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Spectroscopic characterization of recombinant follicle stimulating hormone

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Abstract

Recombinant follicle stimulating hormone (recFSH, Org. 32489) has been characterized by absorption (UV and IR), (polarized) fluorescence, linear-dichroism (LD) and circular-dichroism (CD) spectroscopy. Absorption and fluorescence spectra of the isolated subunits have also been measured. From the spectra the extinction coefficient, fluorescence quantum yield and anisotropy have been calculated. Global analysis is used to characterize the bands in the spectra. The adsorption, CD, LD and fluorescence excitation spectra all contain a band around 300 nm that appears to be a sensitive indicator for the intactness of the protein. Evidence is provided for the involvement of tyrosinate in the fluorescence, and for a close contact between the tryptophan (in the β subunit) with at least one tyrosine of the α subunit. The overall secondary structure of recFSH has been determined from its far-UV CD and its IR absorption spectrum. The secondary structure of recFSH is estimated to contain 15-25% α -helix, 15-25% β -turn and 30-40% β -sheet. The β -sheet in recFSH is almost exclusively antiparallel. The results confirm that recFSH contains significantly more α -helix than the closed related human glycoproteins, chorionic gonadotropin and lutropin; however, the α -helices may be short and distorted.

Keywords: FSH; Global analysis; Gonadotropine; Protein structure; Spectroscopy; Tyrosinate

1. Introduction

Follicle stimulating hormone (FSH) plays an important role in ovulation in the female and in spermatogenesis in the male. FSH in combination with luteinizing hormone (lutropin, LH) is used in treatment of infertility for both men and women [1-3]. It is also applied for in vitro fertilization [4,5]. FSH binds to a specific receptor on granulosa cells in ovaries and to Sertoli cells in testes [6-8]. Binding to the receptor activates adenylate cyclase, resulting in production of the second messenger cAMP.

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FSH belongs to the group of glycoprotein horwhich includes LH, chorionic gomones. nadotropin (CG) (the gonadotropins) and thyroid stimulating hormone (TSH). These proteins are composed of two subunits, a common α subunit and a hormone-specific β subunit, which are noncovalently linked. The amino-acid sequence of these hormones has been determined for a number of species [6,9]. The α and β subunits of human FSH (hFSH) consist of 92 and 111 amino acids, respectively, and the corresponding molecular weights are 15.7 and 19.2 kDa. The α subunit contains five and the β subunit six disulfide bonds [10,11]. Both subunits have two N-linked carbohydrate side-chains each with a molecular weight of approximately 3 kDa [12]. The composition of the carbohydrate side-chains shows considerable variability, especially in sialic acid content [13]. These so-called isoforms of FSH can be differentiated on the basis of their isoelectric point [14]. The function of isoforms is to allow a sensitive fine-tuning of the biological activity of FSH [12.15].

Spectroscopic data on recombinant FSH (rec FSH) are incomplete and inconsistent. Therefore, we have determined the absorption, fluorescence, linear and circular-dichroism (LD and CD) and Fourier transform infrared (FTIR) spectra of recFSH. From the spectra information is extracted concerning the conformation of the protein. In order to interpret the data we have also measured the absorption and fluorescence spectra of the isolated subunits.

2. Experimental

RecFSH was acquired from N.V. Organon (Oss, The Netherlands) in the lyophilized form (Org. 32489), and stored at 4°C. Subunits (α and β) of recFSH were provided by N.V. Organon and stored at – 20°C. For fluorescence, near-UV absorption and near-UV CD measurements, recFSH was dissolved in distilled water just prior to the measurements. For far-UV CD, LD, and far-UV absorption measurements it was necessary to remove the sucrose and citrate from the Org. 32489 product. This was achieved by centrifuging recFSH twice in a Centricon-10 tube (Amicon) at 4°C (Beckman J-21B centrifuge, JA20 rotor, 4000 \times g, 15 min). From IR spectra we estimated that with this approach at least 95% of the sucrose and citrate was removed. More than 95% of the protein was recovered. After removal of sucrose and citrate the samples were stored at – 20°C. All spectra were measured at room temperature (22°C).

UV absorption was measured on a Cary 219 spectrophotometer. Spectra were recorded in a 1 cm pathlength quartz cuvet for near-UV or a 0.1 mm pathlength curvet for the far-UV. The temperature of the cuvet holder was thermostated with a waterbath.

Infrared absorption spectra were recorded on a Perkin-Elmer 1750 Fourier transform infrared (FTIR) spectrometer equipped with a fast recovery TSG detector and a Perkin-Elmer 7300 data station. Samples were placed in a Beckman FH-01 CFT micro-cell fitted with CaF₂ windows and a 6 μ m tin spacer for measurements in H₂O or 50 μ m teflon spacer for measurements in D₂O. The chamber was purged continuously with dried air. Temperature control was achieved by means of a cell jacket of circulating water, and the temperature of the cell was monitored using a thermocouple fixed on the outside of the cell. The concentration of the samples was $5-20 \text{ mg ml}^{-1}$ for the D_2O and 61 mg ml⁻¹ for the H_2O measurements. No aggregation of the samples was observed. For removal of sucrose and citrate the samples were filtrated three times by centrifugation in Centricon-10 tubes as described above. The last filtrate was used for reference measurements. Double-sided interferograms were recorded and apodized using a medium Norton-Beer function prior to Fourier transformation of the data. For each sample 400 scans (in H₂O) or 100 scans (in D₂O) were averaged. All spectra were recorded at a resolution of 4 cm⁻¹ between 400 and 4000 cm⁻⁻¹. Reference and sample spectra were measured separately and subtracted as previously described [16]. The broad amide I bands were analyzed by deconvolution using the Perkin-Elmer DERIV function. Deconvolution of the spectra was performed using the Perkin-Elmer ENHANCE function with a bandwidth of 16 cm^{-1} and resolution enhancement factor of 2.5. Prediction of secondary structure was performed with a multivariate model [16], and by factor analysis [17].

Fluorescence measurements were performed at room temperature on a SLM Aminco SPF500 Nozone fluorimeter in a four-sided 1 cm pathlength quartz cuvet with a volume of 0.8 ml. The difference between the absorption and excitation spectrum of sulforhodamine was used to correct the excitation spectra for the characteristics of the lamp and the monochromator. To avoid possible damage to the protein, the bandwidth for the excitation light was 0.5 nm. The quantum yield $(\phi_{\rm F})$ was calculated from the integrated fluorescence emission spectrum (excitation at 280 and 295 nm), with respect to the quantum yield of tryptophan in solution at pH 7.0 ($\phi_{\rm F} = 0.2$; [18]). The maximal absorption of the samples used for fluorescence measurements was 0.1 at 277 nm. For anisotropy measurements two polarizers were positioned in the sample compartment, and the amount of horizontally (h) and vertically (v) polarized emission (E_m) light due to vertically polarexcitation light was ized measured. The anisotropy A was calculated as

$$A = (E_{mv} - E_{mh})/(E_{mv} + 2E_{mh})$$

The measurements were corrected for the variation in response of the detector to horizontally and vertically polarized light.

CD spectroscopy was performed on a UV-CD/ LD apparatus (built in this laboratory by Dr. B. van Haeringen [19]) with a 0.01 cm pathlength quartz cuvet for the far-UV, and a 1 cm pathlength quartz cuvet (volume 0.8 ml) for the near-UV wavelength region. The bandwidth was 2 nm. The equipment was calibrated with 20 mM camphor sulfonic acid (CSA) ($\Delta \varepsilon$ is – 4.95 M⁻¹ cm⁻¹ at 192.5 nm and 2.37 M⁻¹ cm⁻¹ at 290.5 nm [20]). The experimentally determined ratio of the CD bands of CSA at 192.5 nm and 290.5 nm was 2.2. The protein concentration was 1 mg ml⁻¹. Prediction of secondary structure from the CD spectra was performed with a locally linearized model [21].

LD spectroscopy was performed on the above mentioned UV-CD/LD apparatus. The protein

was dissolved in a 15% acrylamide (29:1 acrylamide/bisacrylamide) solution when was then polymerized [22]. The protein had an absorbance of approximately 0.3 at 280 nm. The resulting gel was carefully inserted into a laboratory built LD cuvet (pathlength 0.5 cm), and squeezed in a direction perpendicular to the light path. The spectra were corrected for base-line contributions by measuring gels without protein [23,24].

3. Results

3.1. UV absorption

The UB absorption spectrum of recFSH dissolved in distilled water was measured both in the presence and absence of sucrose and citrate which were added prior to lyophilization. Fig. 1(a) shows the absorption spectrum from 190 to 340 nm (five spectra were averaged). The spectrum has a band around 230 nm, due to aromatic amino acids, histidine, methionine and cysteine. The peak around 195 nm could be due to an artefact caused by stray light, or could be the major $\pi - \pi^*$ transition of the peptide bond, which would suggest a major contribution of random structures [25]. Because this spectrum was measured in a cuvet with a pathlength of 0.1 mm, the absorption bands of the aromatic amino acids at 250-300 nm are barely visible. Fig. 1(b) shows the aromatic absorption spectrum of recFSH between 240 and 320 nm, measured in a 1 cm pathlength cuvet (14 spectra were averaged). The spectrum, which is in good agreement with that of Manjunath et al. [26], shows a maximum at 277 nm, a shoulder at 284 nm, and a red tail around 300 nm which extends to 320 nm. Assignment of these bands is discussed below. The ratio of the shoulders at 284 nm and 300 nm with the maximum at 277 nm is 0.879 ± 0.013 and 0.119 ± 0.012 (mean and SD, n = 21), respectively. Furthermore, the spectrum shows several small shoulders on the blue side of the 277 nm band due to phenylalanine. The position of these shoulders can be more readily seen in the second derivative of the spectrum, as shown in Fig. 1(a) (inset). The second derivative shows minima at 253, 258, 264 and 268 nm probably due to phenylalanine, and at 273, 283, 292 and 297 nm due to tyrosine or tryptophan. Neither removal of sucrose and citrate, nor subsequent addition of 5 mM sodium phosphate buffer in combination with 25–90 mM NaCl (pH 7) significantly altered the absorption spectrum of recFSH.

We have determined the extinction coefficient of recFSH by measuring the absorption spectrum



Fig. 1. The absorption spectrum of recFSH at room temperature ($T = 22^{\circ}$ C) from 190 to 340 nm with 0.1 mm pathlength (a), and fom 220-320 nm (b, ——). The second derivative of the latter is shown as an inset of (a). The spectrum calculated on the basis of tyrosine, phenylalanine and tryptophan in the ratio 11:7:1 is shown in (b, — — —).

of a sample in the presence of 0 and 6 M guanidine-HCl. At 277 nm the absorption of the native protein was $6.3 \pm 1.2\%$ less than the absorption of the denatured protein. The extinction coefficient at 277 nm of the denatured protein, calculated from the contributions of the aromatic amino acids, is 24 000 M⁻¹ cm⁻¹ [18]. Therefore, we conclude that the extinction coefficient of the native protein at 277 nm is 26 600 M⁻¹ cm⁻¹ (20 540 M⁻¹ cm⁻¹ at 280 nm).

We modeled the aromatic region of the absorption spectrum of FSH as a sum of the absorption spectra of phenylalanine, tyrosine and tryptophan (as free amino acids, at pH 7.0) in the ratio 7:11:1, being the numbers of these amino acids in FSH (Fig. 1(b), dashed line). Below 260 nm the absorption spectrum of the native protein contains a contribution of the peptide backbone. Because free amino acids were used, this contribution is absent in the calculated spectrum. Furthermore, in comparison with the absorption spectrum of recFSH the spectrum of the amino acids is 3 nm blue-shifted, and the shoulder at 300 nm is absent. The blue-shift is because the environment of the amino acids is less polar in the protein than in solution. The absence of the band around 300 nm in the simulated spectrum implies that this band, most likely due to tryptophan and/or tyrosinate (see below), is characteristic for the native form of FSH. Fig. 2(a) shows the absorption spectrum of the isolated α subunit of recFSH, which contains four phenylalanines, four tyrosines and no tryptophan. The spectrum shows a maximum at 275.5 nm and a shoulder around 282.5 nm. In comparison with the native protein, the maximum is 1-2nm blue shifted and the shoulder around 284 nm is less pronounced. Furthermore, the intensity of the absorption is diminished on the red flank of the spectrum, and slighly increased on the blue side. The same effects can be seen in the excitation spectrum (not shown). Fig. 2(b) shows the second derivative of the absorption spectrum. It contains bands around 252, 258, 264 and 268, 273 and 281 nm. The bands with maxima at 292 and 297 nm in the native protein are absent in the α subunit.

The absorption spectrum of the β subunit, which contains three phenylalanines, seven tyrosines and one tryptophan, shows a maximum



Fig. 2. (a) The absorption spectra of α recFSH (----) and β recFSH (-----). (b) The second derivatives of these spectra. The original spectra were normalized (maximum absorption in the near-UV range is 1.0).

around 276 nm and a shoulder around 283 nm (Fig. 2(a)). The second derivative of the absorption spectrum (Fig. 2(b)) shows minima at about 252, 264, 273, 281 and 292 nm. The absorption spectrum of the β subunit is 1–2 nm blue shifted with respect to the spectrum of the intact protein, as is the case with the α subunit. Furthermore, comparison between the (second derivative of the) absorption spectrum of the intact protein and the β subunit shows that the band around 297 nm is absent or only weakly present in the subunit (Fig.

2). This again demonstrates that the 300 nm band is specific for the intact $\alpha\beta$ structure of the protein.

3.2. IR absorption

The amide I and II bands, that provide most information on protein structure in solution, can be found between 1500 and 1800 cm⁻¹ (6.67–5.56 μ m). Because this wavelength region contains an intense OH absorption band, baseline subtraction in aqueous samples is sometimes awkward. Hence it is common practise in IR spectroscopy to compare spectra measured in H₂O with those measured in D_2O . Small band shifts may occur in D_2O due to H/D exchange. Fig. 3 shows the original and deconvoluted spectra of recFSH in H_2O and D_2O . Assignment of the bands in the deconvoluted spectra, based on Jackson et al. [27] and Arrondo et al. [28], is given in Table 1. From quantitative analysis of the amide I band in the H_2O spectra from 1600-1700 cm⁻¹, the percentage of each of the secondary structure elements in the protein has been estimated. The method of Pribić et al. [16] yielded $25 \pm 2\% \alpha$ helix, $45 \pm 3\%$ β sheet, $20 \pm 3\%$ β turn and $13 \pm 2\%$ undefined structures. The method of Lee et al. [17], which uses a different set of reference proteins, gives $23 \pm 6\% \alpha$ helix, $34 \pm 5\% \beta$ sheet, $16 \pm 4\% \beta$ turn and $27 \pm 5\%$ undefined structures.

3.3. Circular dichroism

In Fig. 4(a) we present the aromatic CD spectrum of recFSH (40 spectra were averaged). The CD signal is expressed as the difference in extinction coefficient (M^{-1} cm⁻¹) of left- and righthanded circularly polarized light ($\Delta \varepsilon$). The CD signal of the background (water) was subtracted. The aromatic CD spectrum shows several characteristic bands and shoulders between 250 and 320 nm, namely a positive maximum around 300 nm with a shoulder around 291 nm, two negative minima around 276 and 284 nm, and a negative inaximum around 260 nm with a slight shoulder around 268 nm. The almost conservative nature of the CD spectrum suggests that at least part of the spectrum is due to an interacting pair of aromatic residues. The relatively high intensity of the signal suggests that these residues must be in close contact in a relatively rigid environment [18].

Fig. 4(b) shows the far-UV CD spectrum of recFSH. Despite extensive averaging (n = 32), the spectrum contains considerable noise. This is partially due to background absorption, and partially due to the low CD intensity of proteins with little α helix. The spectrum does not extend below 190



Fig. 3. The infrared absorption spectrum of recFSH in H_2O (a) and D_2O (b). Shown are the original spectrum (——) and the deconvoluted spectrum (——–). The concentration of the sample was 61 mg ml⁻¹ in H_2O and 20 mg ml⁻¹ in D_2O .

Table 1

Assignment of the bands in the IR absorption spectrum of recFSH, measured in H_2O and D_2O (all band positions are given in cm⁻¹)

D ₂ O bands	Assignment	H_2O	Assignment
1516	Tyrosine	1517	Tyrosine
1529, 1543	Amide II	1536	Amide II
1565, 1580,	Side chains	1563	Side chains
1605			
1633	β Sheet	1634	β sheet
1642	Undefined structure	1647	α helix/undefined
1667	β turn	1657	α helix/undefined
1682	β turn/ β sheet	1688	β turn/ β sheet

nm: below this wavelength the carbohydrate sidechains of the protein and the remaining sucrose absorb too much light to allow measurement of the CD signal. The CD spectrum has a minimum around 200 nm and two shoulders, around 210– 220 nm and 225–235 nm. We have checked the effect of removal of sucrose and citrate on the secondary structure of the protein by comparison of the far-UV CD spectra between 205 and 250 nm. No difference could be detected between spectra recorded in the presence and absence of these compounds.

The far-UV CD spectrum contains information on the secondary structure of the protein, and secondary-structure calculations according to Van Stokkum et al. [21], yielded $14 \pm 3\% \alpha$ helix, $28 \pm 10\%$ antiparallel β -sheet, $1 \pm 3\%$ parallel β sheet, $24 \pm 4\% \beta$ turn and $31 \pm 8\%$ undefined structures.

CD spectra have been measured for isolated human pituitary FSH [9,29–32]. The positions of the bands in our near-UV spectrum correspond very well with those reported by Bhalla and Reichert [31], although the intensity of the bands is higher in our spectrum (Table 2). This could be due to differences in calibration of the instrument or to inaccuracies in the determination of the protein concentration. Bhalla and Reichert [31] have used the Lowry method to determine the protein concentration. This method is unreliable for small proteins with a low molecular weight and may underestimate protein concentration by 20% [33,34]. Our experimental setup was calibrated using camphor sulfonic acid and the calibration was confirmed by the good agreement of our CD spectrum of hemoglobin with that reported by Toumadje et al. [35]. The two spectra reported by Giudice and Pierce [9] and Giudice et al. [32] are not identical, and differ from the spectrum of Bhalla and Reichert [31]: the negative minimum is found around 283 nm (instead of 280 nm), the shoulder at 269 nm is missing, whereas in Giudice et al. [32] an additional shoulder is visible



Fig. 4. The UV CD spectrum (in units of $\Delta \epsilon$ /residue) of recFSH in water: (a) the near-UV CD spectrum; (b) the far-UV CD spectrum. The original and the smoothed spectra (-----) are shown.

Table 2

Comparison of the CD spectra from Fig. 4 with data from the literature^a

Min around 210 nm	Min around 280 nm	Max around 300 nm	Ref.
-1.2×10^{4} -0.7×10^{4} to 12×10^{4b}	- 70		[29] [30]
-0.45×10^4	-25	25	[9,32]
-0.50×10^4	-28	14	[31]
$-0.75 imes10^4$	- 74	26	This paper

^a All data have been converted to residual molar ellipticity, in deg M⁻¹ cm⁻¹. Min, minimum; Max, maximum. ^b Depending on protein concentration.

at 280 nm and in Giudice and Pierce [9] the spectrum shows an additional shoulder at 257 nm. The minimum in the far-UV CD spectra shown in Refs. [9,31,32] is located at about 210 nm. However, their spectra were measured down to approximately 205 nm. Our spectra, which could be measured accurately down to 190 nm, show that the minimum actually lies at about 200 nm.

3.4. Linear dichroism

The LD spectrum of FSH is shown in Fig. 5. The proteins were oriented by introducing them



Fig. 5. The linear dichroism spectrum of recFSH. The spectrum was measured with a squeezing factor of 1.8.



Fig. 6. The emission spectrum of recFSH: (a) the emission spectrum with excitation at 280 and 295 nm; (b) the contribution of tyrosine (tyr) and tryptophan/tyrosinate (trp) to the emission spectrum with excitation at 280 nm.

into a polyacrylamide gel and squeezing this gel in one direction (perpendicular to the light path). The spectrum was measured at two different degrees of squeezing (factor 2.25 and 1.8), in order to check for possible distorting effects of the squeezing. The degree of squeezing altered the absolute but not the relative intensity of the bands. The spectrum shows two maxima, around 280 nm and 286.5 nm, and a broad shoulder between 292 and 308 nm. These bands are due to tyrosine and tryptophan and will be discussed below.

3.5. Fluorescence

Fig. 6(a) shows the emission spectrum of recFSH, recorded with excitation wavelengths of 280 and 295 nm. On several occasions the 305/345 emission ratio increased during a series of measurements on the same sample. Therefore, not more than two measurements were performed on the same sample. At 280 nm tyrosine and tryptophan are excited. At 295 nm tryptophan and (if present) tyrosinate are excited. The emission spectra of tryptophan and tyrosinate cannot be discriminated [36]. Generally, tyrosinate emission is weak due to its low quantum yield ($\phi_F = 0.015$). However, as noted by Lakowicz [36], the possibility of tyrosinate emission should be considered.

When recFSH was excited at 280 nm, the spectrum showed emission from tyrosine (maximum around 307 nm) and tryptophan (slight shoulder around 345 nm). When recFSH was excited at 295 nm, the emission spectrum had a single maximum around 345 nm, due to tryptophan/tyrosinate fluorescence (Fig. 6(a)).

The emission maxima of tyrosine and tryptophan/tyrosinate in the protein are determined by their environment (e.g. its polarity). The emission maximum of tryptophan/tyrosinate within the protein lies at 345 nm, whereas the free amino acid emits at 355 nm. This indicates that the environment of the tryptophan residue and/or tyrosinate, if present, is rather non-polar, i.e. that the residues lie buried within the protein.

At wavelengths above 350 nm tyrosine does not fluoresce. To separate the contributions of tyrosine and tryptophan/tyrosinate to the emission spectrum (excitation at 280 nm), we fitted a region of the emission spectrum with excitation at 295 nm, namely from 350 to 400 nm, to the same region of the emission spectrum with excitation at 280 nm. The 295 nm excited emission spectrum was then multiplied by the factor, so obtained, and subsequently subtracted from the 280 nm excited emission spectrum to determine the tyrosine contribution (Fig. 6(b)). We have extrapolated the spectrum to 280 nm and integrated the area of the tryptophan/tyrosinate and tyrosine contribution to the fluorescence between 280 and 400 nm. The ratio of the areas due to emission of



Fig. 7. The excitation spectra of recFSH. The emission wavelengths were 305 nm (a) and 345 nm (b). In (b) the excitation spectrum (emission at 345 nm) is compared with the absorption spectrum of recFSH (---).

tryptophan/tyrosinate and tyrosine was 0.89 ± 0.14 (n = 8).

The fluorescence quantum yield (ϕ_F) of FSH was 0.015 ± 0.004 (n = 10), irrespective of the excitation wavelength (280 and 295 nm). This value is surprisingly low compared to that of free tryptophan (0.2), free tyrosine (0.14), and other proteins with only one tryptophan (about 0.25) [18,37]. However, it is in the same order of magnitude as that of free tyrosinate (0.015). The anisotropy A of recFSH is 0.05 + 0.03 (between 310 and 340 nm) when excited at 280 nm, and 0.08 +0.03 when excited at 295 nm. These values are low but can be explained by excitation transfer. Moreover, the lowest excited state of tryptophan is close to degenerate with two perpendicular transition dipoles, resulting in a lowered anisotropy. Overall motion of the protein with respect to the fluorescence lifetime is too slow to cause depolarization. The value for the rotation correlation time, which is a measure of the overall motion of the protein, estimated from the molecular weight, is about 10 ns for recFSH (1 ns per 2.4 kDa). The estimated value for the observed fluorescence lifetime τ is 7.5 ps (calculated from $\phi_{\rm F} = \tau/\tau_{\rm f}$, with $\tau_{\rm f} \approx 10^{-4} / \varepsilon_{\rm max}$) [18].

The excitation spectrum of recFSH was determined for two emission wavelengths (305 nm and 345 nm) which are the emission maxima of tyrosine and tryptophan/tyrosinate, respectively (three spectra were averaged). Fig. 7(a) shows the excitation spectrum of the protein from 220 nm to 290 nm, with 305 nm as detection wavelength. At this wavelength there is little emission due to tryptophan/tyrosinate (Fig. 6). The spectrum shows a maximum around 275 nm and a shoulder around 281 nm, apparently due to tyrosine. Fig. 7(b) shows the excitation spectrum from 220 nm to 320 nm with 345 nm as detection wavelength (no tyrosine emission, see Fig. 6). Again the spectrum shows a maximum around 275 nm, but compared to Fig. 7(a) the shoulder around 281 nm is more pronounced. Furthermore, a band around 300 nm is clearly visible.

A comparison between the excitation spectrum with emission at 345 nm and the absorption spectrum of recFSH (Fig. 7(b)) shows that the spectra have a similar shape. Differences between the excitation and absorption spectra on the blue side of the band around 275 nm are mainly because the contribution of the peptide bonds is not present in the excitation spectrum. The difference between the red side of the excitation spectrum (emission at 345 nm) and the absorption spectrum is probably caused by a different contribution of tyrosine to the excitation spectrum as compared to the absorption spectrum. Because there is no tyrosine emission at 345 nm, the similarity between the excitation and absorption spectra seems to suggest a high degree of excitation transfer, which can only take place when donor and acceptor are in close contact. However, it is highly



Fig. 8. (a) The fluorescence emission spectra of isolated α subunits of recFSH, with excitation at 280 nm (-----) and 295 nm (· · · ·). (b) The contribution of tyrosine (tyr) and tryptophan (trp) to the emission spectrum with excitation at 280 nm.

unlikely that one tryptophan residue is in close contact with the majority of the 11 tyrosines. Therefore, we attribute the similarity between the absorption and excitation spectra to involvement of tyrosinate in the fluorescence of recFSH.

According to Manjunath et al. [26] excitation of human FSH (hFSH) at 280 nm yields an emission spectrum with a single maximum at 329 nm. This wavelength is intermediate between the emission wavelengths of tyrosine and tryptophan/tyrosinate. In contrast, Sanyal et al. [38] report an emission spectrum with a maximum around 335 nm due to tryptophan and a shoulder around 305 nm due to tryptophan and a shoulder around 305 nm due to tyrosine. Both spectra are very different from ours. However, the absence of the 300 nm band in the corresponding absorption spectrum suggests that the material used by Manjuth et al. [26] is not fully intact. The high emission at 335 nm reported by Sanyal et al. [38] could be due to extensive tyrosinate formation (vide infra).

Figs. 8 and 9 show the fluorescence emission spectra of the subunits of recFSH. Excitation of the α subunit at 280 nm yielded an emission spectrum with a maximum around 305 nm and a shoulder around 345 nm. When the subunit was excited at 295 nm, the spectrum contained a single band with a maximum around 345 nm. We deconvoluted the spectrum with excitation at 280 nm into contributions of tryptophan/tyrosinate and tyrosine. The spectrum was extrapolated to

280 nm. Integration of both the tryptophan/tyrosinate and the tyrosine contributions (between 280 and 400 nm) yielded an area ratio of 0.92.

Excitation of the sample of β -recFSH at 280 nm yielded an emission spectrum with a maximum around 345 nm and a shoulder around 305 nm (Fig. 9). Excitation at 295 nm yielded an emission spectrum with two maxima, around 350 nm and around 430 nm. This spectrum is obviously different from that of the intact protein, and shows emission by a component different to tyrosine, tyrosinate and tryptophan. Hence deconvolution of the tyrosine and tryptophan contributions to the emission spectrum excited at 280 nm, as performed for native and α -recFSH, is not possible in this case. However, the intensity ratio of the emission at 350 and 305 nm (1.3:1) compared to that for the native and α -recFSH (0.43:1) indicates significantly stronger tryptophan fluorescence in β -recFSH. The inset of Fig. 9 shows the excitation spectrum of the additional component (detection wavelength 420 nm). A comparison with the excitation spectrum of intact recFSH (Fig. 7) shows that it has a protein-like excitation spectrum with an additional band around 346 nm. Possibly this arises from an altered amino acid.

We also determined the excitation spectra of the β subunit with detection at 305 nm and 345 nm (not shown). Emission at 345 nm yielded an excitation spectrum with a higher intensity than



Fig. 9. The fluorescence emission spectra of isolated β subunits of recFSH with excitation at 280 nm (---) and 295 nm (· · ·). The inset shows the excitation spectrum of β -recFSH with detection at 420 nm.

emission at 305 nm, whereas in the intact protein the reverse is true. This is due to a higher tryptophan/tyrosine ratio in the β subunit than in the intact protein. Furthermore, the excitation spectrum with $\lambda_{em} = 345$ nm did not have the shoulder around 300 nm which is always present in the absorption spectrum of the native protein.

4. Discussion

4.1. Band assignments

By combination of the results of the various techniques for the intact protein and for the subunits, we are now able to identify and assign the different bands and shoulders. Table 3 summarizes the results from absorption, fluorescence excitation, CD and LD, in and slightly below the near-UV wavelength region. The table does not contain the shoulders at 248, 253, 258 and 264 nm, which are most probably due to phenylalanine. Band I can be due to any of the aromatic amino acids. Band II, present in the absorption and CD spectra, is probably due to phenylalanine. Bands III and IV can be due to either tyrosine or tryptophan, which both have two maxima in this wavelength region [18]. The strong overlap between the bands may cause the discrepancies in the exact band positions between the various techniques. Band V can only be due to tryptophan. This band is present in all spectra, except in the absorption spectrum of the α subunit that does not contain any tryptophan residues.

Band VI is present in spectra of the native protein, but is absent in the absorption spectrum of the subunits (Fig. 2(a)), in denatured protein samples (to be published), and in a calculated absorption spectrum (Fig. 1(b)). Therefore, band VI could be due to tryptophan because it is situated in the native protein. The near-UV CD spectrum indicates that the tryptophan residue is

Table 3

Some of the bands (in nm) present in the near-UV wavelength region of the absorption, CD, LD and fluorescence excitation spectra of recFSH

	Absorption	CD	Fluorescence excitation	LD	Global analysis
I	230	sh	d	d	
Н	268 ^b	268 sh	с	c	266
Ш	273 ^b	276 min	275 max	с	275
IV	283 ^b	284 min	281 sh	284 min	284
v	292 ^ь	291 sh	c	292 ^ь	291
VI	297 ^b	300 max	300 sh	300 sh	300

^a Max, maximum; Min, minimum; sh, shoulder.

^b From 2nd derivative.

^c Below detection limit.

^d Outside measurable range.

in close contact with a second aromatic amino acid, most probably a tyrosine. The absence of band VI in the absorption spectra of the β subunit suggests that this tyrosine is located in the α subunit. This is supported by the location of the tryptophan at the interface of the subunits [32].

4.2. Tyrosinate

Alternatively, band VI may at least partly be due to tyrosinate. One tyrosine residue in the α subunit is in close contact with the tryptophan residue in the β subunit. The electronegativity of the tryptophan residue may induce ionization of this particular tyrosine to tyrosinate, resulting in an (increased) absorption around 300 nm and emission around 345 nm. Consequently, excitation at 295 nm yields emission from tryptophan and tyrosinate. Tyrosinate has a much lower quantum yield than tryptophan. Furthermore, protonation of tryptophan reduces its quantum vield [39]. These two factors may explain the low intensity of the emission band at 345 nm. The relatively low value for the anisotropy would be caused by excitation transfer from tyrosinate to tryptophan. In isolated subunits the interaction between tryptophan and tyrosinate is lost and the tyrosinate becomes tyrosine. The emission band in the β subunit around 345 nm is now due to an isolated tryptophan and perhaps excitation induced tyrosinate. This results in an increase of the intensity of this emission band. The induction of tyrosinate in the excited state could be facilitated by the low isoelectric point of the protein, which is between pH 3 and 5.5 [14].

Tyrosinate formation may also explain the similarity between the excitation spectrum and the absorption spectrum of the intact protein (see Section 3). Moreover, the observed increase in the 305/345 emission ratio during a series of measurements on the same sample could be caused by an increased tyrosinate formation as a result of the excitation light. Perhaps the presence of high amounts of tyrosinate due to the intensity of the excitation light may also explain why the emission spectrum of FSH (excitation at 280 nm) reported by Manjunath et al. [26] and Sanyal et al. [38] is located at higher wavelength, namely 329 nm and 335 nm.

The second derivative of the UV-adsorption spectrum of the α subunit (Fig. 2(b)) shows that bands V and VI (maxima at 292 and 297 nm) of the native protein are absent. This can be explained by the absence of tryptophan in the α subunit. However, the emission spectra of the α subunit can hardly be distinguished from that of the native protein (Figs. 6 and 8). Noticeably, an emission spectrum was obtained by excitation at 295 nm. The ratio between the contribution of tryptophan/tyrosinate and tyrosine (0.915), is indistinguishable from that of the native protein (0.89 ± 0.14) . Because the single tryptophan residue of the protein is located in the β subunit, the emission spectrum of the α subunit cannot contain any contribution from tryptophan. Two explanations are possible. Firstly, a small amount of β subunit may be present in the sample. The amplitude of the fluorescence emission of a tryptophan residue strongly depends on the environment of the fluorophores [39]. Hence, exposure of tryptophan to the solvent may increase the amplitude of its fluorescence emission drastically. Therefore, a small amount of highly fluorescent β subunit which is not detectable in the absorption spectrum could be present in the sample. An alternative expla-



Fig. 10. The results of the global analysis of the absorption, CD, LD and fluorescence excitation spectra of recFSH. For each spectrum the Gaussian bands in the calculated ratio and the residuals (inset) are shown.

nation would again be the presence of tyrosinate, possibly induced by the excitation light [36].

4.3. Global analysis

In order to check the consistency of our spectral results, we have simultaneously fitted the absorption, CD, LD fluorescence excitation spectra in the wavelength region 265–330 nm by global analysis with a single set of bands [40]. Below 265 nm there was too much contribution from the absorption bands of the peptide bonds and the absorption of the residual acrylamide (present in the gels used for LD measurements). To allow reliable fitting, the fit was performed with the minimal number of Gaussian bands, to obey the following criteria: absorption or fluorescence bands do not have a negative amplitude, no bands show 100% overlap, and the residuals are as random as possible.

An optimal fit was achieved with five Gaussian bands, with maxima at 265.5, 275.0, 283.5, 291.0 and 300.0 nm (Fig. 10). The positions of the bands are in good agreement with the posi-

tions of the minimum/maxima in the original spectra (see Table 3). Note that one of the bands has a maximum around 300.0 nm. In the CD spectrum several bands have a negative amplitude, whereas these bands have a positive amplitude in the absorption, LD and fluorescence spectrum. The root mean square for all fits was 3.5% of the maximum signal. The residuals (shown in the insets in Fig. 10) still show some structure. This may be due to vibrational fine structure.

4.4. Secondary structure

The amount of secondary structure in a protein can be determined from its far-UV CD and from its IR spectrum. FTIR and CD measure different aspects of secondary structure, namely hydrogen bonding and dihedral angles, respectively [41]. RecFSH is a glycoprotein, and this causes two problems for CD determination of secondary structure. Firstly, the carbohydrate side chains most probably contribute to the CD spectrum below 215 nm. Bewley et al. [42] have reported that this contribution is approximately

α Helix %	β sheet %	β turn %	Undefined structures %	
25 ± 2	45 ± 3	20 ± 3	13 ± 2	
23 ± 6	34 ± 5	16 ± 4	27 ± 5	
14 ± 3	29 ± 13	24 <u>+</u> 4	31 ± 8	
19	28	n.d. ^g	57	
5-8	25-40	n.d. ^g	52-70	
0-5	~ 35	~25	~ 50	
	α Helix % 25 ± 2 23 ± 6 14 ± 3 19 5-8 0-5	$\begin{array}{c cccc} \alpha \ \text{Helix} \ \% & \beta \ \text{sheet} \ \% \\ \hline 25 \pm 2 & 45 \pm 3 \\ 23 \pm 6 & 34 \pm 5 \\ 14 \pm 3 & 29 \pm 13 \\ 19 & 28 \\ 5-8 & 25-40 \\ 0-5 & \sim 35 \\ \hline \end{array}$	α Helix % β sheet % β turn % 25 ± 2 45 ± 3 20 ± 3 23 ± 6 34 ± 5 16 ± 4 14 ± 3 29 ± 13 24 ± 4 19 28 n.d. ^g 5-8 25-40 n.d. ^g 0-5 ~35 ~25	

Table 4Secondary structure of recFSH.

^a Predicted according to Lee et al. [17] from FTIR measurements in H₂O.

^b Predicted according to Ref. [16] from FTIR measurements in H₂O.

^c Predicted according to Ref. [21] from far-UV CD measurements.

^d From Ref. [15].

^e From Ref. [44].

^f From Ref. [49].

^g n.d., Not determined.

5% betweeen 205 and 215 nm. Secondly, predictions of secondary structure are based on a set of reference proteins. The most reliable results are obtained for proteins that have conformation that is close to one or more of the proteins in the reference set. However, the set of reference proteins contains few glycoproteins. FTIR, however, requires a high protein concentration, which may induce aggregation. As shown by Pribić et al. [16] most reliable results are obtained by a comparison of secondary structures deduced from infrared and CD spectra. Table 4 compares the secondary-structure calculations of recFSH based on UV-CD and FTIR with literature data for FSH and also for LH and hCG. Our CD results are in good agreement with the data from Ryan et al. [43] for hFSH. However, the latter do not discriminate between β turn and random structures. The IR spectra indicate a higher content of α helix. This may indicate short and/or distorted structural elements [41]. In view of the noise of the spectra and the uncertainity of the calculations we propose the following estimation of the secondary structure of recFSH: $15-25\% \alpha$ helix, $15-25\% \beta$ turn and $30-40\% \beta$ sheet. Our results confirm that FSH contains significantly more α helix than hCG and LH [44–48]. The amount of β turn has not been determined before. The CD results indicate that the β sheet in recFSH is almost exclusively antiparallel.

It has been postulated, that because of the common α subunit and the degree of similarity in amino acid sequence in the β subunit, the conformation of the three gonadotropins (FSH, CG and LH) should be rather alike [49]. Because the prediction of the secondary structure of hCG based on CD analysis and on X-ray results are comparable [49], we would expect a low amount of α helix in recFSH. Table 4 shows that recFSH contains a much higher amount of α helix, namely 15-25%. than hCG. However. the three-dimensional structure of the α subunit of FSH, LH and hCG may not be identical, e.g. recombination of β hCG with three α subunits of different origin (from human CG, and from ovine and porcine LH) yields three different far-UV CD spectra [50], which suggests that the conformation of the α subunit influences the conformation of the β subunit and vice versa. Furthermore, the hCG used in the crystallographic study was deglycosylated The deglycosylation [49]. procedure may have altered its conformation. According to Ryan et al. [15], CD spectra indicate that deglycosylated FSH (deglyFSH) has more random structure and less α helix than the native hormone. Bewley et al. [42] and Calvo et al. [51] based on CD spectra report differences in secondary structure between FSH and deglyFSH. Also the thermostability of FSH is affected by deglycosylation [52].

5. Conclusion

In this paper we have characterized the absorption, fluorescence, LD and CD spectral properties of native recFSH. Some conclusions about the structure of recFSH are drawn. In a forthcoming article, we shall use the spectroscopic data to evaluate the effects of thermal and chemical denaturation.

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