Kinetics of Recombination of the Subunits of Human Chorionic Gonadotropin. Effect of Subunit Concentration

K. C. Ingham,* B. D. Weintraub, and H. Edelhoch

ABSTRACT: The rate of formation of human chorionic gonadotropin from its α and β subunits has been measured at neutral pH and 37 °C as a function of subunit concentration, using the fluorescence probe, 1,8-anilinonaphthalenesulfonate (ANS), to monitor the reaction. The subunits were prepared by acid dissociation of the intact hormone (pH ≤2, 37 °C). Following neutralization, the rate of appearance of ANS fluorescence was identical with the rate of recovery of receptor binding activity and both of these properties were completely recovered. Kinetic data obtained over a 100-fold range of subunit concentrations (1.5 to 146 μM) were not compatible with a simple second-order reaction scheme, but required at least one additional step. The data were best fit by a model in which the subunits reversibly form an intermediate complex ($\alpha + \beta \rightleftarrows \alpha\beta$) which then undergoes a conformational rearrangement to form the native structure ($\alpha\beta \rightarrow$ H). Ultraviolet difference absorption measurements suggest that most of the change in the environment of the tyrosyl residues occurs during this second step.

The placental hormone, human chorionic gonadotropin (hCG[1]), is a glycoprotein comprised of two nonidentical subunits, α and β, held together by noncovalent bonds (Morgan and Canfield, 1971; Canfield et al., 1971). The subunits, which can be dissociated in acid or in concentrated solutions of urea, have been purified and their amino acid sequences determined (Carlsen et al., 1973; Bellisario et al., 1973; Morgan et al., 1975). The individual subunits have little or no biological activity (Catt et al., 1973) but can be recombined at neutral pH to yield biological activity comparable to that of the native hormone (Swaminathan and Bahl, 1970; Aloj et al., 1973a; Morgan et al., 1974). The rate of recombination is enhanced by elevated temperature and low ionic strength.

There is extensive homology between the primary structures of the subunits of hCG and those of the corresponding subunits of three pituitary hormones, luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) (Ward et al., 1973; Shome and Parlow, 1974a,b; Pierce et al., 1971a, 1973). Within a given species, the α subunits are essentially identical, whereas the β subunits, although highly homologous, are sufficiently different to confer target organ specificity. By combining the α subunit of a given hormone with the β subunit of another, one can prepare hybrids which have biological properties characteristic of the hormone from which the β subunit was derived (Reichert et al., 1969, 1974; Pierce et al., 1971b; Rathnam and Saxena, 1971). Consequently, these hormones provide an interesting system for investigating subunit interactions. In addition, knowledge of the kinetics of subunit recombination may assist efforts to elucidate the biosynthetic pathway. This mechanism is important since free α and β subunits have been found to circulate in normal patients (Kourides et al., 1975) as well as in patients with various pathological conditions (Rosen and Weintraub, 1974).

It has been proposed (Bewley et al., 1974; Ingham et al., 1974; Reichert et al., 1974) that the mechanism of formation of the native hormone from their subunits involves at least two steps: a bimolecular association of the subunits to form a complex, $\alpha\beta$, followed by a first-order rearrangement in which the complex acquires the conformation of the native hormone. Thus, at sufficiently low subunit concentrations, the first step would be rate limiting and the kinetics would approach those of a second-order bimolecular reaction. At sufficiently high concentration, the rate of association of the subunits would be rapid and the first-order refolding step would become rate limiting. At intermediate concentrations, the kinetics would be intermediate between first and second order.

Previous studies of the kinetics of recombination have not adequately explored the effects of subunit concentration on the rates. Such an approach is necessary in order to establish the order of the reaction. A further complication stems from the fact that, in many cases, the recovery of the properties of the native hormones was substantially less than 100%. Therefore, we have focused our attention on the effect of concentration on the rate of recombination of the subunits of hCG, a hormone for which 100% recovery of the physical and biological properties is routinely obtained. The rate data have been analyzed in terms of several kinetic models in an effort to find the simplest model compatible with the experimental observations.

Materials and Methods

Highly purified preparations of hCG and its subunits (CR117) were obtained from Dr. R. Canfield of Columbia University through the Center for Population Research, National Institute of Child Health and Human Development, NIH. The hormone had a biological potency of 10600 IU/mg by the rat ventral prostate weight assay and ca. 14000 IU/mg by radioligand receptor assay, both based on the 2d international reference preparation. The α and β

† From the Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received September 3, 1975.*

* To whom correspondence should be addressed: Blood Research Laboratory, American National Red Cross, Bethesda, Maryland 20014.

[1] Abbreviations: hCG, human chorionic gonadotropin; LH, luteinizing hormone; TSH, thyroid stimulating hormone; FSH, follicle stimulating hormone; the prefixes b, h, and o, refer to bovine, human, and ovine, respectively; ANS, 1,8-anilinonaphthalenesulfonate.

subunits had potencies less than 0.5% of that of the intact hormone. A second source of hCG was a commercial preparation (Sigma) which was purified to a potency comparable to the CR117 preparation by gel chromatography (see below). Concentrations of hCG were based on the absorbance at 276 nm using ϵ 1.2×10^4 l./mol/cm (Ingham et al., 1975).

ANS (Eastman) was used without further purification and its concentration was determined from the absorbance at 350 nm using ϵ 6240 l./mol/cm (Ferguson et al., 1975). Fluorescence measurements were made on a Perkin-Elmer MPF3 fluorometer 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 was measured at 470 nm using excitation at 410 nm. The corresponding wavelengths used in measuring tryptophan fluorescence were 340 and 280 nm.

Gonadotropic activity was measured by radioligand receptor assay using [^{125}I]hCG (40 μCi/μg) as a tracer and a crude rat testis homogenate as a source of gonadotropin receptor, according to Catt et al. (1972). In the recombination experiments, aliquots were withdrawn periodically and diluted at least tenfold into a solution of 250 μM ANS. These samples were stored at 0 °C until completion of the reaction, at which time they were warmed to 25 °C and the ANS fluorescence was measured. These same samples were then further diluted into a solution containing 0.1% ovalbumin and 0.05 M sodium phosphate, pH 7.4, and frozen until time for the receptor assay. A control experiment indicated that the receptor binding activity was not affected by the low level of ANS present after dilution.

Two methods were employed to measure the rate of recombination of hCG subunits by ANS fluorescence. The aliquot method is similar to that previously described (Aloj et al., 1973a), except that the ANS concentration was increased to 250 μM in order to remove the small deviation from linearity previously observed in the plot of ANS fluorescence vs. hCG concentration. With the *direct* method, ANS (250 μM) was included in the reaction vessels, which were 0.5-cm diameter quartz cuvettes, kept in the fluorometer at the desired temperature. In this way, the ANS fluorescence could be monitired as frequently as desired and some of the random errors inherent in the aliquot method were avoided. All of the recombination experiments and ANS fluorescence measurements utilized 0.01 M potassium phosphate buffer, pH 7.0.

Most of the recombination experiments utilized subunits which were prepared by incubating the intact hormone in acid (pH $\leq$2) for 45 min or longer at 37 °C (Aloj et al., 1973a). This approach has the advantage of ensuring that the subunits will be present in 1:1 ratio for the recombination. Futhermore, part of the sample can be removed prior to acidification to serve as a reference for accurately assessing the extent of recovery of ANS fluorescence, receptor activity, and other properties.

Difference absorption spectra were measured on a Cary-15 spectrophotometer using the methods of Herskovits (1967) and Donovan (1969). Measurement of the rate of recombination by this method was complicated by the appearance of a small amount of turbidity which introduced curvature into the baseline. Accurate evaluation of the magnitude of the absorption difference required a correction for this effect. From the position of the baseline between 350 and 320 nm, where the protein does not absorb,

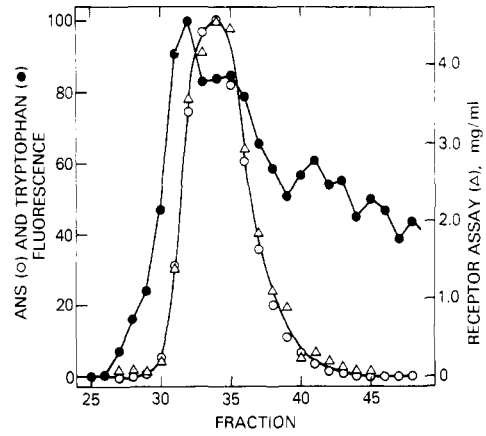

FIGURE 1: Chromatography of commercial grade hCG on a column of Sephadex G-100 (1.2 × 123 cm) in 0.01 M potassium phosphate buffer, 0.3 M KCl, pH 7.0, at room temperature. Approximately 70 mg of material was applied and the 2.0-ml fractions were assayed for protein by tryptophan fluorescence (●) and for hCG by ANS fluorescence (O) and receptor assay (Δ).

an extrapolation was made to 287 nm (the position of the peak) assuming an inverse 4th power dependence on wavelength in accord with Rayleigh's law (Tanford, 1961).

Sedimentation velocity measurements were made on a Beckman Model E analytical ultracentrifuge using scanner optics at 280 nm. The temperature of the rotor was controlled at 37 °C and solutions were scanned at intervals of 8 min. Sedimentation coefficients were calculated by standard procedures and reduced to water at 20 °C (Tanford, 1961).

Kinetic data were analyzed with the SAAM (simultation, analysis, and modeling) program of Berman et al. (1962a,b). A numerical approach was used in which the reactants are placed in compartments at the desired initial concentration and then allowed to pass into additional compartments representing the various intermediates along the reaction path. The rate of flow between compartments is determined by the initial estimates of the various rate constants. The rate of accumulation of material in the product compartment is compared with the experimental rate, and the rate constants are allowed to vary in an iterative fashion until the sum of the squares of the residuals, S, was minimized.

Purification of Commercial hCG. Figure 1 illustrates the elution profiles obtained when commercial (Sigma) hCG was applied to a column of Sephadex G-150 at 25 °C. Fractions were analyzed for tryptophan fluorescence, enhancement of ANS fluorescence, and gonadotropic activity as measured by the radioreceptor assay. The agreement between the profiles obtained by the latter two methods suggests that enhancement of ANS fluorescence is specific for biologically active hCG and indicates the absence of contaminating proteins which interact with ANS. The use of high KCl concentration (0.3 M) in this experiment was found to minimize the self-association of hCG. Similar experiments in the absence of KCl produced broader, less symmetrical, elution bands, which were skewed toward the void volume. The elution profile obtained by tryptophan fluorescence indicates the presence of contaminating proteins. It should be mentioned that hCG contains no tryptophan so that the presence of tryptophan fluorescence in any preparation of hCG reflects protein contamination. This test is particularly sensitive since tryptophan has a higher extinc-

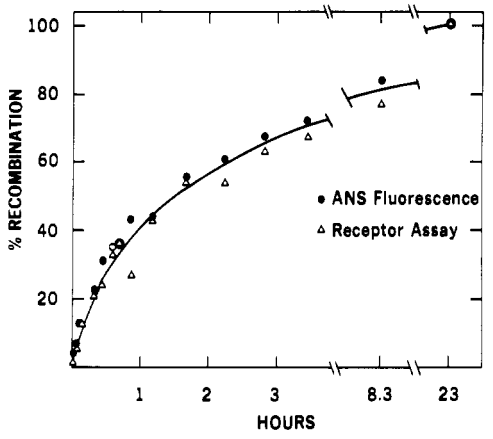

FIGURE 2: Rate of recovery of ANS fluorescenc and receptor binding activity following neutralization of a 70 μM solution of hCG (CR117) which had been dissociated into its subunits by incubation at pH 2.0 and 37 °C for 45 min. The recombination was also at 37 °C.

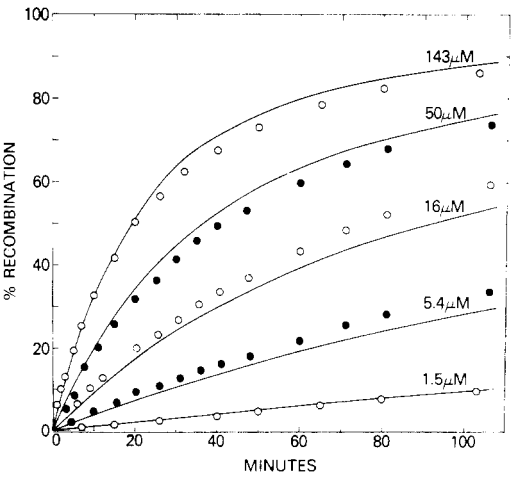

FIGURE 3: Effect of subunit concentration on the rate of formation of hCG at 37 °C. Subunits were prepared by incubation of the purified commercial hCG at pH 1.7 and 37 °C for 90 min. The solid curves are theoretical best fits of the data to model IV in Table II (see text).

tion and, in most proteins, fluoresces more strongly than tyrosine (Teale, 1960; Longworth, 1971).

Fractions 32–36 from Figure 1 were pooled and concentrated to 1 ml. After adding 30 μl of a solution of ANS in acetone (10 mg/ml), the sample was reapplied to the same column which was equilibrated and eluted with 100 μM ANS (0.01 M phosphate, pH 7.3, no KCl). Under these conditions, hCG self-associates to form higher oligomers (Ingham et al., 1975) and the partition coefficient, K_{av}, was reduced from 0.31 to 0.11. This allows hCG to be separated from other proteins of similar molecular weight which do not self-associate in the presence of ANS. The appropriate fractions were combined, dialyzed against 2 M urea to remove ANS, further dialyzed against 0.01 M $(NH_4)_2CO_3$, pH 7.7, and lyophilized to yield hCG having a receptor binding activity and ANS fluorescence enhancement equal to 109% of that of the CR-117 preparation.

Results

Validation of the ANS Fluorescence Assay. It was previously shown that ANS fluorescence is strongly enhanced by hCG but not by its subunits (Aloj et al., 1973a,b). The concentration of ANS required for half-maximal saturation was ~20 μM. However, subsequent studies of this interaction revealed that hCG self-associates to form dimers ($\alpha_2\beta_2$) and tetramers ($\alpha_4\beta_4$) in the presence of ANS and that enhancement of ANS fluorescence was due to binding by these oligomers (Ingham et al., 1975). This finding compelled us to confirm that ANS fluorescence is enhanced only by the native intact form of the hormone and that the presence of ANS in the recombination mixture does not influence the rate of that reaction.

Figure 2 illustrates the results of an experiment in which the recombination of acid-dissociated subunits was monitored by the recovery of both ANS fluorescence and receptor binding activity. The data have been normalized to 100% recombination at 23 h. The actual extent of recovery was 106% by ANS fluorescence and 103% by receptor assay. The agreement between the two methods indicates that only the completely refolded biologically active form of the hormone is capable of enhancing ANS fluorescence.

Since ANS causes the final product of the recombination reaction to self-associate, one might predict that its presence would accelerate the reaction. This possibility was eliminated by an experiment in which ANS was included in the reaction mixture at several concentrations (0, 100, and 250 μM). Aliquots were periodically withdrawn and diluted into an appropriate volume of 250 μM ANS such that the final ANS concentration was essentially identical in all of the assays. The rate of appearance of ANS fluorescence was identical in all three cases.

Sedimentation Velocity Measurements. There are reports in the literature that the subunits of luteinizing hormone can aggregate under various conditions (Bewley et al., 1972, 1974; Bishop and Ryan, 1973). Because of its extensive homology with LH, one might expect similar behavior for hCG, a result which would obviously complicate kinetic analysis of the recombination data. Therefore, we investigated the sedimentation behavior of hCG subunits under conditions (0.01 M potassium phosphate, pH 7.5, 37 °C) similar to those used for the recombination study. The concentrations used (α = 135 μM, β = 160 μM) were comparable to the highest concentrations used in the kinetic experiments (see below). The measured values of the sedimentation rates were 2.36 for hCG-α and 3.17 for hCG-β which, when corrected to standard conditions, give $s_{20,w}{}^0$ = 1.60 and 2.17 respectively. Both values are somewhat low when compared with sedimentation rates of globular proteins of similar molecular weight, in accord with the view that the isolated subunits are at least partially unfolded (Bewley et al., 1972; Garnier et al., 1975). The low ionic strength of the solvent will also tend to reduce the sedimentation rate because of the primary charge effect. The value obtained for hCG-α is in good agreement with published values obtained at lower protein concentration (de La Llosa and Jutisz, 1969; Bishop and Ryan, 1973) using homologous α subunits of similar molecular weights. The value obtained for the β subunit is substantially lower than the value of 2.89 obtained by Bahl (1969) for the intact hormone. Since a $\beta\beta$ dimer would have a molecular weight (46 000) greater than that of the intact hormone (38 000), we conclude that neither subunit is appreciably associated under the conditions used for the recombination.

Effect of Preincubation of Subunits. Most of the recombination experiments employed subunits prepared by acid dissociation of the intact hormone (see Methods). It is conceivable that exposure to acid might damage one or both subunits thereby altering the rate of recombination. If such

Table I: Summary of Parameters Describing the Effect of Subunit Concentration on the Rate of Recombination.

Subunit Concn (μM)	Initial Rate (% per min)	$t_{1/2}$ (min)
1.5	0.10	
5.4	0.41	215
16.1	1.0	75
50.0	1.8	40
143.0	3.2	20

damage occurs, it must be reversible since complete recovery of the physical and biological properties was routinely achieved. Further evidence against such a possibility was obtained using purified, isolated subunits. Solutions of α and β subunits were mixed to give a final concentration of 65 μM each. Part of the mixture was stored at 0 °C where no recombination occurs. The remainder was acidified to pH 2.1 and, after incubating at 37 °C for 40 min, was neutralized. The rate of appearance of ANS fluorescence (*direct* method) was indistinguishable between the two samples.

A second preincubation experiment was designed to detect any slow conformational change which might potentially be required of one or both subunits prior to their reassociation. Separate solutions of α and β subunits were incubated under recombination conditions for 0, 1, and 2 h before mixing and following the recombination (aliquot method). The results indicated that all three samples recombined at identical rates. If a slow conformational change were required of either subunit, preincubation would be expected to accelerate the reaction. These results also diminish the possibility of contamination of either subunit preparation with its complementary subunit since they would have recombined during preincubation.

Effect of Subunit Concentration. In order to provide data with which to test various kinetic models, the rate of recombination of acid-dissociated subunits was measured over a 100-fold range of subunit concentrations. The results are illustrated in Figure 3 where the circles are experimental points and the solid lines are theoretical best fits based on the model presented below. For these experiments, a sample of the purified commercial hCG (150 μM) was dissociated into its subunits by incubation at pH 1.7 and 37 °C for 90 min. This resulted in a reduction in the ability to enhance ANS fluorescence to less than 3% of that of the undissociated sample. This mixture of subunits was then kept at 0 °C while the pH was adjusted to 7.0 and appropriate dilutions made. ANS was then added to a final concentration of 250 μM and the recombination was monitored at 37 °C by the *direct* method. The reaction was allowed to proceed for 20 h. A comparison of the final ANS fluorescence with that of the control sample indicated that the 16, 50, and 143 μM samples had recombined completely, while the 1.5 and 5.4 μM samples had respectively undergone 37 and 90% recombination. When the final fluorescence values were plotted against the concentration, the points corresponding to 16, 50, and 143 μM fell on a straight line through the origin indicating that, under these conditions, the proportionality between ANS fluorescence and hCG concentration extends to the highest concentration employed.

The initial rates and times required for 50% recombination are summarized in Table I. At the lowest concentration range the initial rate was approximately proportional to the subunit concentration, as expected for a simple second-order combination reaction. However, at the highest range a threefold increase in subunit concentration caused only about a twofold change in the initial rate and half-time indicating that at least one additional step is involved in the reaction, in agreement with previous conclusions based on studies with homologous hormones (Bewley et al., 1974; Ingham et al., 1974; Riechert et al., 1974).

Table II: Summary of Various Models Used to Fit Recombination Data.

Model	Mechanism	Parameter Values	S^a
I	$\alpha + \beta \xrightarrow{k_1} H$	$k_1 = 4.4 \times 10^2$ M^{-1} min^{-1}	599
II	$\alpha + \beta \xrightarrow{k_1} \alpha\beta$	$k_1 = 7.1 \times 10^2$ M^{-1} min^{-1}	355
	$\alpha\beta \xrightarrow{k_2} H$	$k_2 = 0.41$ min^{-1}	
III	$\alpha + \beta \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \alpha\beta$	$k_1 = 6.6 \times 10^3$ M^{-1} min^{-1}	
		$k_{-1} = 1.1$ min^{-1}	189
	$\alpha\beta \xrightarrow{k_2} H$	$k_2 = 0.14$ min^{-1}	
IV	$\alpha + \beta \overset{K}{\rightleftharpoons} \alpha\beta$	$K = \frac{k_1}{k_{-1}} = 7.0 \times 10^3$, M^{-1}	
		$k_{-1} = 10.0$ min^{-1} (fixed)	144
	$\alpha\beta \xrightarrow{k_2} H$	$k_2 = 0.11$ min^{-1}	

[a] Sum of squares of residuals.

Several models were tested for their ability to explain the effects of concentration on the rate of recombination, simultaneously fitting all 5 sets of data in Figure 3. The models are summarized in Table II along with the values of the parameters and a quantitative indication of the relative quality of the fits, as expressed by the quantity S, the sum of the squares of the residuals. It was not possible to obtain a reasonable fit with a simple one-step, second-order mechanism (model I). Although any one of the experimental curves in Figure 4 could be reasonably fit to this mechanism, it was not adequate to explain the effects of concentration. We next tried a two-step mechanism (model II) in which the subunits associate irreversibly to form an intermediate complex, $\alpha\beta$, followed by a first-order molecular rearrangement in which the comformation of the native hormone, H, is acquired. This led to a substantial improvement in the quality of the fit, as reflected by the two-fold reduction in S, but the fit was still poor. We then introduced a third parameter, k_{-1}, corresponding to the dissociation of the $\alpha\beta$ complex (model III). This led to a further two-fold reduction in S. The value obtained for k_{-1} was an order of magnitude larger than that obtained for k_2, suggesting that it might be possible to replace k_1 and k_{-1} by an equilibrium constant $K = k_1/k_{-1}$, with the added restriction that the equilibrium be fast compared with the second step. This was accomplished by fixing $k_{-1} = 10.0$ min^{-1} and allowing k_1 and k_2 to vary (model IV). This led to a slightly better fit and gave the theoretical curves shown in Figure 4. Considering the broad (100-fold) range of concentration covered and allowing for a 5-10% error in the end points of each curve, the fit seems very satisfactory. The slightly better fit obtained with model IV, which is a special case of III, is probably due to

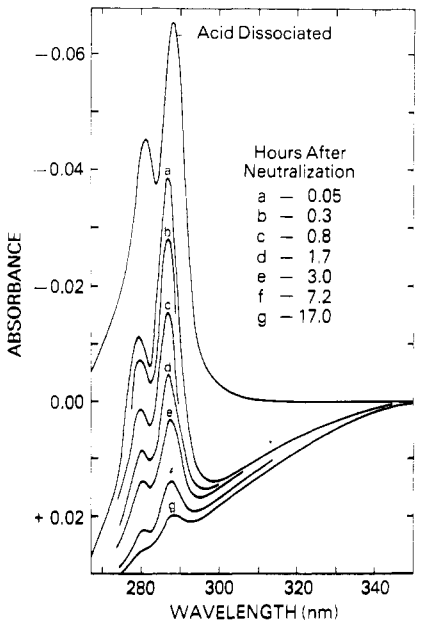

FIGURE 4: Differential absorption measurements of hCG (CR117) at 37 °C. The solution in the sample compartment was at pH 7.0. The hCG concentration was 40 μM. The upper spectrum was obtained after keeping the reference solution at pH 3.0 for 3.0 h. The remaining spectra were obtained periodically after neutralization of the reference solution. The solvent contained 0.01 M potassium phosphate and 0.01 M glycylglycine.

the reduced number of parameters and the consequent greater ease with which the program locates the true minimum in the error surface.

In order to investigate the uniqueness of the best fit obtained with model IV, a study was made of the interdependency of K and k_2. This was accomplished by arbitrarily fixing these parameters, one at a time, at values above and below the best fit values, and determining the effect on the quality of the fit. The results, illustrated in Table III, indicate that either parameter can be increased or decreased by 30–40% with very little effect on S. However, if either parameter is changed by a factor of ~3, the other parameter is unable to compensate and the quality of the fit is drastically reduced.

Absorption Difference Measurements. The acid dissociation of hCG into its subunits is accompanied by the appearance of a large negative difference absorption (blue shift), as illustrated by the upper spectrum in Figure 4. The difference spectrum exhibits sharp peaks at 280 and 287 nm which are characteristic of tyrosyl perturbations (Donovan, 1969). Neutralization of the acid-dissociated sample results in the immediate appearance of a small amount of turbidity, followed by a gradual loss of the spectrum. When the absorption difference at 287 nm was corrected for baseline curvature (see Methods), the recombination curve in Figure 5 was obtained. Extrapolation of the corrected absorption difference at 287 nm to zero time indicated that virtually no instantaneous change in absorption occurred following neutralization. The dashed curves in Figure 5 are taken from the data in Figure 3 at 16 and 50 μM for comparison with the absorption data at 40 μM.

Discussion

The results presented here confirm the validity of the ANS fluorescence assay for hCG, which, under appropriate conditions, offers a convenient alternative to bioassay, radioimmunoassay, and radioreceptor assay. Although the latter assays have a greater sensitivity and specificity, they require more elaborate preparation. For some applications, the ANS fluorescence method may be more specific than the radioimmunoassay, e.g., for distinguishing between the intact hormone and its subunits as illustrated in this report. The use of ANS fluorescence to locate hCG in a series of column fractions is illustrated in Figure 1. The excellent agreement between the radioreceptor assay and the ANS assay suggests that the latter is specific for the native, biologically active form of the hormone. This conclusion is further supported by the agreement between the rates of recombination as measured by the two assays (Figure 2).

Table III: Uniqueness Test of "Best Fit" Values Obtained with Model IV.[a]

K (10^3 M^{-1})	k_2 (min^{-1})	S
2.*	0.83	6175
5.0*	0.15	164
(7.0)	(0.11)	(144)
9.0*	0.093	152
20.0*	0.082	7135
34	0.03*	825
12	0.07*	208
(7.0)	(0.11)	(144)
5.1	0.15*	163
2.1	0.33*	299

[a] After fixing either K or k_2 at the values indicated with the asterisks, the calculation was repeated, allowing the remaining parameter to vary. The "best fit" values are included in parentheses. For these calculations, k_{-1} was fixed at 10.0 min^{-1}.

The fact that hCG self-associates to form dimers ($\alpha_2\beta_2$) and tetramers ($\alpha_4\beta_4$) in the presence of ANS (Ingham et al., 1975) was utilized in the present report as an aid in the purification of commercial hCG by gel chromatography (see Methods). A similar approach might be useful for relieving subunit preparations of contamination by intact hormone. The complicated nature of the ANS binding compelled us to ensure that the ligand-induced self-association did not interfere with the interpretation of the kinetic data. The lack of effect of ANS on the rate of recombination suggests that ANS does not interact with the postulated $\alpha\beta$ intermediate and that the rearrangement of this intermediate to form the native structure is an irreversible process. If the final step involved an equilibrium ($\alpha\beta \rightleftarrows$ H), then ANS should have enhanced the rate by mass action. Furthermore, we have never observed a loss of physical or biological properties of native hCG upon standing for up to 24 h at 37 °C and neutral pH.

There have been several reports dealing with the rates of recombination of the subunits of hCG and other homologous hormones. Reichert et al. (1973) measured the rates of recombination of hLH and hCG subunits and concluded that the reactions followed second-order kinetics. In a later study (Reichert et al., 1974), data on the rate of recombination of hFSH-β with various α subunits could be fit equally well by first- or second-order rate equations, and it was concluded that the combination step must be followed by at least one unimolecular refolding step. A two-step mechanism was also suggested by us (Ingham et al., 1974) based on observation of the effect of subunit concentration on the

rate of formation of bTSH. Bewley et al. (1974) followed the recombination of oLH subunits using near ultraviolet circular dichroism and sedimentation velocity measurements. They found that the time required for the sedimentation coefficient to reach its final value was much less than that required to regenerate the circular dichroic spectrum of the native hormone, again suggesting the existence of at least two steps in the reaction scheme.

Our results confirm and extend these conclusions regarding the multi-step nature of the recombination mechanism. The effect of subunit concentration on the kinetics of recombination could not be explained on the basis of a simple second-order reaction. We obtained a substantial improvement in the fit of our data when a second step was incorporated (model II, Table II). However, a better fit was obtained when the initial step was allowed to be reversible (model III). By incorporating a rapid preequilibrium into model IV, it was possible to obtain a reasonable fit over the entire range of concentration, using only two parameters. It is of interest that Merz et al. (1973) reported the appearance of a *transient* species in mixtures of hCG-α and hCG-β which had immunological and circular dichroic spectral properties unlike those of the native hormone or either subunit. It is possible that this species could be the postulated $\alpha\beta$ intermediate.

A quantitative analysis of the large negative difference absorption (Figure 4) indicates that at least two tyrosyl residues are exposed to a more polar environment upon dissociation of the subunits (Ingham et al., 1976). Similar results have been reported for other homologous hormones (Ingham et al., 1974; Bishop and Ryan, 1975; Garnier et al., 1975), and are supported by chemical studies using iodine and other tyrosine specific reagents (Canfield et al., 1972; Hum et al., 1974). There was no measurable change in the absorption difference immediately following neutralization of the acid-dissociated sample and the rate of disappearance of the absorption difference was not significantly different than the rate of recovery of ANS fluorescence (Figure 5). Using $K = 7.0 \times 10^3\ M^{-1}$ (model IV), about 19% of the subunits should initially be present as the $\alpha\beta$ complex at the subunit concentrations employed (40 μM). Thus, in terms of the proposed mechanism, the absorption data suggest that the first step (formation of the $\alpha\beta$ complex) does not lead to a significant change in the environment of the tyrosyl residues, but that most of this change occurs during the subsequent acquisition of the native tertiary structure.

There are several reasons for exercising caution in the interpretation of our results, especially with respect to the proposed kinetic model, whose ability to adequately explain the kinetic data does not prove its validity. Other conceivable models might provide an equally good fit of the data, especially if additional parameters are introduced. One possibility is that one or both subunits might be required to undergo a conformational change prior to recombination. However, preincubation of the individual subunits under recombination conditions had no effect on the rate. Thus, any obligatory conformational change prior to reassociation would have to involve a rapid equilibrium. Another reasonable possibility is that the subunits reversibly form several distinct $\alpha\beta$ complexes, only one of which allows the native conformation to develop. Such reactions, if present, would obviously alter the meaning of K in model IV. Finally, self-association of the individual subunits would also complicate the kinetic analysis. Our sedimentation velocity measurements indicate that this is not a problem with hCG.

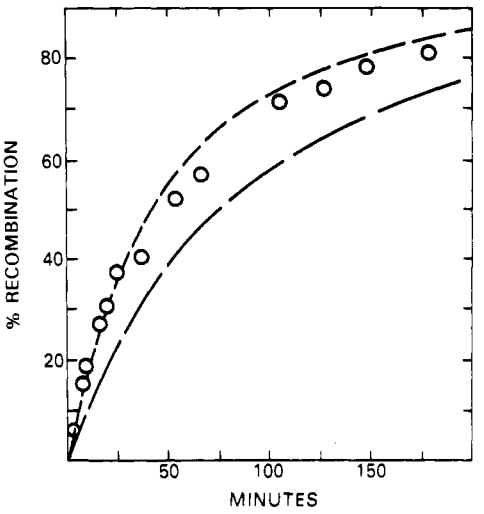

FIGURE 5: Rate of recombination of subunits of hCG as measured by absorption difference at 287 nm. Data obtained from experiment in Figure 4. The lines are the experimental curves corresponding to 16 μM (——) and 50 μM (- - -) in Figure 3.

One unexplained factor regarding the elucidation of the recombination mechanism is the observation of small but measurable differences in the rates of recombination for different subunit preparations. The order of rates for the preparations which we have used is: CR117 (isolated subunits) > purified commercial (acid dissociated) > CR117 (acid dissociated). As yet we have no explanation for this discrepancy. The isolated subunits were prepared by urea dissociation followed by ion-exchange chromatography (Morgan and Canfield, 1971), whereas most of our experiments utilized subunits prepared by acid dissociation of the intact hormone. The possibility that acid dissociation might damage the subunits was eliminated by the observation reported here that incubation of the isolated subunits under similar conditions had absolutely no effect on their rate of recombination. Furthermore, all three preparations yield 100% recovery of the properties of the native hormone. It is unlikely that these discrepancies seriously detract from the adequacy of the proposed mechanism to account for the effects of subunit concentration on the rates, although the values of the constants will vary for the different preparations. This is supported by the fact that, using an earlier preparation of hCG (CR116), model IV gave an excellent fit to recombination data obtained at 23 and 70 μM with $K = 4.7 \times 10^3\ M^{-1}\ min^{-1}$ and $k_2 = 0.055\ min^{-1}$.

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