that the parameters K_d and P_0 appear only as a product in eq 2. If this product is sufficiently large, R will be large relative to unity and the first term in eq 1 will vanish. Under such conditions, all of the protein is in the dimer form and one observes statistical binding with 50% saturation at $L = K_2^{-1}$. On the other hand, if K_dP_0 is sufficiently small, R approaches unity and the second term in eq 1 vanishes. In this case one observes statistical binding to the monomer with 50% saturation at $L = K_1^{-1}$. For intermediate values of K_dP_0 the binding curve will fall between these limiting cases. The fraction of protein in the monomer form under all conditions is given by $1/R$.

$$\bar{\nu} = \left[\frac{N_1K_1L}{1 + K_1L}\right]\left(\frac{2}{R+1}\right) + \frac{1}{2}\left[\frac{N_2K_2L}{1 + K_2L}\right]\left(\frac{R-1}{R+1}\right) \quad (1)$$

$$R = \left\{1 + 8K_dP_0\frac{(1 + K_2L)^{N_2}}{(1 + K_1L)2N_1}\right\}^{1/2} \quad (2)$$

Figure 1A illustrates a family of curves generated with eq 1 for a "half-of-the sites" model (Levitzki et al., 1971; Bernhard and MacQuarrie, 1973) in which $N_1 = N_2 = 1$, i.e., the number of sites per mole of monomer is 1 for the monomer and 0.5 for the dimer. At high values of K_dP_0, all of the protein is present as dimer and one obtains a statistical curve centered at $L = K_2^{-1}$. As K_dP_0 is reduced, by dilution for example, the fraction of protein initially present as dimer is lowered. However, addition of L shifts the equilibrium back toward the dimer since the latter has a higher affinity for ligand. At sufficiently high L, the dimers dissociate into monomers, which have a greater number of sites, and a second rise in the binding curve is obtained. At sufficiently low values of K_dP_0, one again observes a statistical curve at $L = K_1^{-1}$. It is interesting to note that all of the curves intersect at a point corresponding to $\bar{\nu} = N_2/2$ and $L = K_1^{-1}$. Furthermore, there is a rather broad range of protein concentration (10^{-2}–10^{-4} M) over which the binding curve is extremely flat, requiring as much as a 10^4-fold change in L for saturation, a manifestation of negative cooperativity.[3] The curve corresponding to $K_dP_0 = 10^{-2}$ is characterized by a Hill coefficient of 0.42 (Brown and Hill, 1923).

Figure 1B and C illustrates the kinds of curves obtained when binding to the monomer is sufficiently weak to be neglected ($K_1 \ll K_2$). In both cases, for large values of K_dP_0 one again obtains statistical binding to the dimer. As K_dP_0 is lowered the curves shift to the right and become broader (Figure 1B) or steeper (Figure 1C), both the shape and the magnitude of the shift depending on the value of N_2. In general, for a monomer–dimer model with $K_1 = 0$, a change of 1 log unit in K_dP_0 will produce a maximum shift of N_2^{-1} log units along the abcissa. The effect of N_2 on the limiting shape of the binding curve for such a model is summarized in Figure 2. It can be seen that for $N_2 = 1$, negative cooperativity prevails whereas for $N_2 \geq 2$, increasing degrees of positive cooperativity are observed. The corresponding Hill coefficients for $N_2 = 1$, 2, and 8 are 0.7, 1.4, and 5.0, respectively.

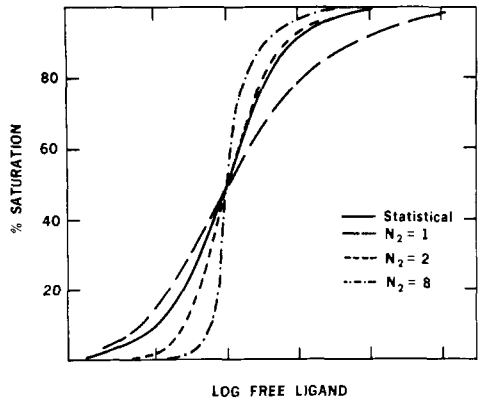

FIGURE 2: Effect of N_2 on the shape of the saturation curve for a monomer–dimer model in which the binding of ligand to the monomer is sufficiently weak to be neglected. Values of K_dP_0 for the above curves are sufficiently small that the protein is initially present as monomer. The curves have been arbitrarily superimposed at their midpoints for easy comparison of their shapes and the ligand concentration scale is arbitrary.

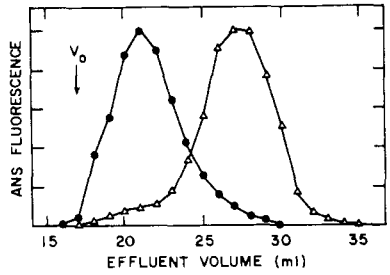

FIGURE 3: Gel filtration of hLH on Sephadex G-100 at 4°C in the presence (●) and absence (Δ) of 80 μM ANS. Fractions were analyzed for hLH using the ANS fluorescence as described in the Experimental Section. The arrow indicates the void volume as determined with 19S thyroglobulin. Bovine serum albumin elutes as a single band centered at fraction 19.

Results

Ligand-Induced Self-Association. Evidence for self-association of hLH was obtained by gel filtration on Sephadex G-100 at 4°C. As shown in Figure 3, in the absence of ANS, most of the hormone eluted in a band centered at $V_e/V_0 = 1.6$. The small amount of material eluting ahead of the main peak in the absence of ANS suggests that the hormone is slightly associated under these conditions. When the column was equilibrated with 80 μM ANS, the hormone eluted earlier at $V_e/V_0 = 1.2$ indicating that the effective molecular radius had increased. Bovine serum albumin (molecular weight 65,000) eluted slightly ahead of the hLH–ANS complex suggesting that the latter involves a dimer of hLH.

Preliminary experiments indicated that at an hLH concentration of 0.6 mg/ml, the sedimentation coefficient, $s_{20,w}$, increased from 2.5 to 3.1 in the presence of 32 μM ANS, in qualitative agreement with the gel filtration results. In order to confirm the dimerization of hLH in the presence of excess ANS, a sedimentation equilibrium experiment was performed in 80 μM ANS at 25°C. The data were fit to a monomer–dimer model using a value of 28,800 for the monomer molecular weight (Bishop and Ryan,

[3] By negative (positive) cooperativity we simply mean binding curves which are less steep (more steep) than statistical. Such curves are characterized by Hill coefficients (Brown and Hill, 1923) which are less than (greater than) unity and exhibit upward (downward) concavity when plotted according to Scatchard (1949). Frieden (1967) refers to cooperativity arising from protein association as "apparent cooperativity" to distinguish it from the more classical type of cooperativity as described by the models of Monod et al. (1965a,b) and Koshland et al. (1966).

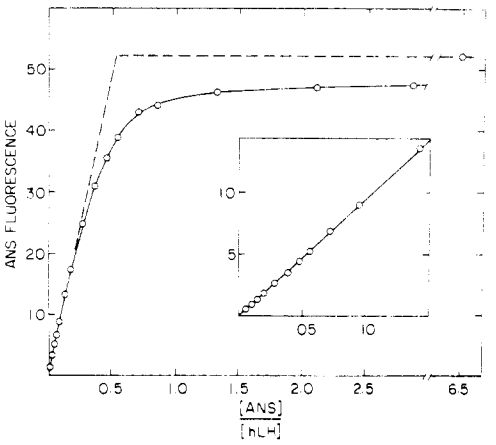

FIGURE 4: ANS fluorescence titration of hLH (20 μM) with ANS at 25°C. Excitation and emission wavelengths were 400 and 480 nm, respectively. The intersection of the extrapolated initial slope (inset) with the saturation level gives an ANS/hLH ratio of 0.5. The background fluorescence (in the absence of hLH) was negligible on this scale.

1973). The calculations indicated that 95% of the hormone was in the dimer form under these conditions with no evidence of higher molecular weight species. By contrast, Bishop and Ryan (1973) found 90% monomer under very similar conditions in the absence of ANS. Their value of 28,800 for the monomer molecular weight was in good agreement with that calculated from the amino acid and carbohydrate composition.

Stoichiometry of the ANS–hLH Complex. An estimate of the stoichiometry of the hLH–ANS complex was obtained by gel filtration using the method of Hummel and Dreyer (1962), as modified by Fairclough and Fruton (1966). The Sephadex G-100 column was equilibrated with near saturating levels of ANS (80 μM). A known amount (1.95 mg) of hormone was dissolved in 1 ml of the same ANS solution and applied to the column. The fractions were analyzed for ANS by measuring the absorbance at 350 nm. The resulting elution profile exhibited an increased absorption in the region where the hormone elutes followed by a decrease of approximately equal area near the salt volume. From the latter area, it was calculated that 0.46 mol of ANS was bound per mol of hormone (0.92/mol of dimer).

An additional estimate of the stoichiometry was obtained by ANS fluorescence titration at high hormone concentration (20 μM). The results are presented in Figure 4. Extrapolation of the linear ascending portion of the curve to the saturation level indicates 0.5 mol of ANS bound/mol of hormone, in reasonable agreement with the gel filtration results. Since the sedimentation studies indicate that the hormone is primarily in the dimer form in excess ANS, we conclude that the dimer has one site for binding the dye.

ANS Fluorescence Measurements. The addition of small amounts of ANS to a dilute solution of hLH leads to time-dependent increases in ANS fluorescence. The time required for the fluorescence to reach a steady value depends on the concentration of the reactants. At low ANS and hLH concentrations (≤ 1 μM) the initial rate of increase in ANS fluorescence is approximately first order with respect to dye concentration and second order with respect to hormone concentration. As much as 45 min may be required to reach equilibrium. Under these dilute conditions there is little or no instantaneous increase in ANS fluorescence. Assuming that hormone dimerization is the slow step, this observation suggests that binding to monomers is much weaker than binding to dimers and that the concentration of dimers in dilute hormone solutions is negligible in the absence of ANS. At much higher ANS (40 μM) or hLH (20 μM) concentration, the fluorescence rapidly (within 30 sec) reaches 90–95% of its final value which is then established in less than 5 min. The presence of 0.1 M KCl diminishes the time required to establish equilibrium but does not affect the final fluorescence.

Measurements of polarization of ANS fluorescence were also carried out in order to distinguish between binding of ANS by monomers and dimers of the hormone. The polarization at 25°C was essentially constant ($P = 0.215 \pm 0.02$) over the entire range of ANS binding (hLH = 1 μM, ANS = 0–80 μM). Furthermore, the polarization showed no variation with time, even under conditions where the fluorescence intensity increased with time. The invariance of the polarization suggests that ANS binds exclusively to dimers. If ANS were bound first to monomers which then associated to form dimers, one would expect to see an increase in polarization as the system shifts from monomers to dimers. From the Perrin equation (Perrin, 1934; Chen et al., 1969) one would predict the fluorescence polarization of ANS bound to an hLH dimer to be about 30% greater than when bound to a monomer. The latter prediction is based on a measured value of $17.7 \pm 0.1 \times 10^{-9}$ sec for the fluorescence decay time of ANS (40 μM) bound to hLH (1 μM) and the assumption that monomers and dimers are both rigid spheres. If the dimer is less symmetric than the monomer, an even greater difference in polarization would be expected. Since a 30% change in polarization would have been easily detected, we conclude that ANS binds exclusively to dimers under the conditions investigated.

A key feature of the proposed monomer–dimer model is its prediction of a shift in the position of the binding curve with changes in protein concentration (Figure 1). Thus, hLH was titrated with ANS at two different hormone concentrations as described in Materials and Methods. The results (Figure 5) indicate that a fivefold increase in hLH concentration shifts the binding curve about 0.25 log unit to lower ANS concentration. The solid curves were obtained by simultaneously fitting both sets of data to the monomer–dimer model of eq 1 with $N_1 = 0$ and $N_2 = 1$. This resulted in the values of K_d and K_2 indicated in the figure. The dashed curve is the statistical curve which would prevail at infinite protein concentration for the same value of K_2. The experimental curves are seen to be less steep than the statistical curve (negative cooperativity), as expected for this model (Figure 1B). The rather large scatter in the experimental points is due primarily to problems of adsorption of hLH on glass and quartz surfaces. However, the general shape and the shift with hLH concentration were qualitatively reproducible.

In order to assess the uniqueness of the parameter values obtained by fitting the fluorescence binding data, a parameter dependency study was performed as described in Materials and Methods. The results (Figure 6) indicate that arbitrarily fixing K_d at values above or below the "best fit" value has very little effect on the standard deviation (within experimental error). Similarly, K_2 can be fixed at values above the "best fit" value with very little effect. However, if $\log K_d \leq 6$, the quality of the fit is seriously diminished. This is because K_2 must be at least this large in order to obtain binding at ANS concentrations in the 1 μM range. Any combination of K_d and K_2 such that $\log K_d K_2 = 11.8 \pm 0.2$

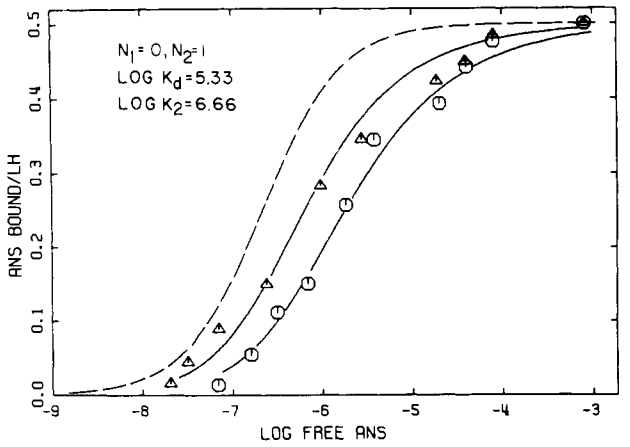

FIGURE 5: Effect of protein concentration on the binding of ANS to hLH at 25°C. Protein concentrations were 3.5 μM (Δ) and 0.75 μM (O). The concentration of bound ANS was calculated from the fractional saturation and the known hormone concentration assuming 0.5 mol of ANS bound per mol of hLH monomer at the end point (10^{-3} M ANS). The small amount of background fluorescence which exists in the absence of hLH was subtracted. The lines through the points are theoretical curves obtained by simultaneously fitting both sets of data to the monomer-dimer model with $N_1 = 0$ and $N_2 = 1$. The resulting K values are shown in the figure. The dashed curve is the theoretical "statistical" curve generated with these parameters at infinite hormone concentration.

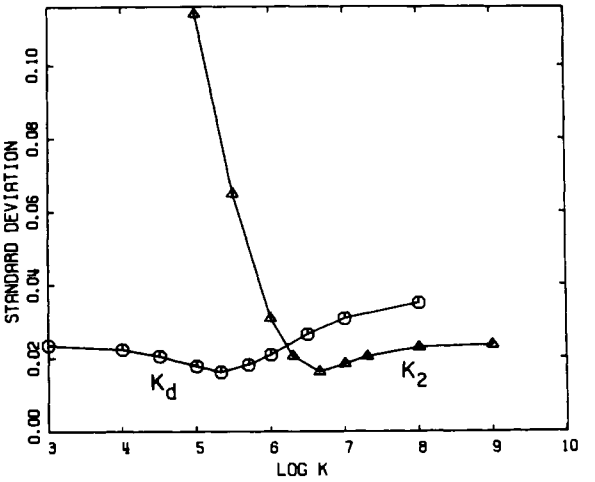

FIGURE 6: Uniqueness test for the parameters K_d and K_2 whose values were obtained by fitting the data in Figure 5. Each parameter was arbitrarily fixed at the values shown and the calculation repeated, allowing the other parameter to vary, to determine the effect on the quality of the fit as measured by the standard deviation. The minima correspond to the "best fit" values of K_d and K_2 (see Discussion).

will give a reasonable fit providing log $K_2 \geq 6$.

Discussion

The above results indicate that hLH self-associates to form dimers and that this process is enhanced by ANS. The data are compatible with a model in which the hLH dimer has a single high affinity site for ANS whereas binding of ANS to the monomer is sufficiently weak to be neglected. The binding curves exhibit negative cooperativity in that they are less steep than the statistical curve, a result which is theoretically predicted for a monomer–dimer model with $N_1 = 0$ and $N_2 = 1$ (Figures 1 and 2). It should be pointed out that it is impossible from equilibrium measurements alone to distinguish between (1) a high affinity of dimer for the ligand and (2) a strong tendency for a liganded monomer to associate with another free monomer. The two possibilities are thermodynamically equivalent.[4] However, the time effects suggest a kinetic model in which ANS binds rapidly to the small amount of dimer present at low hormone concentrations. The monomer $\rightleftarrows$ dimer equilibrium then slowly shifts, due to mass action, generating additional dimer, in a manner analogous to the hysteretic enzyme concept developed by Frieden (1970). An alternative kinetic route in which ANS binds rapidly to a monomer which then slowly associates to form a dimer appears to be ruled out by the fact that the ANS fluorescence polarization was essentially constant with time and/or ANS concentration. Furthermore, there was no instantaneous rise in fluorescence upon addition of ANS to a dilute solution of hLH.

The values of K_d and K_2 obtained by fitting the fluorescence binding data (Figure 5) should be regarded with caution. Although the dependency curves for both parameters exhibit minima (Figure 6), the minima are too shallow to be significant within experimental error. The explanation of this behavior lies in the tendency for K_d and K_2 to compensate because of their multiplicative relationship in eq 2. The nature of the model is such that extremely precise data would be required to establish both constants from binding studies alone. We thus report a precise value for their product (log $K_dK_2 = 11.8 \pm 0.2$) and a lower limit for K_2 (log $K_2 \geq 6$).

The finding that the enhancement of ANS fluorescence by hLH is due to dimers of the hormone does not alter the conclusions of a previous study (Ingham et al., 1973) in which ANS was used as a probe to follow the rates of dissociation and recombination of the subunits. With the assay conditions employed in that study, the ANS concentration was sufficiently high to cause all of the hormone to dimerize rapidly. Thus, the ANS fluorescence was directly proportional to the concentration of hLH whereas the subunits had a negligible effect. Although the subunits have been reported to aggregate (Bishop and Ryan, 1973), they apparently do not form species which bind ANS. The previously reported ability of low concentrations of urea to diminish the fluorescence of ANS–hLH solutions (Ingham et al., 1973) can now be attributed to the expected ability of urea to interfere with self-association of the hormone.

To our knowledge, hLH is the first example of a polypeptide hormone whose state of association is strongly influenced by a relatively small ligand. However, there are several other proteins whose states of aggregation have been reported to influence the binding of sulfonate dyes. For example, Birkett et al. (1971) followed the AMP induced dimer–tetramer transition of phosphorylase B by exploiting the preferential ability of the tetramer to enhance TNS fluorescence. Laurence (1966) observed that the binding of

[4] The following reactions are all potentially involved in the hLH–ANS system.

$$P + P \rightleftarrows P_2 \qquad K_d$$
$$P_2 + L \rightleftarrows P_2L \qquad K_2$$
$$P + L \rightleftarrows PL \qquad K_1$$
$$PL + P \rightleftarrows P_2L \qquad K_d'$$

It can be easily shown that $K_dK_2 = K_d'K_1$. Thus, if $K_2 \gg K_1$, then $K_d' \gg K_d$. The fact that the hormone is completely dimerized in excess ANS proves that K_2 is significantly greater than K_1. However, since the dimer has only one site, the presence of even a weak site on the monomer would require the equilibrium to shift back to the monomer form at sufficiently high ANS, analogous to the half-of-the-sites model in Figure 1A. Apparently the affinity of hLH monomers for ANS is sufficiently low that this does not occur in the range of ANS concentration investigated (up to 1 mM).

ANS to calf thymus histone fractions was favored by conditions favoring aggregation. Anderson (1971) reported that the lactic dehydrogenase tetramer (M_4) undergoes extensive aggregation in the presence of bis(ANS), a covalently linked dimer of ANS. Finally, Beyer et al. (1972) observed sigmoidal fluorescence titration curves with TNS and tyrocidine B, a result which they suggested might be due to cooperative effects. However, in none of these studies was an attempt made to analyze the possible cooperative effects in detail.

There are numerous multisubunit enzymes whose oligomeric state is known to depend on the concentration of substrates and/or effectors (see reviews by Koshland, 1970; Frieden, 1971; Phillips, 1974). As pointed out by Levitzki and Schlessinger (1974), many of the more "cooperative" enzymes fall into this category. However, many of these same enzymes exhibit cooperative ligand binding even in the absence of changes in oligomeric state. Thus it has been difficult to isolate the cooperative effects arising from protein association. The problem is further complicated by the fact that much of the pertinent data on these systems stem from enzyme kinetic measurements as opposed to direct binding studies. The present data on the hLH–ANS system provide an experimental illustration of cooperative effects which arise exclusively from ligand coupled protein association in a system where the above mentioned complications are not present. In the following paper (Ingham et al., 1975) we report the results of a similar study with human chorionic gonadotropin which, although functionally and structurally similar to hLH, exhibits marked differences in its ANS binding properties.

Phillips (1974) and earlier Frieden (1967) have discussed the extension of the model of Monod et al. (1963, 1965) to include the effects of protein association. We find it instructive to view an associating protein system in which the various oligomers have different affinities and/or capacities as a limiting case of the more classical models (Monod et al., 1963, 1965; Koshland et al., 1966) such that the subunit interactions in one conformational state become so weak that the subunits physically dissociate in the absence of ligand. The extent of binding and the degree of cooperativity then become dependent on protein concentration. Such a model lends additional flexibility in terms of control since the need to switch an enzyme on or off might reasonably be expected to vary with the level of enzyme present. The curves in Figures 1 and 2 illustrate that, depending on the protein concentration, almost any type of cooperative effect is theoretically possible, given the appropriate combination of ligand affinities and/or capacities. Furthermore, it is not necessary to invoke tertiary conformational differences between oligomers. Although such differences may exist and may in fact be responsible for differences in binding, they need not be included in the formalism since the cooperative effects arising from protein association can be explained entirely on the basis of mass action through eq 1 and 2.

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Ligand-Induced Self-Association of Human Chorionic Gonadotropin. Positive Cooperativity in the Binding of 8-Anilino-1-naphthalenesulfonate†

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ABSTRACT: Human chorionic gonadotropin (hCG) self-associates to form higher molecular weight species in the presence of the fluorescence probe 8-anilino-1-naphthalenesulfonate (ANS). Sedimentation equilibrium and fluorescence titration data have been analyzed in terms of a monomer–dimer–tetramer model in which the various oligomers have different affinities and/or capacities for the ligand. The results indicate that the ligand affinities are in the order tetramer > dimer > monomer whereas the numbers of ligand binding sites per mole of hCG are in the reverse order. Consequently, addition of ANS first shifts the equilibrium from monomer to tetramer and gives rise to positive cooperativity in the titration curves. At sufficiently high ANS concentration (~0.5 mM), the equilibrium shifts back to the dimer because of its greater binding capacity. This is manifested by a second phase in the titration curve and a decrease in the polarization of ANS fluorescence. The results are discussed in terms of the general problem of ligand controlled protein association and are contrasted to results reported in the previous paper for the homologous protein, human luteinizing hormone.

Human chorionic gonadotropin (hCG)[1] and human luteinizing hormone (hLH) are glycoproteins comprised of two nonidentical subunits, α and β, held together by noncovalent bonds (Canfield et al., 1971; Morgan and Canfield, 1971; Ward et al., 1973). There is extensive homology between the primary structures of the two hormones and they have similar biological properties. They have been shown to compete with each other for binding to receptor sites on target tissue membranes (Catt, et al., 1972; Lee and Ryan, 1973; Rao, 1974).

In the previous paper (Ingham et al., 1975) we reported the results of a detailed study of the interaction of the fluorescence probe, 8-anilino-1-naphthalenesulfonate (ANS), with hLH. It was shown that hLH self-associates to form dimers $(\alpha\beta)_2$ in the presence of ANS, due to the presence of a single high affinity site ($K > 10^6\ M^{-1}$) on the dimer while binding of ANS to the monomer was too weak to be observed. This led to negative cooperativity in the ANS binding and to a dependence of that binding on hormone concentration. In this paper we report the results of a similar study with hCG which, in spite of its extensive homology with hLH, exhibits marked differences in its interaction with ANS.

Materials and Methods

Highly purified hCG (CR117) was obtained from Dr. R. Canfield of Columbia University through the Center for Population Research, National Institute of Child Health and Human Development, NIH. hCG concentrations were determined optically using ϵ 1.2×10^4 l. per mol per cm at 276 nm. This molar extinction coefficient was calculated from the amino acid composition (Bellisario et al., 1973; Carlsen et al., 1973; Morgan et al., 1973) as described previously (Ingham et al., 1975). Concentrations determined in this manner were within 10% of those determined either by refractive index measurements or by the weight of lyophilized hormone assuming a molecular weight of 37,900 (Bellisario et al., 1973; Carlsen et al., 1973). The fluorescence spectra of hCG solutions had maxima near 305 nm with no indication of any tryptophan emission indicating that preparations were free of contaminating proteins which contain tryptophan.

The magnesium salt of ANS was obtained from Eastman

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[1] Abbreviations used are: hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; ANS, 8-anilino-1-naphthanesulfonate.