Vol. 81, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1978 Pages 725-733

CIRCULAR DICHROISM OF MAMMALIAN FOLLITROPINS AND THE EFFECTS OF TREATMENT WITH N-BROMOSUCCINIMIDE

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Received February 16,1978

SUMMARY: The circular dichroism of the tryptophan containing glycoprotein hormone, follitropin, displays bands in the near ultraviolet which are absent in homologous, tryptophan-free hormones. In the far ultraviolet, the dichroism is very similar to the other glycoprotein hormones with little or no indication of α-helix. The single tryptophan of follitropin is in a domain of the β subunit sequences of these hormones which is highly conserved from hormone to hormone. Without prior dissociation of the follitropin into subunits, no change is seen in circular dichroism, absorption at 280 nm, fluorescence emission or hormonal activity after treatment with N-bromosuccinimide. In contrast, these properties change when intact human lutropin is studied; its tryptophan residue is a position different than in follitropin. These results support the proposal that the domain containing the tryptophan in follitropin is in or near a region of subunit-subunit contact in the glycoprotein hormones.

Of the mammalian glycoprotein hormones (thyrotropin, lutropin (LH)[1], follitropin (FSH) and human chorionic gonadotropin) (see ref. 1 for a review of sequences), only the follitropins and human lutropin contain tryptophan. A single residue is in their β subunits, in human LH at position 8 (e.g. 2,3) and in human FSH at position 27 (4)[2]. The latter is in a region of eight highly conserved residues which has been proposed to be in contact with the common, α, subunit (5,6). Thus it is interesting to compare the reactivities of FSH and human LH towards N-bromosuccinimide, an oxidizing agent with selectivity towards tryptophan residues (7). In the present study the reaction was monitored by absorption spectra, effects on hormone

1. Abbreviations: LH, lutropin; FSH, follitropin; CD, circular dichroism; NBS, n-bromosuccinimide.

2. A tryptophan is found at the analogous position in porcine FSH (Closset, J., Maghuin-Rogister, G., Hennen, G., and Strosburg, A.D., personal communication).

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activity, measurement of CD spectra and tryptophan fluorescence. With regard to circular dichroism, spectra for bovine and porcine FSH have not been reported. Extensive studies on LH from several species have been made (ref. 8 for complete references). Some data for human FSH and human LH are in conflict (9,10).

MATERIALS AND METHODS: Human FSH, LH, bovine FSH and subunits were prepared as previously described (11-15). Porcine FSH was obtained from acetone-dried pituitary powder by ethanol-salt extraction, ethanol-ammonium sulfate fractionation, DEAE-cellulose chromatography and gel filtration[3]. It is homogeneous by disc gel electrophoresis, gel filtration, analytical ultracentrifugation and immunoprecipitation.

CD spectra were also determined as previously described (16). Protein concentrations were based on dry weight assuming 15% moisture, and molar ellipticities were calculated using molecular weights of 27,000 for human LH, 34,000 for human and porcine FSH, 30,000 for bovine FSH, 14,000 for human LH-β and 16,400 for bovine FSH-β. Ultraviolet difference spectra were determined at room temperature with a Cary Model 14 recording spectrophotometer. NBS, recrystallized from glacial acetic acid and dissolved in a buffer at pH 4.5 which contained 0.05 M sodium phosphate and 0.15 M NaCl, was added in 5 or 10 µl aliquots to 1.2 ml of protein solution so that the final molar ratio of NBS to tryptophan was either 2:1 or 4:1.

Tryptophan fluorescence of dissociated and undissociated human FSH and of bovine FSH-β in the pH 4.5 buffer was monitored by an Aminco Bowman fluorimeter before and after treatment of the proteins (0.09 mg/ml) with a two or five fold molar excess of NBS in the same buffer. The wavelength of the exciting light was 260 nm, and the relative intensity of the emitted light was determined at 340 nm.

To study the effects of reaction with NBS on hormonal activity, FSH and LH were treated at room temperature for 30 min in the phosphate-NaCl buffer at pH 4.5. Molar ratios of NBS to protein tryptophan were 4:1. Unreacted NBS was destroyed by the addition of a 50 molar excess of tryptophan. For controls, NBS and tryptophan were added in reverse order to the hormone preparations. Biological activities of human and porcine FSH preparations were measured with the Steelman-Pohley assay (17). Each assay was a 3 x 3 design with 4 animals at each dose level and thrice daily injections. Human LH activity was tested in the *in vitro* radioreceptor assay described by Lee and Ryan (18). Data were analyzed by parallel line bioassay statistics making a direct comparison between the control and NBS treated samples. Data were also analyzed in terms of NIH LHS18 and NIH FSHS12 which were assayed concurrently.

RESULTS AND DISCUSSION: The CD spectra of intact human and porcine FSH are shown in Fig. 1A. The negative extrema at 282-285 nm and 290-292 nm have been assigned to oriented tyrosines (10), and the positive bands at 287-289 and 295-298 nm to the tryptophan residue (8,10); the latter are not seen in the spectra of tryptophan-free glycoprotein hormones (e.g. 16,19). The

3. Whitley, R. and Ryan, R.J., unpublished work.

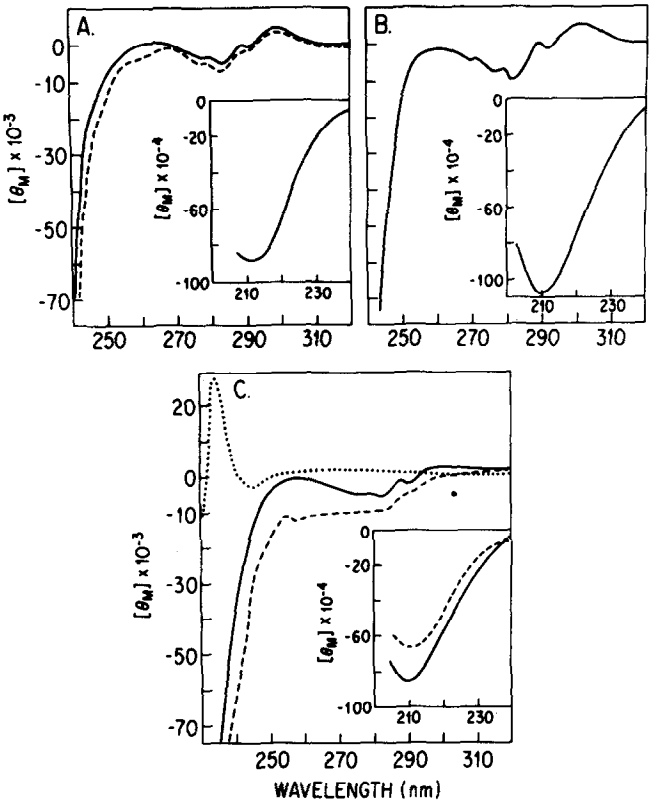

Fig. 1. CD spectra of FSH and subunits before and after treatment with NBS at pH 4.5. A; the solid line (——) shows the near ultraviolet spectra of human FSH at pH 7.5, pH 4.5 and at pH 4.5 after addition of a two-fold molar excess of NBS. No change was seen in the spectra 1 and 33 min after addition of the reagent. Protein concentration was 3.6 mg/ml and measurements above 240 nm were in cells of 0.5 cm pathlength; those below 240 in cells of 0.1 cm with a protein concentration of 0.22 mg/ml. The inset shows the far ultraviolet spectra of human FSH as above. The dashed line (----) shows the analogous near ultraviolet spectra of porcine FSH (1.8 mg/ml). B; Near ultraviolet spectra of bovine FSH (0.9 mg/ml) at pH 7.0, pH 4.5 and at pH 4.5 five min after addition of a two-fold excess of NBS. The inset shows the far ultraviolet spectra under the same conditions. C; Near ultraviolet spectra of FSH-β at pH 7.0 and pH 4.5 (——) and at pH 4.5 after addition of a two-fold excess of NBS (----). The dotted line (....) is the spectrum of bovine FSH-α at pH 7.0. A sample of bovine LH-α (not shown) gave the expected similar spectrum. The inset shows the far ultraviolet spectra.

spectra in the far ultraviolet region are very similar to those of LH and TSH with little or no indication of α helix (20). The spectrum of bovine FSH is very similar (Fig. 1B) and no change was observed in the FSH spectra after addition of a two or four fold excess of NBS. The near ultraviolet

spectra of the isolated β subunit of bovine FSH (Fig. 1C, solid line) is very similar to that of intact hormone but, after addition of NBS, a rapid alteration occurred with disappearance of the inflections above 287 nm (Fig. 1C). The spectrum of the α subunit (Fig. 1C) is essentially identical to those of bovine LH and TSH (16) and no change in spectra was found when bovine LH-α was treated with NBS[4]. The fact that no changes were seen after treatment of bovine α subunits with NBS is good evidence that the exposed tyrosines present in α subunits do not react under the conditions used. Substitution or perturbation of these tyrosines causes changes in the CD of the α subunit (8,16).

The lack of reactivity of the tryptophan in intact FSH was further demonstrated by determination of difference absorption spectra. The addition of NBS to intact porcine FSH did not result in a difference spectrum at pH 4.5 as would have been expected if reaction had occurred (7). If, however, the FSH was allowed to dissociate by standing at pH 2.0 for 1 hour (21) before being brought to pH 4.5, a change in absorption occurs because of dissociation, and when NBS is added to the dissociated preparation, a further change occurs, with a loss of absorbance centering at 280 nm. Calculation of the tryptophan oxidized (7) showed 0.7 residues reacting in dissociated human or porcine FSH.

In contrast to FSH, intact human LH reacts with NBS. CD spectra are given in Fig. 2A; that at pH 7.5 agrees with those previously reported (8,10) with positive bands at 285 and 292 nm attributed to tryptophan and the negative bands centering at 268 and 282 nm assigned to tyrosine and cystine chromophores. In contrast to FSH, the near ultraviolet spectrum of human LH at pH 4.5 (Fig. 2A, solid line) is different than at pH 7.5 (dashed line) in that a small positive extremum centered at 233 nm is increased at pH 4.5. The change cannot be attributed to dissociation, because its rate

4. Bovine LH-α was used because of limited amounts of bovine FSH-α available. There are no data to indicate any differences in amino acid sequences between α subunits from the same species.

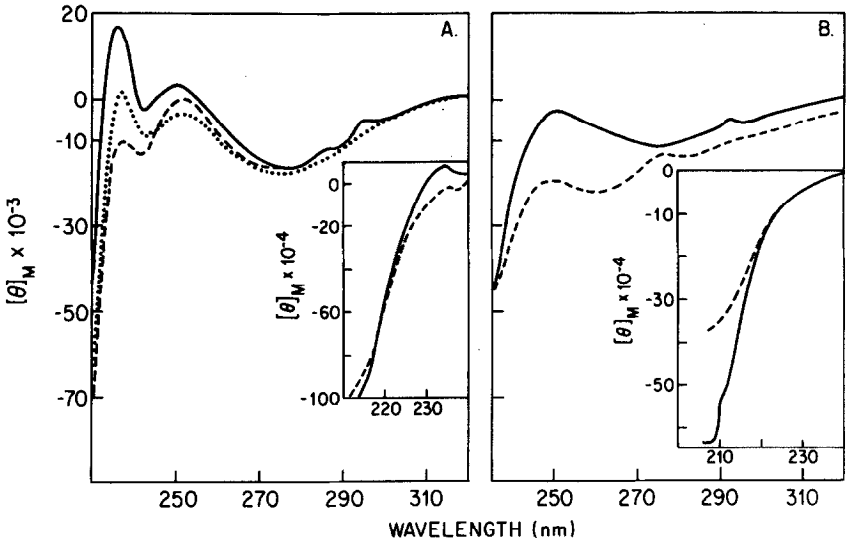

Fig. 2. CD spectra of human LH and its β subunit. A; The near ultraviolet spectra which show the change between pH 7.5 (----) and pH 4.5 (——) and the change 1-5 min after the LH was treated with a four-fold excess of NBS (....) (the maximum at 295 began to disappear after addition of a two-fold excess of reagent). Further diminution of the maxima at 248 and 234 nm occurred during the next 15 min. Protein concentration 1.8 mg/ml; other conditions as in Fig. 1. The inset shows the far ultraviolet spectra at pH 4.5 before and after addition of the NBS. B; The near ultraviolet CD spectrum of human LH-β (1 mg/ml) at pH 7.4 and 4.5 (——) and at pH 4.5 after addition of a two-fold excess of NBS (----). The inset shows the far ultraviolet spectra.

under the conditions used (pH 4.5, 25°) is slow (20-22). Additionally the spectra between 270-310 nm at pH 4.5 and pH 7.5 are identical, whereas, upon dissociation of LH, a major decrease of dichroism in this region is found (8). When NBS is added to human LH at pH 4.5, an immediate change in the near ultraviolet dichroism occurs with the earliest effect being a diminution of the band at 293 nm (attributed to tryptophan) when a two-fold excess of reagent is added. More pronounced effects are seen 15 min after addition of a four-fold excess (Fig. 2A, dotted line). The CD spectrum of isolated LH-β was not affected by the change in pH from 7.5 to 4.5 but a change in both the near and far spectra was seen directly after addition of NBS as shown in Fig. 2B. Upon treatment of intact human LH with NBS, the ultraviolet absorption spectra showed a loss in absorbance at 280 nm analogous to

TABLE I. The effect of NBS treatment upon the biologic activity of human and porcine FSH and human LH. Untreated preparations were regarded as the standard.

		Relative Potency
hFSH*	control	1.00
hFSH	treated	0.99
pFSH**	control	1.00
pFSH	treated	0.88
hLH***	control	1.00
hLH	treated	0.67

* hFSH in the Steelman-Pohley assay was 170 times the potency of NIH FSH S12

** pFSH in the Steelman-Pohley assay was 20 times the potency of NIH FSH S12

*** hLH in a radioreceptor assay was 2.6 times the potency of NIH LH S18 standard.

that found with dissociated FSH with 0.4 residues apparently reacting after addition of a two-fold excess of NBS[5].

The differences in the reactivity towards NBS of intact FSH vis-a-vis its β subunit were confirmed by observation of the disappearance of tryptophan fluorescence. Approximately 80% of the emission at 340 nm remained after treatment of human FSH with a five-fold excess of NBS; the dissociated (or partly dissociated) FSH preparations retained 46% and isolated bovine FSH-β only 16%.

The biological activities of human and porcine FSH and human LH after NBS treatment are shown in Table I. No significant change is seen with FSH; some loss may have occurred with human LH in agreement with the partial reactivity of its tryptophan residue as detected by spectroscopic measurements, though more detailed studies are needed to determine if the tryptophan at position 8 plays a direct role in expression of hormonal activity.

5. The reason that all molecules did not react is not clear; the preparation may contain more than one conformer. Such differences in ovine LH preparations have been reported (20).

The above results show that the CD spectra of human, bovine and porcine FSH are very similar and also closely resemble that of human LH despite the fact that the latter has its tryptophan in a different position. The spectrum of human LH agrees with a recent study (8); it is somewhat different than given in an earlier report (9) which also described an inflection at 220 nm in human FSH, indicative of some α helix. The spectra of FSH given herein agree with that previously noted in a second report (10). Some helix has also been reported to be present in a preparation of ovine FSH (23); whether FSH can assume conformations which contain a detectable percentage of α helix should be further investigated.

Most important is that, in contrast to human LH, the tryptophan in intact FSH is inaccessible to reaction with NBS. These results are consistent with several studies on the reactivity of a tyrosine residue (24) in the same region of β subunit sequences and further support the proposal that the region is one of subunit-subunit contact in intact hormones. Similarity of sequence has been noted (25) between this region and a region near the amino terminus of the B protein of cholera toxin (26,27), and it was proposed that, in the glycoprotein hormones as well as in cholera toxin, it may play a role in binding to surface receptors (25). If this be correct, the data concerning the inaccessibility to chemical modification of both tryptophan and tyrosine residues in the region must be taken into account. Thus for this region of the glycoprotein hormones to bind to receptor, dissociation of intact hormone or a major change in conformation at the cell surface after initial contact elsewhere on the hormone would seem to be necessary. With human LH the data show its tryptophan to be exposed, although perhaps only partially, to solvent (28) and to modification. In bovine-ovine LH-β the residue corresponding to human LH-β's tryptophan is leucine, and in bovine and human TSH-β it is phenylalanine; however, in hCG-β and human FSH-β these residues are arginine and serine, respectively (1). Since both hydrophobic and hydrophilic residues are at the same posi-

tion, it is less likely that this position is in a contact region between subunits.

ACKNOWLEDGMENTS: Appreciation is expressed to Ms. Tiiu Reimo for preparation of human LH, to Dr. Norman Simmons for use of the CD apparatus and to Dr. D.S. Sigman and Mr. Vito D'Aurora for assistance with measurement of fluorescence. This work was supported in part by USPHS grants 9R01 AM 18005, HD 8322, HD 9140, the Mayo Foundation and the Medical Research Council of Canada, MA-5110.

REFERENCES

1. Pierce, J.G. (1977) in Endocrinology, Proceedings of the V International Congress (James, V.H.T., ed.) Vol. 2, pp 99-103, Excerpta Medica, Amsterdam-Oxford.
2. Closset, J., Hennen, G., and Lequin, R.M. (1973) Fed. Eur. Biochem. Soc. Lett. 44, 224-228.
3. Shome, B. and Parlow, A.F. (1973) J. Clin. Endocrinol. Metab. 36, 618-621.
4. Saxena, B.B. and Rathnam, P. (1976) J. Biol. Chem. 251, 993-1002.
5. Pierce, J.G., Liao, T.-H., Howard, S.M., Shome, B., and Cornell, J.S. (1971) in Recent Progress in Hormone Research (Astwood, E.B., ed.) Vol. 27, pp 165-212.
6. Liu, W.-K., Nahm, H.S., Sweeney, C.M., Holcomb, G.N., and Ward, D.N. (1972) J. Biol. Chem. 247, 4365-4381.
7. Spande, T.F. and Witkop, B. (1967) Methods Enzymol. XI, pp 498-532.
8. Puett, D., Nureddin, A., and Holladay, L.A. (1976) Int. J. Peptide Protein Res. 8, 183-191.
9. Rathnam, P. and Saxena, B.B. (1973) in Structure-Activity Relationships of Protein and Polypeptide Hormones (Margoulies, M. and Greenwood, F.C., eds.) Excerpta Medica, Amsterdam, pp 320-324.
10. Bhalla, V.K. and Reichert, L.E., Jr. (1974) J. Biol. Chem. 249, 7996-8004.
11. Ryan, R.J. (1968) J. Clin. Endocrinol. Metab. 28, 886-896.
12. Cornell, J.S. and Pierce, J.G. (1973) J. Biol. Chem. 248, 4327-4333.
13. Cheng, K.W. (1976) Biochem. J. 159, 651-659.
14. Bishop, W.H. and Ryan, R.J. (1973) Biochemistry 12, 3076-3084.
15. Cheng, K.W. (1978) Biochem. J. (in press).
16. Cheng, K.W., Glazer, A.N., and Pierce, J.G. (1973) J. Biol. Chem. 248, 7930-7937.
17. Steelman, S.L. and Pohley, F.M. (1953) Endocrinology 53, 604-616.
18. Lee, C.Y. and Ryan, R.J. (1973) Biochemistry 12, 4609-4615.
19. Bewley, T.A., Sairam, M.R., and Li, C.H. (1972) Biochemistry 11, 932-936.
20. Garnier, J., Pernollet, J.-C., Tertrin-Clary, C., Salesse, R., Casteing, M., Barnaron, M., De La Llosa, P., and Jutisz, M. (1975) Eur. J. Biochem. 53, 243-254.
21. Reichert, L.E., Jr. and Ramsey, R.B. (1975) J. Biol. Chem. 250, 3034-3040.
22. Ingham, K.C., Aloj, S.M., and Edelhoch, H. (1973) Arch. Biochem. Biophys. 159, 596-605.
23. Ekblad, M., Bewley, T.A., and Papkoff, H. (1970) Biochim. Biophys. Acta 221, 142-145.
24. Pierce, J.G., Faith, M.R., Giudice, L.C., and Reeve, J.R. (1976) in Polypeptide Hormones: Molecular and Cellular Aspects, pp 225-250, Ciba Foundation Symposium 41, Elsevier, Excerpta Medica, North Holland, Amsterdam.

25. Ledley, F.D., Mullin, B.R., Lee, G., Aloj, S.M., Fishman, P.H., Hunt, L.T., Dayhoff, M.O., and Kohn, L.D. (1976) Biochem. Biophys. Res. Commun. 69, 852-859.
26. Lai, C.Y., Mendez, E., Chang, D., and Wang, M. (1977) Biochem. Biophys. Res. Commun. 74, 215-222.
27. Kurosky, A., Markel, D.E., Peterson, J.W., and Fitch, W.M. (1977) Science 195, 299-301.
28. Bishop, W.H. and Ryan, R.J. (1975) Biochemistry 14, 4083-4087.