REVERSIBLE FOLDING OF HUMAN CHORIONIC GONADOTROPIN AT ACID pH OR UPON RECOMBINATION OF THE α AND β SUBUNITS

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1. Introduction

Gonadotropic hormones share the property of being made of two non-covalently bound subunits, the α and β subunits. Isolated subunits have been found to be inactive either by biological or by radioligand receptor assays [1,2]. Circular dichroism (CD) measurements performed by several authors [3–5] on native ovine luteinizing hormone (oLH) and its isolated subunits strongly suggest at least a partial unfolding of the polypeptide chains upon dissociation and refolding upon reassociation and subsequent recovery of the biological activity.

Recently it has been found by Aloj et al. [6] that native human chorionic gonadotropin (hCG) and hLH, but not their subunits, enhance ANS fluorescence, which seems to be a sensitive probe of the associated state. However, this property is so far limited to these luteinizing hormones since, for example, native oLH exhibits only a weak binding affinity for ANS (7×10^3 M^{-1} at 37°C) and a very low enhancement (ten times less than hCG) of the quantum yield of fluorescence as found by Aloj et al. [6] and by Garnier (unpublished).

It was suggested by studies of the chemical reactivity towards tetranitromethane or iodine that some tyrosine residues were exposed by dissociation of the subunits of ovine, bovine and porcine LH [7–10]. The same conclusion was reached by difference spectroscopy [4] and CD measurements of oLH [3–5]. Evidence is presented here that acid dissociation of hCG also resulted in a reversible unfolding of the subunits during which tyrosine residues were perturbed as in other luteinizing hormones. Good agreement was observed between rates of unfolding and refolding when these processes are followed either by difference spectroscopy or by ANS fluorescence. Extent of unfolding was estimated from computed periodic (secondary) and aperiodic structures from observed CD.

2. Material and methods

Purified hCG, hCGα and hCGβ subunits were obtained from NIH, batch CR 117 of a biological potency of 10 600 IU/mg, 0.8 IU/mg and 40.3 IU/mg respectively.

Protein concentrations were calculated from the absorbance at 276 nm by using $A^{1\ mg/ml}_{1\ cm} = 0.39$ for hCG [11] and 0.55 and 0.28 for hCGα and hCGβ, respectively, computed from the absorbance of hCG on a basis of 4 Tyr residues in hCGα and 3 Tyr residues in hCGβ for a calculated molecular weight of 14 961 and 21 763 respectively from sequence data [11].

Acid unfolding was performed on hormone preparations dissolved in 0.1 M NaCl and adjusted with 0.1 M NaOH to pH 7.0 ± 0.1. The pH was dropped to pH 2.1 ± 0.03 by adding an aliquot of N HCl (i.e. 10 μl to 750 μl of hormone or subunits solutions), followed by quick mixing (mixing time approx. 10 sec.). Some acid unfoldings were also performed by adding 200 μl of a solution of 30 μM hCG in 0.1 M NaCl (pH 7.0) to 1.6 ml of a solution of 20 mM phosphate, 0.1 M KCl previously adjusted with N HCl to pH 2

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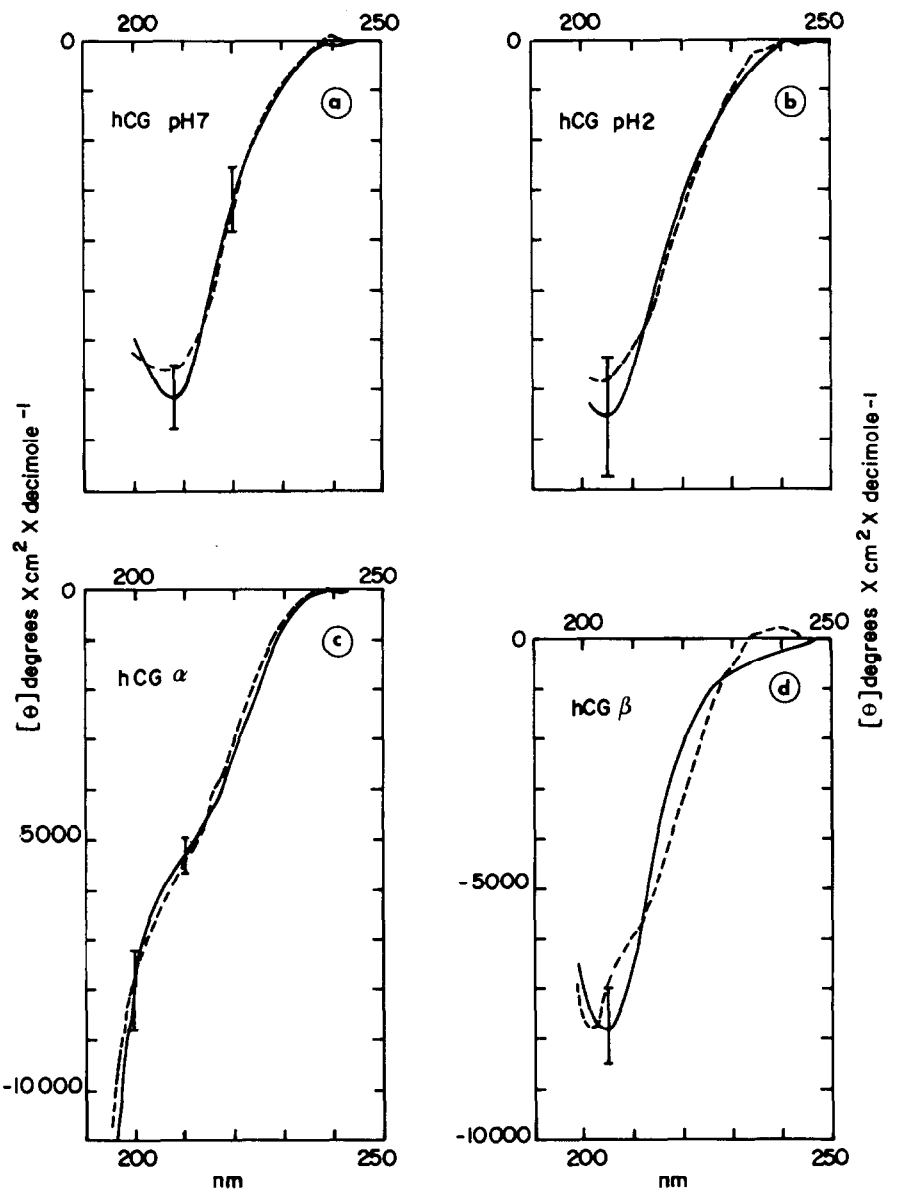

Fig. 1. (a) CD of recombined hCG (——). Protein concentration: 1.3 mg/ml in 5 mM KCl, 20 mM phosphate 6.9, and computed CO: 3% α helix, 34% β structure and 62% aperiodic structure (- - - -). (b) CD of hCG at pH 2, 0.1 M NaCl (——) and computed CD: 1% α helix, 28% β structure and 70% aperiodic structure (- - - -). (c) CD of hCGα at pH 6.5, 0.1 M NaCl (——) and computed CD: −1% α helix, 27% β structure and 72% aperiodic structure (- - - -). (d) CD of hCGβ at pH 6.5, 0.1 M NaCl (——) and computed CD: −0.3% α helix, 32% β structure and 68% aperiodic structure (- - - -). Protein concentration: 1.1 mg/ml for both subunits. Room temperature Estimated experimental errors are indicated by a vertical line.

Refolding at neutral pH was carried out hCG solutions, which were previously acid unfolded, as mentioned above, by adding an aliquot of 1 M phosphate buffer pH 6.9 (i.e. 25 μl to 760 μl of a 0.1 M NaCl (pH 2.1 ± 0.3) hCG solution), resulting in a final pH 6.45 ± 0.1.

Recombinations of subunits were followed by difference spectroscopy by using double-sector cells (2 X 0.437 cm path length), with the reference cell containing hCGα in one compartment and hCGβ in the other. Both compartments of the sample cell contained a mixture of an equal volume of these two subunit solutions. The solutions were made in 20 mM phosphate buffer (pH 7.4) and 5 mM KCl.

All experiments were carried out at 37°C.

Difference spectroscopy was made in a Cary 14 spectrophotometer and absorbance changes with time were followed at 286.5 nm. Spectra were usually corrected for light scattering which never exceeded 10% of the maximum of absorbance change at 286.5 nm.

For fluorescence studies, ANS was used as the magnesium salt, recrystallized from the commercial sodium salt (K and K Lab.). Measurements were carried out in a Farrand MKI spectrofluorometer using a 1 cm path length cell. ANS concentration was obtained from absorbance at 350 nm (ϵ = 5000, ref. [12]), an aliquot of the ANS stock solution (2.4 X 10^{-2} M) was added to a solution of hCG (0.5 to 30 μM) and emission intensity of ANS was measured at 480 nm with excitation at 360 nm [6]. Acid unfolding and refolding at neutral pH was performed as mentioned above and in the presence of ANS (20–105 μM).

Rate constants were calculated by curve fitting of the experimental data according to a least squares analysis adaptated for a multilinear regression (ITER 04 and 05) and computed with Univac 1108 or 1110.

Ultracentrifuge experiments were performed on a Spinco Beckman ultracentrifuge model E, equipped with a laboratory-made single beam photoelectric scanner. CD was measured on a Jouan dichrograph II with 0.5, 0.1 and 0.01 cm path length cells. Ellipticities were calculated with a mean residue molecular weight of 151, 156 and 148 for hCG, hCGα and hCGβ, respectively. Periodic structures were computed by least squares fitting to observed CD according to Chen et al. [13].

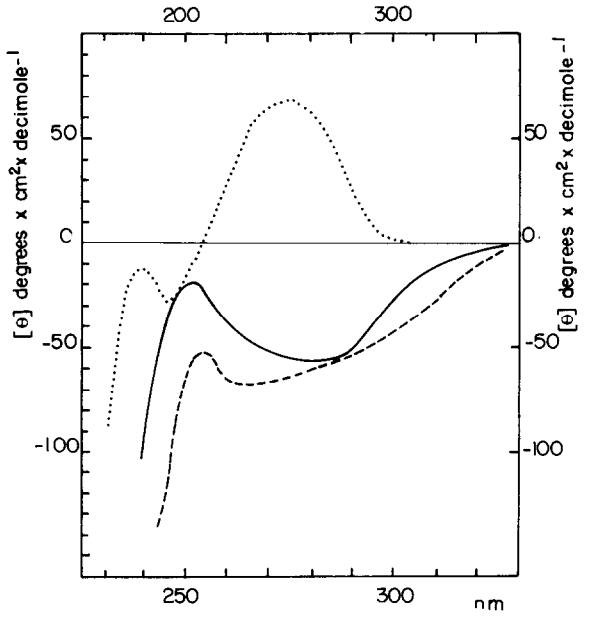

Fig. 2. Near UV CD of recombined hCG (——), hCGα (. . . ...) and hCGβ (- - - -) at pH 6.5, 0.1 M NaCl. Room temperature.

3. Results and discussion

3.1. *Folding state of hCG and its subunits*

CD of recombined α and β subunits (figs. 1 and 2) was identical to already published CD of native hCG [14,15]. Conformations of the native hormone and its subunits could be solved with only α helix, β structure and aperiodic (unordered) structure (fig. 1). They are characterized by a sizable fractional content of β structure (about one third) and a low α helical content. Acid unfolding or separation of the subunits led to a significant decrease of the β structure and subsequent increase of aperiodic structure, the ratio of β structure/aperiodic structure was 0.56 at pH 7 and 0.41 at pH 2 or 0.43 for α and β subunits all together (fig. 1). Those structural changes are small but apparently enough to tremendously decrease the binding affinity constants of the two subunits for each other and for the receptor. Similar results were found with oLH (to be published).

3.2. *Unfolding of hCG at acid pH*

Acid treatment of hCG at pH 2 not only leads to the dissociation of the subunits ($s_{20,w}$ = 2.7 at pH 2 against $s_{20,w}$ = 3.5 at pH 6.4 at a protein concentration of 0.6 and 1 mg/ml, respectively), but also to an alteration in the folding of the polypeptide chains similar to that observed for oLH [3,4] i.e. a blue shift of the 208 ± 1 nm CD band to 205 ± 1 nm and a significant decrease of the 275–280 nm CD band in a region where tyrosine residues are expected to contribute. This is confirmed by the observation of a difference spectrum between hCG at pH 2 versus

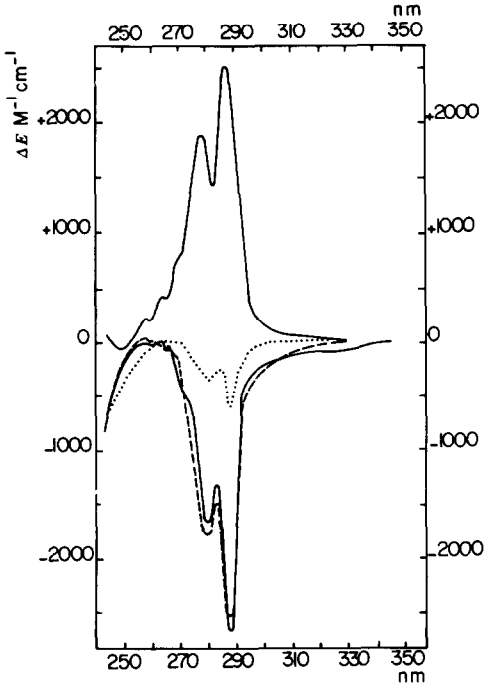

Fig. 3. Difference spectra of hCG at 37°C after recombination or at pH 2. Upper half: Recombined hCG (samples cell) versus separated hCGα (75 μM) and hCGβ (63 μM) subunits (reference cell) at pH 7.4 after 16 hr of contact at 37°C (20 mM phosphate buffer, 5 mM KCl). Lower half: hCG pH 2.1 (sample cell) versus hCG pH 7.0 (reference cell) 0.1 M NaCl (——) hCG concentration = 30 μM; hCG (30 mM phosphate pH 6.45, 0.1 M NaCl) in the sample cell versus hCG pH 7.0, same buffer in the reference cell. The difference spectrum was scanned 15 hr after having adjusted the pH from 2–6.45 in the sample cell (. . . . .), hCG concentration = 30 μM; hCG (30 mM phosphate pH 2.1, 0.1 M NaCl) versus hCG pH 7.0 same buffer after renaturation from pH 2.1 to 6.45 (- - - -). Spectra are corrected from light scattering.

Table 1
Macroscopic rate constants of folding of hCG at 37°C

Unfolding at pH 2 (sec^{-1})	Refolding at pH 6.4 (CM^{-1} sec^{-1})
3.5×10^{-3} [a]	0.8 [a]
3.3×10^{-3} [b]	2.2 ± 1.8 [b,d]
3.6×10^{-3} [a,c]	3.[e]

a As followed by difference spectrophotometry at 286.5 nm (30 μM hCG, 0.1 M NaCl for unfolding and 0.1 M NaCl 30 mM phosphate for refolding).

b As followed by enhancement of ANS fluorescence at 480 nm (1 and 3.3 μM hCG, 20–25 μM ANS, 20 mM phosphate, 0.1 M KCl for unfolding and 0.5 to 30 μM hCG, 25–105 μM ANS, 0.1 M NaCl 30 mM phosphate for refolding).

c After renaturation to pH 6.4 (29 μM hCG, 0.1 M NaCl, 30 mM phosphate).

d 0.95 confidence limit.

e Recombination of the subunits 75 μM hCGα, 63 μM hCG in 5 mM KCl, 20 mM phosphate pH 7.4. Followed by difference spectrophotometry at 286.5 nm.

hCG at pH 7 (fig. 3) which corresponds to a blue shift of the absorption band of tyrosine and phenylalanine residues by, for instance, the exposure of new aromatic chromophore to the solvent. No CD changes or significant difference spectra could be detected in the isolated subunits upon acidification.

3.3. *Reversibility of the unfolding*

This was shown firstly by observing the disappearance with time of the difference spectrum by raising the pH from 2 to 6.4 (fig. 3). Secondly by denaturing the refolded hormone again at pH 2: an identical spectrum versus the native form was observed which develops at the same rate (fig. 3 and table 1). Thirdly the refolded hormone exhibited the same CD features as the native hormone.

Upon recombination of the separated subunits at pH 7.4, a difference spectrum corresponding to a red shift of the absorption bands of the aromatic amino acid residues was observed which is the mirror image of the difference spectrum observed at pH 2 (fig. 3). The CD of the recombined subunits was very close, if not identical, to the native conformation with the characteristic 208 and 280 nm dichroic bands (figs. 1 and 2).

3.4. *Rates of unfolding and refolding of hCG*

Conformational changes were followed at the maximum of the difference spectra (286.5 nm) and by enhancement of ANS fluorescence.

At 37°C the time course of unfolding could be described essentially by only one exponential term with an apparent (macroscopic) rate constant of $(3.5 \pm .1) \times 10^{-3}$ sec^{-1} at pH 2, independent of concentration within a 30-fold range. Both methods gave the same rate constants (see table 1). These rates are in very good agreement with Aloj et al. [16] under the same conditions of ionic strength, ANS concentration and pH. However, we observed that rates of unfolding at higher ANS concentration (80 to 100 μM for 30 μM hCG) are significantly lower ($1 \pm 0.3 \times 10^{-3}$ sec^{-1}), as if ANS would stabilize the native form to which it binds.

The unfolding rates of hCG are about one order of magnitude smaller than the rates observed for oLH [4,17] under the same experimental conditions. On the other hand, at least two first order steps can be detected for oLH unfolding and essentially only one for hCG.

Apparent second order rate constants for refolding were found to be independent of the concentration from 0.5 to 60 μM (table 1) within the experimental errors and whatever method is used. They are in good agreement with the rate measured by Aloj et al. [16]. From this a possible reaction scheme for refolding would be first a quick equilibrium, possibly diffusion controlled, of subunit pairing, with a low association constant K_{eq} ($K_{eq} \leqslant 10^4$ M^{-1}) followed by a rate limiting first order step, k_3, towards the native state. The observed rate constant would then be pseudo-second order and equal to $K_{eq} \times k_3$ with $k_3 \geqslant 10^{-4}$ sec^{-1}

The similar conformational behaviour of the two biologically and structurally related hormones, hCG and oLH, is worthy of note if one considers that their synthesis occurs in two different tissues: the chorion and the anterior pituitary gland, and that the content and nature of their carbohydrates are quite different [11,18]. Although the hCGβ subunit contains an extra C terminal peptide of 28 amino acid residues compared to oLHβ, the same conformational permanence is encountered.

If a similarly low rate of folding occurs in vivo to form the native conformation after the synthesis of the polypeptide chains on the ribosome, the necessity of a storage of the native preformed hormone in the secretory cells becomes evident both to increase rates of subunit pairing and to allow a rapid increase in release after stimulation by the releasing factor.

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