Thermal Stability of Human Chorionic Gonadotropin

REVERSIBLE DISSOCIATION OF SUBUNITS AT NEUTRAL PH*

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Measurements of the polarization of tyrosyl fluorescence of gonadotropin in 0.01 M KPO₄, pH 7, as a function of temperature revealed two distinct reversible transitions. The first exhibited a midpoint near 22 °C, independent of protein concentration or the presence of 0.15 M NaCl. The second transition occurred at higher temperature and the midpoint increased with increasing hormone concentration and decreasing ionic strength. Analysis by high performance size exclusion chromatography confirmed that this transition corresponds to a reversible dissociation of α and β subunits. Equilibrium constants determined by this method were consistent with those determined by interpolation between polarization values of the initial and final states and by monitoring the ability to enhance the fluorescence of 1,8-anilinonaphthalene sulfonate. Linear van't Hoff plots of ΔH with and without 0.15 M NaCl. Extrapolation to 37 °C provided estimates of ΔH = 61 ± 3 kcal mol⁻¹ with and without 0.15 M NaCl. Extrapolation to 37 °C provided estimates of ΔH = 5 ± 10⁻⁸ M and 2 ± 10⁻⁷ M in the absence and presence of 0.15 M NaCl, indicating that human chorionic gonadotropin is thermodynamically unstable under conditions encountered in vivo. Measurements of the rate of subunit dissociation as a function of temperature were consistent with an activation energy of 40 ± 3 kcal/mol and extrapolation to 37 °C provided an estimate of approximately 40 h for the half-life under physiological conditions. Given the greater metabolic clearance rate of dissociated subunits relative to intact hormone, spontaneous subunit dissociation could have a regulatory effect on the levels of circulating gonadotropins.

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The placentalf hormone human chorionic gonadotropin is one of several homologous glycoprotein hormones, each consisting of two nonidentical subunits, α and β, held firmly together by noncovalent bonds (Canfield et al., 1971; Morgan and Canfield, 1971; Bahl, 1973; Pierce and Parsons, 1981). The isolated subunits exhibit little or no biological activity (Catt et al., 1973), but can be reassociated at neutral pH to yield intact hormone with activity comparable to that of native hormone (Swaminathan and Bahl, 1970; Aloj et al., 1973). The functional significance of the unique two-subunit nature of these hormones remains to be elucidated. An improved understanding of the molecular properties of hCG* is important for understanding its mechanism of action and may also facilitate the development of new approaches to fertility control.

Although the subunits of hCG can be dissociated at low pH or in concentrated solutions of urea (Aloj et al., 1973), the intact hormone is considered to be stable under physiological conditions. Indeed, kinetic studies of subunit recombination at neutral pH and 37 °C revealed substantial formation of intact hormone at subunit concentrations as low as 1.5 μM (Ingham et al., 1976). However, the thermodynamic stability of the hormone at much lower concentrations, such as those which prevail in the circulation, has not been characterized. The fact that isolated subunits are cleared from the circulation much faster than the intact hormone (Wehmann and Nisula, 1979) suggests that spontaneous dissociation, if it occurs, could have important regulatory consequences.

Studies of the equilibrium between the intact and dissociated states of hCG at concentrations in the submicromolar range are complicated by the slow rate of both the forward and reverse reactions and by the difficulty of quantitating the state of association under such conditions. An alternative approach is to examine these reactions at elevated temperatures in order to obtain, by extrapolation, an estimate of the dynamic state of equilibrium to be expected at 37 °C. An earlier study with the homologous hormone ovine lutropin provided evidence for the reversible dissociation of subunits at elevated temperatures and suggested that polarization of tyrosyl fluorescence might be a useful tool for this purpose (Ingham and Bolotin, 1978). The present study was conducted with hCG because it was available in larger quantities. In addition to polarization of tyrosyl fluorescence, we have used the ability to enhance ANS fluorescence to monitor the rate and extent of dissociation and have confirmed the results by high performance liquid chromatography.

MATERIALS AND METHODS

Highly purified hCG (CR 123) having a biological potency of 12,780 was obtained from Dr. R. Canfield of Columbia University via the Center for Population Research, National Institute of Child Health and Human Development, National Institutes of Health. Polycrylamide disc gel electrophoresis of the hormone in the presence of 8 M urea (Swank and Munksres, 1971) exhibited two bands corresponding to its subunits. Fluorescence spectra indicated that the preparations were free of tryptophan-containing impurities. Protein concentrations were determined from the absorbance at 276 nm using 12,000 as the molar extinction coefficient for hCG (Ingham et al., 1975).

Polarization measurements were made as previously described (Forastieri and Ingham, 1980a) on a Perkin-Elmer MPF4 fluorometer equipped with Polacoat polarizing filters and thermostated cell holder.

1 The abbreviations used are: hCG, human chorionic gonadotropin; hLH, human lutropin; oLH, ovine lutropin; ANS, 1,8-anilinonaphthalene sulfonate; HPLC, high performance liquid chromatography.
Excitation and emission wave lengths of 270 and 310 nm, respectively, were used in order to avoid interference by Raman scattering. The observed intensities were corrected for buffer background, which never exceeded 10% of the sample intensity.

For the determination of melting curves, the temperature was changed in increments of approximately 5 °C, and the samples were maintained at each temperature until a stable value for P was obtained. The extent of dissociation or recombination was also determined by the ability to enhance ANS fluorescence (Aloj et al., 1973; Ingham et al., 1976). Small portions were periodically removed from the reaction mixture and diluted into cold ANS (250 μM in 0.01 M phosphate buffer, pH 7.0, on ice) and subsequently analyzed at room temperature. Enhancement of ANS fluorescence by dissociated subunits is almost negligible compared to that of the intact hormone and the state of association can be readily determined by comparison of a given test sample to that of an undissociated control. Recombination during the time required for measurement is negligible at the dilutions employed (Ingham et al., 1976).

Direct observation of the physical state of association was accomplished by high performance liquid chromatography on a TSK-G 3000 SW exclusion column (0.75 x 60 cm) kindly provided by Toyoda Soda Manufacturing Co., Tokyo, Japan. All elutions were done with 0.01 M KPO₄, 0.15 M NaCl, pH 7.0, at approximately 300 p.s.i. which gave a flow rate of 1 ml/min. The elution time was monitored by the absorbance at 280 nm. This column does not resolve intact hCG from the state of association can be readily determined by comparison of a given test sample to that of an undissociated control. Recombination during the time required for measurement is negligible at the dilutions employed (Ingham et al., 1976).

The hormone concentrations are: 0.15 M NaCl and 133 μM (β, A). The filled symbols represent the recovery of β on cooling. The temperature was changed in increments of approximately 5 °C and maintained until no further variation in P could be detected. The dashed lines represent the extrapolated P values for intact hormone, P₀, and dissociated subunits, Pᵦ.

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Ingham 1980a). It involves a unimolecular conformational change within the intact hormone and is thus insensitive to changes in hormone concentration. Inclusion of 0.15 M NaCl in the buffer had no effect on P in this temperature range.

By contrast, the second higher temperature transition, which involves dissociation of the subunits, is accompanied by a much larger decrease in P and its midpoint is noticeably affected by both the concentration of hormone and the ionic strength of the medium. As the hormone concentration is increased from 20 to 133 μM, the midpoint shifts from 55 °C to approximately 60 °C. This increased stability with increasing concentration reflects the bimolecular nature of the recombination reaction. Increasing the salt concentration from 0 to 0.15 M lowers the midpoint by approximately 2 °C, indicating a shift of the equilibrium between intact hormone and dissociated subunits at each temperature, favoring the dissociated state. The polarization of the isolated subunits decreases smoothly over the entire temperature range with no evidence for structural transitions (not shown).

Analysis by HPLC—Parallel samples of 33 μM hCG in 0.01 M PO₄ buffer, with and without 0.15 M NaCl, were incubated extensively at various temperatures and the extent of subunit dissociation analyzed by exclusion chromatography on a high pressure system at 25 °C as described under "Materials and Methods." At this temperature, no further dissociation would be expected and the rate of recombination following application of the sample is effectively minimized both by the lower temperature and by the dilution which occurs on the column. Therefore, the equilibrium between intact and dissociated states should not be significantly altered during the less than 20 min required for elution.

The results are shown in Fig. 2 where the left and right hand panels correspond, respectively, to samples incubated in the absence and presence of 0.15 M NaCl. Panels A and B represent controls which were incubated at 25 °C and illustrate the profiles obtained for the undissociated hormone. Panels C and D represent samples incubated at 55 °C. Significant amounts of α subunit appear with an elution time near 17.5 min. The next two panels (E and F) correspond to samples incubated at 65 °C where the amount of α subunit can be seen to have increased. At both 55 °C and 65 °C, the extent of dissociation is significantly greater in the presence of salt, as expected from the observed shift in the midpoint of the transition in Fig. 1. The lower panels (G and H) of Fig. 2 represent samples incubated at 80 °C and are consistent with complete dissociation of subunits; the relative areas under the α and β peaks are in good agreement with the relative extinction coefficients of the two subunits.

The β subunit elutes in a position almost identical with that of the intact hormone, a result which is also seen by conventional exclusion chromatography. However, the resolution seen here between α and β subunits is much greater than that observed previously with a Sephadex G-100 column of similar size (Forastieri and Ingham, 1980a), suggesting that such columns might be useful in the purification of subunits. It is also of interest that the profile of the β subunit is consistently narrower than that of the intact hormone, perhaps reflecting a smaller diffusion coefficient.

Thermodynamic Analysis—The apparent two-state melting behavior exhibited by the hormone between 30 °C and 89 °C (Fig. 1) justifies analysis of the transition on the basis of a simple equilibrium between intact hCG and completely dissociated subunits:

$$ H \frac{b_1}{k_{-1}} a + \beta $$

The extent of dissociation at any point within the transition
values thus obtained are compared in Table I to those derived from polarization measurements as well as from ANS fluorescence measurements (see below). At a given temperature, the values obtained by the three methods agree within a factor of 2, providing strong support for the validity of this analysis.

A short extrapolation of the straight lines in Fig. 3 yields an estimate of $K_D = 5 \times 10^{-8} \text{ M}$ at 37°C in the low ionic strength medium and a somewhat higher value of $2 \times 10^{-7} \text{ M}$ in the presence of salt. On this basis, it appears that the hormone is thermodynamically unstable at physiological temperature and pH at concentrations below $10^{-8} \text{ M}$. In the following sections we provide additional support for this conclusion and attempt to estimate the rate at which subunit dissociation might be expected to occur at 37°C.

**Approach to Equilibrium**—Fig. 4 illustrates the time course of the approach to equilibrium as intact hCG is thermally dissociated in 0.01 M PO₄ and neutral pH at elevated temper-

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**TABLE I**

<p>| Subunit dissociation constants for hCG at neutral pH |
|-----------|-----------|-----------|-----------|
| $K_D \times 10^9$ |</p>
<table>
<thead>
<tr>
<th>$T$ (°C)</th>
<th>No NaCl</th>
<th>0.15 M NaCl</th>
<th>No NaCl</th>
<th>0.15 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>9.5</td>
<td>23</td>
<td>12°F</td>
<td>37°F</td>
</tr>
<tr>
<td>53</td>
<td>48</td>
<td>175</td>
<td>8°F</td>
<td>37°F</td>
</tr>
<tr>
<td>55</td>
<td>91</td>
<td>320</td>
<td>8°F</td>
<td>37°F</td>
</tr>
<tr>
<td>60</td>
<td>400</td>
<td>174</td>
<td>8°F</td>
<td>37°F</td>
</tr>
<tr>
<td>65</td>
<td>1260</td>
<td>5200</td>
<td>8°F</td>
<td>37°F</td>
</tr>
</tbody>
</table>

Values determined from data in Fig. 2 corresponding to a hormone concentration of 53 μM.  
Data from separate experiments not shown (54 μM).  
Data taken from Fig. 4A (120 μM).  
Values determined from data in Fig. 2 corresponding to a hormone concentration of 53 μM.

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**Fig. 3**. Subunit dissociation constants for hCG determined from data in Fig. 1 as described in the text and plotted according to the van't Hoff equation. Hormone concentrations were 133 μM (D), 54 μM (C, not shown in Fig. 1), 33 μM with (+) and without (O) 0.15 M NaCl, and 20 μM (C). The dashed lines represent extrapolation to 37°C (arrows) where the predicted values of $K_D$ are $5 \times 10^{-8} \text{ M}$ in the absence and $2 \times 10^{-7} \text{ M}$ in the presence of 0.15 M NaCl.
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**Fig. 4.** Approach to equilibrium at various temperatures starting with intact hCG (A) or dissociated subunits (B) in 0.01 M phosphate, pH 7, in the absence (open symbols) or presence (filled symbols) of 0.15 M NaCl. Small portions were periodically removed and tested for ability to enhance ANS fluorescence as described under “Materials and Methods.” The dashed line in A schematically represents a sample held at 80 °C for 2 h to generate the dissociated subunits which were recombined in B. Panel C presents HPLC elution patterns for the initial and final samples in B, excluding the sample in 0.15 M NaCl.

- **Fig. 5.** The time course for the dissociation of 3.3 μM hCG at pH 7 in the presence (filled symbols) and absence of 0.15 M NaCl at 59 °C (○), 65 °C (□), 70 °C (△), and 80 °C (□). The inset shows the first order kinetic plots for the data. Each point corresponds to a separate 250-μl sample of 3.3 μM hCG which was incubated at the desired temperature in a 0.5 cm diameter cuvette. A small volume of concentrated ANS was subsequently added to give a final concentration of 250 μM and the fluorescence measured at 25 °C.

Kinetic Analysis—First-order kinetic plots of the dissociation data in Fig. 4A are nonlinear. This is to be expected since the reverse reaction, i.e. subunit recombination, is also occurring at a significant rate and only the net rate is observed. Therefore, an additional experiment was designed to measure the rate of subunit dissociation at much lower hormone concentration where the reverse reaction is less significant and dissociation is expected to go to completion. Examples are shown in Fig. 5. Under these conditions, the loss in the ability to enhance ANS fluorescence is essentially complete and first-order plots of the rate data are sufficiently linear to obtain a reasonable estimate of the dissociation rate constant (Fig. 5, inset). The filled symbols correspond to data obtained in the presence of 0.15 M NaCl, which clearly has very little effect on the rate of dissociation. Thus, the effect of salt on the extent of dissociation at equilibrium arises entirely through its influence on the rate of the reverse (bimolecular) reaction.

In Fig. 6, rate constants for subunit dissociation are plotted according to the Arrhenius equation. Also included are dissociation rate constants obtained at pH 3.0 and pH 2.5 using the ANS fluorescence method. In acid, the reaction goes to completion even at higher concentrations of hormone (Aloj et al., 1973; Forastieri and Ingham, 1980a). It is apparent that the activation energy for subunit dissociation, as determined from the slopes of the Arrhenius plot, is relatively insensitive.
shown. The value of K values at pH 2.5 and an estimate of the half-life, although large, is an order of magnitude smaller than that reported by Strickland and Puett (1981). These authors showed that the clearance of hCG-a was about 3-fold more rapid than that of hCG-β and 10 to 20 times more rapid than that observed for intact hCG. The greater clearance rate of dissociated subunits relative to intact hormone suggests that spontaneous dissociation of the latter could have a regulatory effect on the levels of circulating hCG. Intravenous injection of highly purified hCG produced biexponential disappearance curves with rapid and slow components corresponding to average t1/2 values of 6 and 36 h, respectively. The latter value is of the same order as our estimate of 30 h for the t1/2 of subunit dissociation although substantially shorter than the estimate of 37 days obtained by Strickland and Puett (1981). It must be cautioned that the validity of our estimate, which involves an extrapolation from higher temperature down to 37 °C, is based on the assumption that the activation energy is independent of temperature over the entire range. Studies on the kinetics of subunit recombination in a number of different laboratories have provided evidence that the two-state mechanism given by Equation 2 may be an oversimplification (Merz et al., 1973; Bewley et al., 1974; Reichert et al., 1974; Ingham et al., 1974, 1976; Salesse et al., 1975). The recombination mechanism is believed to involve at least one intermediate consisting of an associated complex of α and β subunits which then undergoes a conformational change leading to the biologically active structure. The law of microscopic reversibility would demand that such an intermediate also be involved in the dissociation reaction. The agreement between

FIG. 6. Arrhenius plots of first order rate constants for dissociation of hCG subunits at pH 7 (○), pH 3 (■), and pH 2.5 (△). The values at pH 7 were determined from the data in Fig. 5. The values at pH 2.5 and 3.0 were determined in separate experiments not shown. The dashed line shows extrapolation of the pH 7 data to 37 °C (arrow) where a t1/2 of 40 h is predicted.

dissociation rate constants (Fig. 6) reveals that the half-life of association of hCG, hLH, and oLH during prolonged incubation is extremely long, on the order of 40 h, thus accounting for the failure of this phenomenon to be detected by numerous investigators working at room temperature or 37 °C.

Our estimate of the half-life, although large, is an order of magnitude smaller than that reported by Strickland and Puett (1981). These authors used low temperature sodium dodecyl sulfate polyacrylamide gel electrophoresis to assess the state of association of hCG, hLH, and oLH during prolonged incubation of iodinated hormones at 37 °C in gelatinized phosphate-buffered saline. With all three hormones, they found evidence for two components, a rapidly dissociating component with k = 1.3 to 3.8 x 10^-11 min^-1 comprising between 7 and 18% of the population and a slowly dissociating component with k = 0.1 to 0.4 x 10^-4 min^-1 comprising the remainder. Our extrapolated value of 4 x 10^-4 min^-1 for hCG is close to their value for the rapidly dissociating component. We found little evidence for a slowly dissociating component at elevated temperatures. These differences could be due to the different hormone preparations utilized or to subtle differences in the conditions employed. However, these same authors determined a value of Kd = 6 x 10^-7 M for hCG at 37 °C which agrees quite well with our extrapolated value of 2 x 10^-7 M (in 0.15 M NaCl).

In an earlier study, the ability of the homologous hormone, human lutropin, to enhance ANS fluorescence was found to gradually deteriorate upon incubation at neutral pH and 37 °C, with a half-life of about 1 day (Ingham et al., 1973). No attempt was made to assess the possibility that subunit dissociation had occurred. A similar but much faster loss in ANS fluorescence observed at higher temperature (55 °C) was shown to be partially reversed upon returning to 37 °C and was attributed to recombination of heat-dissociated subunits. Loebier et al. (1978) subsequently provided unequivocal evidence for slow spontaneous dissociation of hLH subunits at 37 °C under physiological conditions. Working at a hormone concentration of 10 µg/ml (approximately 3.3 x 10^-7 M) they found a 50% loss in receptor-binding activity after 100 h, and demonstrated the presence of free β subunit by exclusion chromatography. Although these authors made no attempt to demonstrate reversibility, their observations are consistent with a dissociation constant in the submicromolar range for hLH at 37 °C. Unpublished measurements in our laboratory of the polarization of lutropin fluorescence as a function of temperature yield data similar to that in Fig. 1 for hCG. By extrapolation, a value of Kd = 3 x 10^-7 M was estimated for hLH at 37 °C in 0.01 M phosphate, pH 7 (Forrestieri and Ingham, 1980b). Ovine lutropin also exhibits a drop in tyrosyl polarization at elevated temperatures which was partially recovered at 37 °C and which was shown by exclusion chromatography to involve reversible dissociation of subunits (Ingham and Bolotin, 1978). Taken together, these observations indicate that slow spontaneous dissociation of subunits may be a characteristic of gonadotropins in general and suggest that other members of the glycoprotein hormone family (thyrotropin and follitropin) would be worth examining. Such dissociation could have implications for the mechanism of catabolism of these hormones in circulation or after internalization by target cells.

The metabolic clearance rates of hCG and its subunits in humans have been examined by Wehmann and Nisula (1979, 1981). These authors showed that the clearance of hCG-a was about 3-fold more rapid than that of hCG-β and 10 to 20 times more rapid than that observed for intact hCG. The greater clearance rate of dissociated subunits relative to intact hormone suggests that spontaneous dissociation of the latter could have a regulatory effect on the levels of circulating hCG.
values of $K_D$ determined by different methods suggests that such an intermediate either fails to accumulate to a significant extent or that it cannot be distinguished from the intact hormone by ANS or tyrosyl polarization. The enthalpy of dissociation determined from the equilibrium analysis (60 kcal/mol) is 50% larger than the activation energy determined from the kinetic analysis (40 kcal/mol), an observation which could reflect the occurrence of intermediate species. An even larger discrepancy between $\Delta H$ and $E_a$ was reported for thermal denaturation of ribonuclease, a unimolecular reaction known to involve intermediates (Lee and Timasheff, 1981). Future studies of the kinetics of recombination of glycoprotein hormone subunits should take into account the potential reversibility of the reaction and the possibility that such reversibility could influence the observed rates.

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