THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 252, No. 15, Issue of August 10, pp. 5386-5392, 1977
Printed in U.S.A.

Isolation and Amino Acid Sequence of COOH-terminal Fragments from the β Subunit of Human Choriogonadotropin*

(Received for publication, February 7, 1977)

STEVEN BIRKEN AND ROBERT E. CANFIELD

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032

The amino acid sequence of the unique COOH-terminal region of the β subunit of human choriogonadotropin has been reinvestigated. The desialylated subunit was digested with thermolysin and a 27-residue peptide from positions 115 through 141 isolated in a high yield. Quantitative Edman sequence degradation of this peptide, of another peptide produced by thermolysin digestion containing residues 142 to 145, and of two tryptic peptides (residues 123 to 145, 134 to 145) has established that the amino acid sequence of this region is: Phe-Gln-Asp-Ser-Ser-Ser-Ser-Lys-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-Gln. In addition, the positions of attachment of the carbohydrate moieties to serine residues was established by a direct procedure using alkaline elimination and ^{35}S-labeled sulfite addition, which yields [^{35}S]-cysteic acid residues at the site of a substituted serine. Carbohydrate side chains in the COOH-terminal region have been shown to exist at residues 121, 127, 132, and 138. These studies have also resulted in the development of improved methods for the purification of COOH-terminal peptides of the human choriogonadotropin β subunit.

Human choriogonadotropin (hCG) is a glycoprotein hormone synthesized by trophoblast cells early in pregnancy (1, 2). Primary structure studies of the nonidentical α and β subunits of this hormone (3, 4) have revealed a high degree of homology with the α subunits of the other glycoprotein hormones, *i.e.*, lutropin, thyrotropin, and follitropin; target organ specificity is apparently conveyed by differences in the structures of their β subunits (5). The structural comparison of hLH[1] with hCG is of particular interest since these two hormones bind at the same ovarian or testicular receptors (6-8). Also, antibodies that bind hCG and do not bind hLH have been used as tumor markers and to make the diagnosis of pregnancy as early as several days following fertilization (9, 10).

* This work was supported by National Institutes of Health Contract NO1-HD-0-2251 and Research Grant AM 09579.

[1] The abbreviations used are: hLH, human lutropin; asialo, desialylated; hCG, human choriogonadotropin; hCG β, β subunit of human choriogonadotropin; PTH, 3-phenyl-2-thiohydantoin; RCM, reduced, *S*-carboxymethylated.

Two proposals for the primary structure of hCG have been published (11-13). Both indicate a high degree of homology between the α and β subunits of hLH and hCG with the interesting finding of a unique extension of approximately 30 amino acids at the COOH terminus of the hCG β subunit. However, these two proposals contain significant differences in the amino acid sequence of this region and in sites of carbohydrate attachment to serine residues. Since synthetic peptides containing this unique sequence of the hCG β subunit provide the most promising source of antigens to raise antibodies that distinguish hCG from hLH, it is important to resolve these disagreements about the primary structure. This communication describes the isolation of peptides from this region of the hCG β subunit and structural studies which confirm our initial proposal. The accompanying paper contains the results of an independent study arriving at the same conclusion (14).

EXPERIMENTAL PROCEDURES

Hormone Preparation-Crude human choriogonadotropin was purchased from Organon (Oss, Netherlands) and purified as described earlier (15). The hormone was dissociated into subunits and purified by methods that have also been described previously (16).

Materials and Methods-Details of the material and methods used are given in the supplement to this paper.[2]

RESULTS

Isolation of Peptides-The isolation and designation of nomenclature of the peptides βT(14 + 15) and βT(15) from reduced, carboxymethylated hCG β subunit have been described earlier (13). Additional analyses are listed in Table I. In order to obtain other fragments from the COOH-terminal region of the β subunit, we also investigated other enzymatic digestions of native and asialo β subunit. Since there was a limited supply of material, the conditions for large scale preparation of peptides were established by small scale digestions followed by gel filtration and monitoring of the eluant fractions by absorb-

[2] "Materials and Methods" as well as some of the figures and tables are presented in a miniprint format immediately following this paper. The abbreviations used, in addition to those in the main text, are RIA, radioimmunoassay; DMAA, *N*,*N*-dimethyl-*N*-allylamine; $EtCl_2$, dichloroethane; PITC, phenylisothiocyanate. Figs. S.1 through S.5 and Tables S.I through S.VI are found on pp. 5391-5392. Also, 16 pages of full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request JBC Document Number 77M-180, cite the author(s), and include a check or money order for $2.40 per set of photocopies.

ance at 230 nm and by a sensitive radioimmunoassay to the COOH-terminal region.

The gel filtration patterns following these small scale (3 mg) digestions of the β subunit are illustrated in the supplement. Asialo hCG β was digested with trypsin, chymotrypsin, and thermolysin. Since approximately 0.15 μmol of substrate was employed, the immunoassay technique was of great benefit in determining the degree of cleavage and the location of COOH-terminal peptides in the column effluents. Amino acid analyses and NH_2-terminal sequence studies were also used to characterize the products. Fig. S.1 shows the results of a tryptic digestion of asialo hCG β fractionated on Sephadex G-100. The third peak containing virtually all of the immunoreactive material was identified as peptide βT(14 + 15) by amino acid analysis and NH_2-terminal sequence. Fig. S.2 shows that chymotrypsin, under the conditions used in this study, does not cleave a COOH-terminal peptide from asialo hCG β. The accompanying paper indicates that a COOH-terminal fragment can be derived from chymotryptic digestion of the RCM β subunit (14).

Small scale digestions of both native hCG β (Fig. S.3) and asialo hCG β with thermolysin, followed by chromatography of the digestion products on Sephadex G-75, indicated that a COOH-terminal peptide starting at residue 115 of the β subunit was released. As a result of these studies a large scale preparative digestion of asialo hCG β subunit was performed and the products were separated on Sephadex G-75 (Fig. 1). Effluent fractions were pooled as shown in Fig. 1 and designated βTh-1 through βTh-5. Amino acid analyses of βTh-1 and βTh-2 appear in Table S.I and the NH_2-terminal sequence

TABLE I

Amino acid analyses of COOH-terminal peptides

Given as relative number of residues. Column A gives the analysis obtained for the peptides as isolated, while column B is the analysis obtained after the alkaline β elimination, converting substituted serine residues to cysteic acid (see "Methods"). – = <0.05 residue.

Amino acid	βTh-4		βT(14 + 15)		βT(15)		HV-1
	A[a]	B	A	B	A	B	
Cysteic acid[b]		1.0		1.4		0.5	
Aspartic acid	1.8 (2)	2.0 (2)	1.0 (1)	1.0 (1)	1.0 (1)	1.3 (1)	
Threonine	0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)	0.9 (1)	
Serine	7.5 (8)	5.9	3.7 (4)	2.3	0.9 (1)	0.5	
Glutamic acid	1.0 (1)	0.8 (1)	0.8 (1)	0.8 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Proline	7.1 (8)	9.4 (8)	9.6 (9)	10.7 (9)	3.8 (4)	3.0 (4)	1.0 (1)
Glycine	0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Alanine	0.9 (1)	0.9 (1)	1.0 (1)				
Cysteine							
Valine							
Methionine							
Isoleucine	0.1		1.0 (1)	0.9 (1)	1.0 (1)	0.7 (1)	0.9 (1)
Leucine	1.9 (2)	2.3 (2)	3.3 (3)	3.2 (3)	1.9 (2)	1.8 (2)	1.0 (1)
Tyrosine							
Phenylalanine	0.7 (1)	0.7 (1)					
Histidine							
Lysine	0.9 (1)	1.0 (1)					
Arginine	1.0 (1)	1.0 (1)	0.9 (1)	1.0 (1)	0.9 (1)	1.0 (1)	

[a] Results of 24-, 48-, 72-h corrected analyses.

[b] Cysteic acid results from the β elimination-sulfite addition reaction for determination of carbohydrate-substituted serines (25).

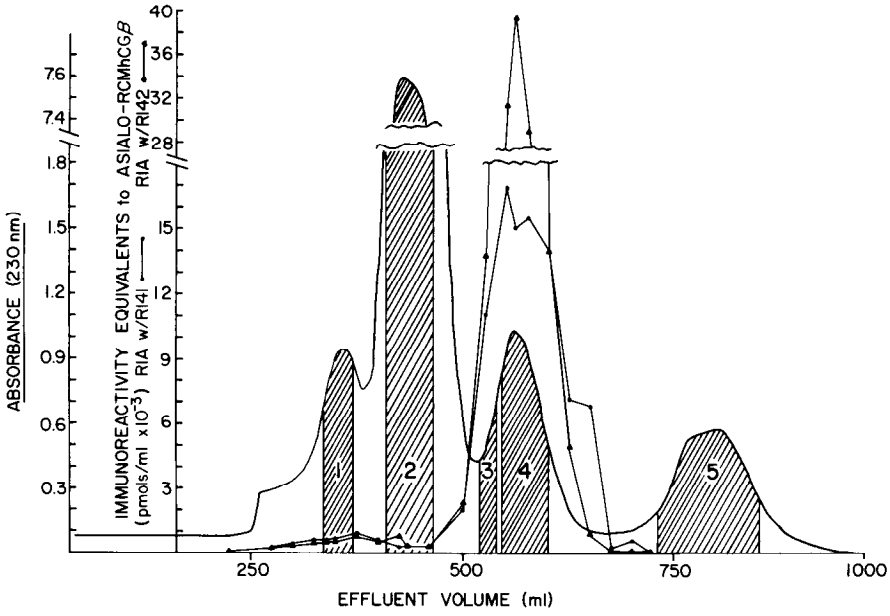

FIG. 1. Separation of products of a thermolysin digest of 200 mg of asialo hCG β. Gel filtration of the digestion products was on Sephadex G-75 (195 × 2.5 cm); eluant, 0.08 M ammonium acetate, pH 5.9; flow rate, 40 ml/h; temperature, 23°. Fractions were 5 ml and pooled as indicated by *shaded areas*. *RIA*, radioimmunoassay.

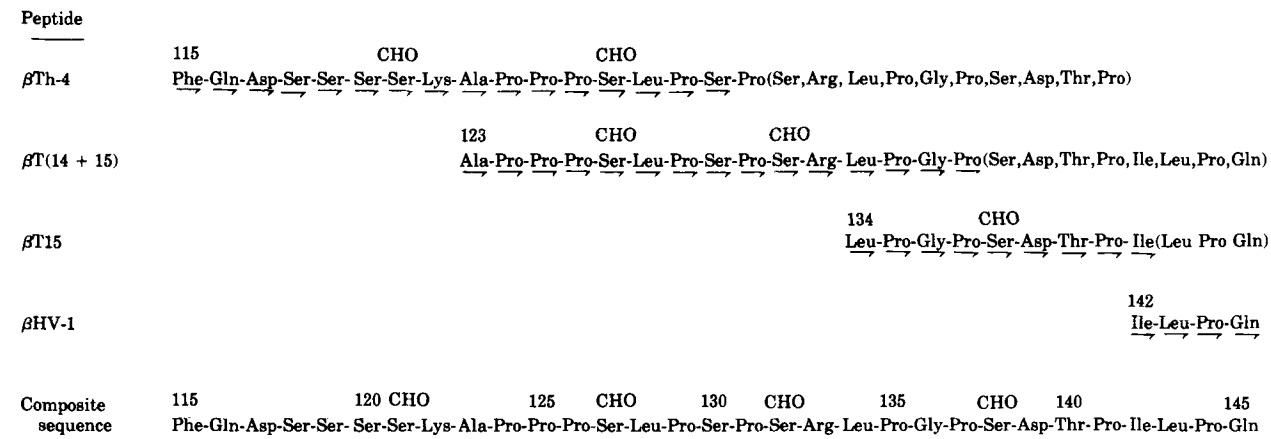

FIG. 2. Quantitative Edman degradation of peptides derived from the COOH-terminal portion of hCG β. Residues sequenced and quantitated by HI hydrolysis (see supplement) are indicated by →. The COOH-terminal glutamine of peptide HV-1 was determined directly. The peptide was subjected to three steps of Edman degradation and the free COOH-terminal glutamine was derivatized to the PTH-derivative of glutamine and identified on thin layer chromatography as well as glutamic acid after HI hydrolysis.

data of βTh-2 appears in Tables S.IIA and S.IIB. Similar but less distinct sequence results were noted with βTh-1. βTh-1 appears to be β subunit which has been partially degraded with thermolysin, but still contains the COOH-terminal fragment. βTh-2 consists of β subunit lacking the COOH-terminal residues 115 to 145 and is also missing amino acids in the region of residues 1 to 4 and 41 to 50. The six disulfide bonds presumably hold the remainder of this "core" fragment together. The βTh-3 and βTh-4 fractions contain a large peptide from the COOH terminus, which was further purified by rechromatography on Sephadex G-25 to remove any contaminating core fragment. The product contains a peptide with an amino acid analysis and NH_2-terminal sequence which indicate that it is comprised of residues 115 to 141 in a yield of 60 to 70%. βTh-5 is a complex mixture of salts and a variety of small peptides excised from the NH_2 terminus and the middle of the core fragment (region of residues 41 to 50) and also Residues 142 to 145 from the COOH terminus of the subunit. This mixture was chromatographed on both Sephadex G-15 and G-25 and the peptides were separated by high voltage electrophoresis and identified by amino acid composition and NH_2-terminal structure. Peptide HV-1 was identified as the COOH-terminal tetrapeptide (Residues 142 to 145) and its amino acid analysis (Table S.III) and NH_2-terminal sequence (Fig. 2; Table S.VI) were determined. The amino acid analyses and sequence of the other small peptides separated from pool βTh-5 are detailed in Table S.III. Fig. S.4 summarizes our results indicating the location of bonds that appear to have been cleaved by thermolysin during the large scale digestion asialo hCG β.

Amino Acid Sequence – The structure of the COOH-terminal region was determined quantitatively by manual Edman degradation followed by HI hydrolysis of the resultant PTH-derivatives. Data for identification of PTH-derivatives were also obtained by gas chromatography and thin layer chromatography as described under "Methods." Four peptides were employed in this procedure: βTh-4; βT(14 + 15); βT(15), and βHV-1. Amino acid analyses for the peptides appear in Table I. The results of quantitative Edman degradation of each peptide are summarized in Fig. 2. The yields of PTH-derivatives at each step of the degradation are summarized in Tables S.IV, S.V, and S.VI, which appear in the supplement. These yields were corrected for losses during hydrolysis with HI by parallel studies with PTH-derivative standards.

Determination of Position of Carbohydrate Moieties – The alkaline sodium [^{35}S]sulfite β elimination-sulfite addition reaction was used to determine the positions of the serines substituted with carbohydrate side chains. Three carbohydrate-containing peptides were subjected to this reaction: tryptic peptides βT(14 + 15) and βT(15) and thermolysin peptide βTh-4. Amino acid analysis indicates that after a 4-h reaction period, approximately 1.5 serine residues were converted to cysteic acid in peptides βTh-4 and βT(14 + 15). In peptide βT(15) one-half of the residues of serine were converted to cysteic acid. Amino acid analyses of these peptides after alkaline elimination appear in Table I. These partial conversions of substituted serines to cysteic acid produced labeled residues of sufficient radioactivity for identification after Edman degradation. Gel filtration of the reaction products in ammonium acetate on Sephadex G-15 separated the peptide (eluting at the void volume) from several other radioactive peaks which were presumed to be partially hydrolyzed peptide fragments, labeled sugar fragments, and the salt peak. In each case subsequent gel filtration on Sephadex G-50 in ammonium bicarbonate produced one symmetrical peak, which represented the only 230 nm absorbing material in the eluant.

Each of the peptides was subjected to sequential manual Edman degradation and the radioactivity of aliquots of both aqueous and organic phases was determined. Part of each organic phase was used for direct identification of the PTH-derivative by gas chromatography and thin layer chromatography and part was hydrolyzed and quantitated by amino acid analysis. Fig. 3*A* indicates the ^{35}S counts observed during each step of the manual Edman procedure of peptide βTh-4. Fig. 3*B* illustrates the same data for peptide βT(14 + 15) and Fig. 3*C* for peptide βT(15). The yield of PTH-derivatives at each step was determined by HI hydrolysis which indicated that heterogeneity was not present in the alkaline-treated peptides.

The results of these alkaline β elimination experments provided direct evidence for carbohydrate side chains attached to serines at position 121 (Fig. 3*A*), positions 127 and 132 (Fig. 3*B*), and position 138 (Fig. 3*C*). In addition, minor amounts of incorporated radioactivity were also noted at serine residues 120 and 130.

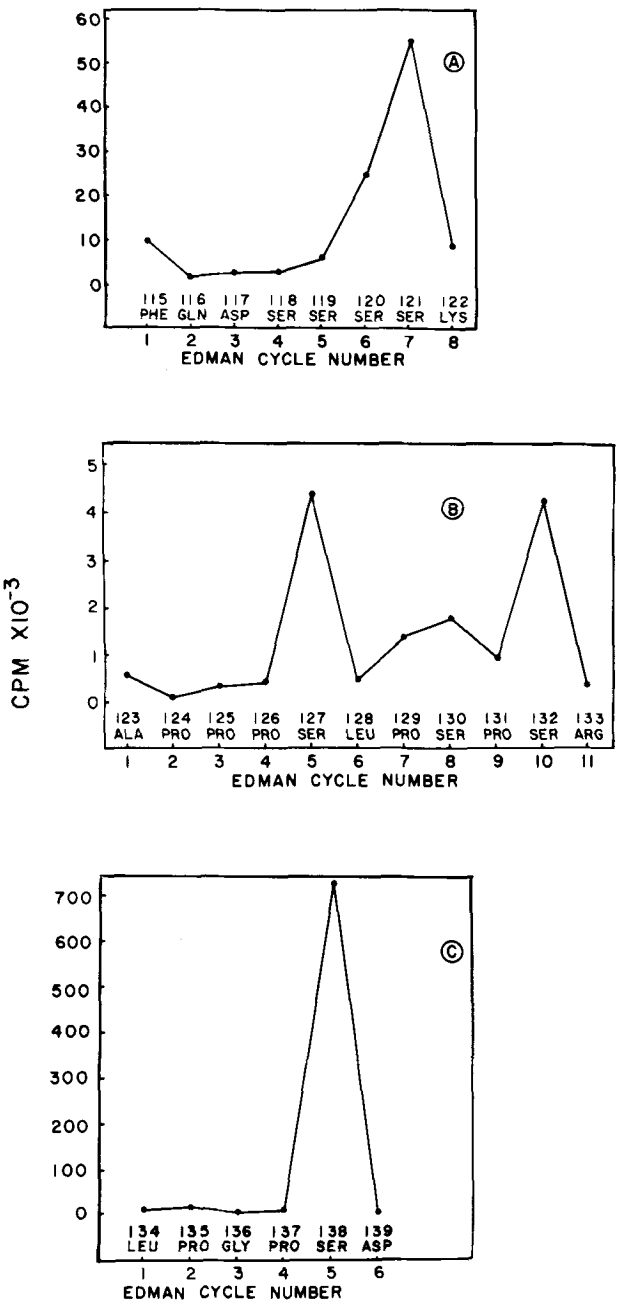

FIG. 3. Identification of the location of carbohydrate groups attached to serines in the COOH-terminal region of the hCG β molecule. Radioactivity of [^{35}S]cysteic acid produced by an alkaline elimination-sulfite addition reaction (see "Methods") is plotted on the *ordinate*. The amino acid sequences identified in a quantitative fashion are plotted on the *abscissa*. *A*, peptide βTh-4; carbohydrate present at position 121 with a trace present at 120. *B*, peptide βT(14 + 15); carbohydrate attached to residues 127 and 132. *C*, peptide βT(15); carbohydrate present at residue 138. The background radioactivity noted at other steps is presumed to be due to extraction of the labeled peptide. The scale of the *ordinate* of *B* is different since a planchette counter has been used to measure radioactivity, whereas a liquid scintillator was used for the other experiments. Different specific activities of the various batches of sodium [^{35}S]sulfite also contributed to variations in the labeling of the peptides.

DISCUSSION

The principal biological role of hCG is to convey a stimulus from the fertilized ovum to the corpus luteum of the ovary to sustain the synthesis of steroid hormones that will maintain the endometrial lining of the uterus and prevent the onset of the impending menstrual period (2). Since hLH, which is synthesized in the pituitary and plays a role along with follitropin in the ovulation process, and hCG appear to act at the same receptor sites (6–8), their structural comparison is of considerable interest. hCG has a higher carbohydrate content than hLH and the hCG β subunit contains an additional 30 amino acids at the COOH terminus, including four additional carbohydrate side chains attached at serine residues in this region. The amino acid composition of this extra structural component is unusual in that 9 of its 30 residues are proline. These features may convey additional resistance to proteolytic degradation and account for the nearly 10-fold increase in the plasma half-life of hCG when compared with hLH (17, 18).

Several practical immunologic applications have centered about the differences in primary structure between the β subunits of hCG and hLH. Antisera raised against hCG β, that have a low degree of cross-reactivity with hLH, have been employed as sensitive tests of pregnancy (9, 10). In addition, it has been shown that many nonpregnant patients with cancer have a detectable amount of hCG in their serum, making this assay a valuable tumor marker (19, 20). Finally, immunization of animals and humans with the hCG β subunit, conjugated to tetanus toxoid, has begun to be studied as a method of altering fertility (21, 22). However, when the entire hCG β subunit is employed as a component of the immunogen, there is the probability that some of the antibodies produced will cross-react with homologous regions of hLH. The 30-residue COOH-terminal region of the hCG β subunit thus becomes a very attractive component of an immunogen since those antibodies that do cross-react with the native hormone will be unique for hCG. For this reason, synthetic fragments from this region have begun to be employed as antigens with varying degrees of success (23, 24). One explanation of a lack of success would be that the synthetic fragment was prepared according to an incorrect sequence.

In view of the fact that there are numerous disagreements about the primary structure of this area of the molecule, we have undertaken to repeat a study of the isolation and amino acid sequence of this region, so that the types of biological experiments described above can proceed with synthetic material that is based on the correct primary structure. The principal differences between the report of the structure of the hCG β COOH terminus by Carlsen *et al.* (12) and that from our laboratory (4, 13) include both amino acid and carbohydrate assignments. Carlsen *et al.* (12) found an extra Ser-Leu-Pro sequence extension at the COOH terminus, but they did not find the serine at position 121. Carlsen *et al.* (12) reported a proline at 138 (our numbering), while we found a serine in that position. Carlsen *et al.* (12) found three carbohydrate side chains at serines in positions 117, 130, and 132 (our numbering), while we reported the major carbohydrate substitutions to be at four serines at residues 121, 127, 132, and 138.

Our earlier report employed peptides produced by trypsin and thrombin cleavage of asialo RCM hCG β (13) while the structural studies of Carlsen *et al.* (12) were performed by digestion of asialo, *S*-carboxamidomethylated hCG β with trypsin, chymotrypsin, and thermolysin. In this study, in addition to repeating the isolation of the tryptic peptides, we evaluated the possibility that trypsin, chymotrypsin, or thermolysin might cleave the asialo β subunit without prior reduction and carboxymethylation. This was accomplished by enzymic digestion of small quantities of the subunit, passing the products through Sephadex G-75, and using a sensitive

radioimmunoassay to detect COOH-terminal antigens in the effluent. The results indicate that trypsin and thermolysin, but not chymotrypsin, under the conditions employed (Figs. S.1 to S.3), will release fragments containing COOH-terminal antigens from the core of the subunit, the latter retaining a high molecular weight presumably due to cross-linking by the six cystine bridges. This new method of preparation is a major improvement over those that employ RCM asialo hCG as a substrate, because it permits a one-step purification with a peptide yield of nearly 70%.

The products of the large scale thermolysin digestion (Fig. 1) indicate that a COOH-terminal fragment arose as a result of cleavage of the peptide bond between residues at positions 114 and 115. This fragment was apparently further cleaved giving rise to a tetrapeptide containing residues 142 to 145, which was recovered from the digestion mixture (HV-1). A summary of residue assignments from Edman degradation studies is given in Fig. 2 and the method of identification and quantitative yields are listed in Tables S.III to S.VI in the supplement. The results are in complete agreement with our earlier proposal (4, 13).

In an effort to re-examine the number and location of serine residues substituted with carbohydrate we used a modification of the method of β elimination and sulfite addition described by Simpson *et al.* (25), employing ^{35}S-labeled sulfite. The results are summarized in Fig. 3. Carbohydrate side chains appear to be located at serines in positions 121, 127, 132, and 138, with a minor amount at position 120. The attachments at positions 127, 132, and 138, all follow a Pro-X-Pro sequence, which has been described before (26).

The reasons for the primary structure inaccuracies reported by Carlsen *et al.* (12) were probably the result of sequencing impure peptides and also lack of quantitation of PTH-derivatives. For example, the erroneous assignment of the extra tripeptide at the COOH terminus, Ser-Leu-Pro, resulted from dansyl(5-dimethylaminonaphthalene-1-sulfonyl)-monitored Edman sequence degradation of the peptide designated, βC-19, T-3, Th-2 (12). This preparation was probably contaminated with other peptides and the sequence of a larger contaminating peptide was apparently added at the point the major peptide had been sequenced to its final residue. With regard to other errors, Residue 138 was reported as proline apparently due to overlap in the Edman degradation since the PTH-derivative of serine, linked to carbohydrate, cannot be observed by the direct Edman identification. Another error appeared in the report of region 118 to 121, *i.e.* the failure to detect the fourth serine at position 121. The peptide βTh-21, T-1 was sequenced through only three serines and the COOH-terminal lysine was not recovered since it was in the form of a dipeptide, Ser-Lys, at that point of Edman degradation. The peptide βC-19, T-2, containing residues 115 to 122, was purified but not subjected to extensive sequence analysis. Had peptide βTh-21 been sequenced through this region to the lysine at position 122, the four serines would have been detected. The carbohydrate residue assignments were primarily confused because of a lack of accurate subtractive Edman data as well as lack of a positive method of identification such as the isotopic substitution method described in this paper.

The accompanying paper (14) describes an independent study of the structure of the β subunit COOH terminus employing a different type of enzymic cleavage and a different method to identify serine substituted with carbohydrate. The results agree completely with those described herein.

Acknowledgments—The technical assistance of Mr. Jeremiah Desmond, Mrs. Gladys Agosto, and Mr. Michael Gabel are gratefully acknowledged. The expert immunization techniques of Dr. Robert Lauer were instrumental in raising the antisera used during the course of these studies. We also gratefully acknowledge the advice of Dr. Francis Morgan, especially in relation to the determination of the positions of substituted serine residues.

REFERENCES

1. Loraine, J. A. (1967) in *Hormones in Blood* (Gray, C. H., and Bacharach, A. L., eds) pp. 313–332, Academic Press, New York
2. Canfield, R. E., Birken, S., Morse, J. H., and Morgan, F. J. (1976) in *Peptide Hormones* (Parsons, J. A., ed) pp. 299–316, Macmillan Press Ltd., London
3. Swaminathan, N., and Bahl, O. P. (1972) *Biochem. Biophys. Res. Commun.* **40**, 422–427
4. Morgan, F. J., Birken, S., and Canfield, R. E. (1973) *Mol. Cell. Biochem.* **2**, 97–99
5. Pierce, J. G. (1971) *Endocrinology* **89**, 1331–1344
6. Kammerman, S., Canfield, R. E., Kolena, J., and Channing, C. P. (1972) *Endocrinology* **91**, 65–73
7. Catt, K. J., Tsuruhara, T., Medelson, C., Ketelslegers, J.-M., and Dufau, M. L. (1974) in *Binding and Target Cell Activation in the Testis* (Dufau, M. L., and Means, A. R., eds) pp. 1–30, Plenum Press, New York
8. Bishop, W. H., Nureddin, A., and Ryan, R. J. (1976) in *Peptide Hormones* (Parsons, J. A., ed) pp. 273–298, Macmillan Press Ltd., London
9. Vaitukaitis, J. L., Ross, G. T., Reichert, L. E., Jr., and Ward, D. N. (1972) *Endocrinology* **91**, 1337–1342
10. Kosasa, T. S., Levesque, L. A., Taymor, M. L., and Goldstein (1974) *Fertil. Steril.* **25**, 211–216
11. Bellisario, R., Carlsen, R. B., and Bahl, O. P. (1973) *J. Biol. Chem.* **248**, 6796–6809
12. Carlsen, R. B., Bahl, O. P., and Swaminathan, N. (1973) *J. Biol. Chem.* **248**, 6810–6827
13. Morgan, F. J., Birken, S., and Canfield, R. E. (1975) *J. Biol. Chem.* **250**, 5247–5258
14. Keutmann, H. T., and Williams, R. M. (1977) *J. Biol. Chem.* **252**, 5393–5397
15. Canfield, R. E., and Morgan, F. J. (1973) in *Methods in Investigative and Diagnostic Endocrinology* (Berson, S. A., and Yalow, R. S., eds) Vol. 2B, pp. 727–733, North Holland, Amsterdam
16. Morgan, F. J., Canfield, R. E., Vaitukaitis, J. L., and Ross, G. T. (1973) in *Methods in Investigative and Diagnostic Endocrinology* (Berson, S. A., and Yalow, R. S., eds) Vol. 2B, pp. 733–742, North Holland, Amsterdam
17. Kohler, P. O., Ross, G. T., and Odell, W. D. (1968) *J. Clin. Invest.* **47**, 38–47
18. Rizkallah, T., Gurpide, E., and Vande Wiele, R. L. (1969) *J. Clin. Endocrinol. Metab.* **29**, 92–100
19. Braunstein, G. D., Vaitukaitis, J. L., Carbone, P. P., and Ross, G. T. (1973) *Ann. Intern. Med.* **78**, 39–45
20. Vaitukaitis, J. L. (1974) *Ann. Clin. Lab. Sci.* **4**, 276–280
21. Stevens, V. C., and Crystle, C. D. (1973) *J. Obstet. Gynecol.* **42**, 485–495
22. Talwar, G. D., Sharma, N. C., Dubey, S. K., Salahuddin, M., Das, C., Ramakrishnan, S., Kumar, S., and Hingorani, V. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 218–222
23. Schneider, C. H., Blaser, K., Pfeuti, Ch., and Gruden, E. (1975) *FEBS Lett.* **50**, 272–275
24. Stevens, V. C. (1975) *Immunization with Hormones in Reproduction Research* (Nieschlag, E., ed) pp. 217–231, North Holland, Amsterdam
25. Simpson, D. L., Hranisavljevic, J., and Davidson, E. A. (1972) *Biochemistry* **11**, 1849–1856
26. Baenziger, J., and Kornfeld, S. (1974) *J. Biol. Chem.* **249**, 7270–7281

SUPPLEMENT

TO: Isolation and Amino Acid Sequence of COOH Terminal Fragments from the Beta Subunit of Human Choriogonadotropin*

Steven Birken and Robert E. Canfield

INTRODUCTION

The preparation characterization and use of antisera to the COOH-terminal region of the hCG β subunit are presented in this supplement. In addition, the results of small scale enzyme digestions of the hCG β subunit and the quantitative Edman degradation results are provided in this section.

EXPERIMENTAL PROCEDURE

Materials and Methods

Materials - Neuraminadase and trypsin were purchased from Worthington (Freehold, N.J.), while thermolysin (Protease X) and chymotrypsin were acquired from Sigma Chemical Co. (St. Louis, Mo.). Sephadex gels were from Pharmacia Fine Chemicals (Piscataway, N.J.). All chemicals were either Fisher or Baker reagent grade except "Sequencer grade" reagents which were purchased from Beckman Instruments or Pierce Chemical Company.

Methods - Removal of sialic acid by incubation with neuraminadase was performed as described earlier (27). Pilot scale proteolytic digestions of the asialo, hCG β subunit with trypsin and chymotrypsin were performed as follows: trypsin or chymotrypsin were dissolved at a concentration of 3 mg/ml in 0.001N HCL. Three mg of desialylated hCG β were dissolved in 300 µl of 0.1M ammonium bicarbonate. Ten µl of the enzyme solution were added to the hormone solution at zero time, and again at 45 min. The digestion was carried out at 25° and terminated by lyophilization after 90 min. The products of trypsin digestion were chromatographed on Sephadex G-100, superfine (1.2 x 95cm) in 0.08M ammonium acetate, pH 5.9, while the chymotryptic digestion products were gel filtered in G-75 Sephadex Superfine employing the same buffer. Fractions of 1 ml size were collected at a flow rate of 8 ml/hr.

Pilot scale digestions of both native and asialo hCG β subunit preparations were performed with thermolysin in 0.1M NaCl, 0.05M Tris, 0.01M $CaCl_2$, pH 7.5 (28). Thermolysin (3 mg/ml) was dissolved in the above buffer. Three milligrams of hCG β or asialo hCG β were dissolved in 300 µl of buffer and 20 µl thermolysin solution (6 units) were added. The mixture was incubated at 25° for 90 min following which 25 µl of glacial acetic acid were added to stop the reaction and the mixture was immediately gel filtered on Sephadex G-75 Superfine (1.2 x 95cm) in 0.08M ammonium acetate, pH 5.9 as outlined above.

Based on results of the pilot scale digestion, a preparative thermolysin digestion was carried out as follows: asialo, hCG β subunit (200mg) was dissolved in 24 ml of 0.1M NaCl, pH 7.5 0.05M Tris, 0.01M $CaCl_2$ buffer. Six milligrams (498U) of thermolysin were dissolved in 1 ml of buffer and added to the hCG β solution. The digestion was carried out for 90 min at 25° and terminated by the addition of 200 µl of glacial acetic acid. The solution was lyophilized, redissolved in 5 ml of 0.08M ammonium acetate, pH 5.9 and applied to a Sephadex G-75 column (190 x 2.5cm).

The column was eluted with the ammonium acetate buffer and 5 ml fractions were collected at a flow rate of 40 ml/hr.

The tryptic peptides βT(14+15) and βT15 were prepared as described earlier (13) from a preparation of reduced, carboxymethyl hCG β subunit.

Preparation of Antisera - The two antisera used to detect the COOH-terminal peptide antigens in column effluent were produced by immunizing rabbits by the conventional toe pad injection method detailed as follows: A conjugate of peptide βT(14+15) to ovalbumin was prepared by the carbodiimide technique (20) with equal parts by weight of peptide and carrier. Salts were removed by dialysis. The resultant product was used as immunogen. The immunogen was emulsified with Freund's complete adjuvant at a concentration of 1 mg/ml. Each of two rabbits was injected with 0.4 ml (0.1 ml in each toe pad) at 10 day intervals for a total of three injections. Antibody titer rose to 1:9000 (final serum dilution to obtain 50% binding of labelled antigen) for R141 and 1:1500 for R142 within four months.

These antisera were characterized for specificity by competitive binding assays (30) employing the following competitors: hCG β, asialo hCG β, asialo, RCM hCG β, peptide βT(14+15) and peptide βTh-4. ^{125}I-labelled-asialo, RCM hCG β was used as tracer. The tracer proteins were iodinated by the chloramine T technique (31). Antibody dilutions to bind 30-50% of the added tracer were used in these assays. Standard curves were developed for each competitor and slopes were calculated by a Wang 2200 programmable calculator using the Rodbard linear-logit system of weighing (32).

Immunoassay of Column Effluents - Aliquots of every 3rd to 5th tube were taken and diluted in a phosphate buffered saline solution (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4) containing 1% normal rabbit serum and subjected to radioimmunoassay using antisera produced to the conjugated tryptic peptide βT(14+15). Antiserum R141 was used at a 1/9000 final dilution and R142 was used at a 1/2500 final dilution in a system consisting of 100 µl EDTA (0.1M), 100 µl ^{125}I-labelled-asialo, RCM hCG β tracer (30-40,000 cpm), 300 µl of 1% rabbit serum in phosphate buffered saline, 200 µl of assay (open competitive) column or (sample) tubes and 300 µl of antisera. This "first antibody" mixture was vortexed and incubated at 4° for 16-20 hr following which 1 ml of sheep anti-rabbit IgG antiserum (diluted 1/15 in phosphate buffered saline) was added to each tube, except total count tubes, vortexed, incubated at 4° for 4-6 hr, centrifuged, decanted, and the pellets counted. Standard curves were prepared in all assays by use of asialo, RCM β competitor and standard curves and "unknown" competitors were calculated on a Wang 2200 programmable calculator described above.

Antiserum Characterization - Antisera produced to the conjugated tryptic peptide βT(14+15) falls into two basic groups: (1) Those that crossreact with both native and asialo, RCM hCG β subunit and (2) those that react mainly with the latter molecule (40,41). The antisera used to monitor the preparation of β subunit COOH-terminal fragments in this study fall into the latter category i.e., they react chiefly with asialo, RCMβ. The antisera do crossreact slightly with native β but the predominant antibody species binds best to the unfolded molecule. Figure S.5 shows the crossreactivity of several competitors to antisera R141 and R142 when ^{125}I-labelled-asialo, RCM hCG β was used as tracer. As expected the peptide βT(14+15), which was part of the coupled immunogen is the best competitor along with the unlabelled tracer itself, asialo RCM β. Peptide βTh-4 and asialo, native β were the next most effective competitors. It appears that sialic acid plays a crucial role in the antibody binding site of these antisera. Native β or RCM β with sialic acid intact are both very poor competitors by two or three orders of magnitude.

However, specificity of these antisera to react with fragments of the COOH-terminal region of hCG β made them very useful for locating these peptides during purification.

High Voltage Paper Electrophoresis - Small peptides were purified by high voltage electrophoresis using Gilson electrophoretors with a 0.05M, pH 3.6 pyridine-acetate buffer (33). A center strip was stained with ninhydrin as a guide for cutting out parallel areas containing unstained peptides. Peptides were eluted from paper by use of Reeve-Angel "spin thimbles" and a sequence of acid-base treatments: 2% acetic acid (2x); 5% NH_4OH (2x), H_2O (2x).

Amino Acid Analysis - Amino acid analyses of proteins and peptides were performed on a Beckman model 121 automatic amino acid analyzer by a single column method (13).

Amino Acid Sequence Determination

Automatic Edman Degradation - Automated Edman degradation was performed in an "updated" Beckman 890B sequencer using Beckman program No. 60275, which is a single coupling, single cleavage program. Conversion and PTH-amino acid direct identifications were performed as described earlier (13). Quantitation of PTH-amino acids was performed by a modification of the methods of Smithies (34) and of Inglis (35) as follows: The organic phase containing the PTH amino acid was divided into equal parts immediately after dissolving in EtCl2. One-half of the sample was identified by thin layer chromatography (36) and gas chromatography (37), and the other half was mixed with a norleucine internal standard in a hydrolysis tube and dried down in a vacuum oven. PTH-Arg was identified by the phenanthrenequinone fluorescent paper spot test (38). This PTH-amino acid fraction was then hydrolyzed with HI (Baker 47-51%) under vacuum at 140° for 24 hr, and the HI was then removed by vacuum in a desiccator over NaOH. The amino acid content of the residue was analyzed in a Beckman model 121 amino acid analyzer by use of a "rapid" single column program (Beckman No. 969972).

Manual Edman Degradation - Manual Edman degradation was performed as described by Morgan et al. (13) using diethyl-ether for thiazolinone extraction (39). Peptides less than five amino acids in length were sequenced by elimination of the benzene and ethyl acetate washes. The peptides were coupled in the DMAA (0.5M) - pyridine/water buffer, the PITC and buffer removed by evaporation under high vacuum (20 microns) for 1 hr at 60°, followed by cleavage and subsequent extraction with diethyl-ether. The last amino acid of these peptides was identified directly following cleavage, evaporation, and immediate conversion and extraction of the PTH-amino acid which was then identified by the methods described above. This procedure was especially useful for peptide HV-1 in order to confirm its COOH-terminal glutamine by thin layer chromatography.

Alkaline β-Elimination - The following is an adaptation of a method described by Simpson et al. (25) to convert serine residues that are substituted with carbohydrate side chains to ^{3}⁵S-labelled cysteic acid. Sodium (^{3}⁵S) sulfite was obtained from New England Nuclear (NE X-030) and was dissolved in H_2O at a concentration of 10 mCi/ml. Approximately 500 nanomoles of each peptide were lyophilized, redissolved in a solution consisting of 100 µl of sodium (^{3}⁵S) sulfite (1 mCi) in H_2O and 100 µl of 0.1N NaOH, 0.1M Na_2SO_3 (cold), pH 12.8. The final pH after mixing was approximately 12.5. This peptide solution was incubated 4 hr at 25°. The reaction was stopped by the addition of 10 µl of glacial acetic acid and 5% of the volume was removed for amino acid analysis to measure the extent of serine conversion to cysteic acid. The remainder was lyophilized and then filtered through Sephadex G-15 (0.7 x 10cm) in 0.08M ammonium acetate, pH 5.9. The protein-containing effluent emerging in the void volume was lyophilized. This material was again chromatographed in Sephadex G-50 fine (0.7 x 18cm) in 0.1M ammonium bicarbonate, freeze-dried, and subjected to manual Edman degradation.

REFERENCES

Note: References 1-26 are in the parent paper.

27. Van Hall, E.V., Vaitukaitis, J.L., Ross, G.T., Hickman, J.W., and Ashwell, G. (1971) Endocrinology 88, 456-464
28. Blumberg, S., and Valle, B.L. (1973) Biochemistry 14, 2410-2419
29. Goodfriend, T.L., Levine, L., Fasman, G.D. (1964) Science 144, 1344-1346
30. Odell, W.D., and Daughday, W.H., Eds (1971) Principles of Competitive Protein-Binding Assays, Lippincott Co., Philadelphia
31. Greenwood, F.C., Hunter, W.M., and Glover, J.J. (1971) Biochem. J. 89, 114-123
32. Rodbard, D., and Lewald, J.E. (1970) in Steroid Assay by Protein Binding, Karolinska Symposia, 2nd Symposium (Diczfalusy, E., Ed) pp.79-103, Bogtrykkeriet Forum, Stockholm
33. Katz, A.M., Dreyer, W.J., and Anfinsen, C.B. (1959) J. Biol. Chem. 234, 2897-2905
34. Smithies, O., Gibson, D., Fanning, E.M., Goodfliesch, R.M., Gilman, J.G., and Ballantyne, D.L. (1971) Biochemistry 10, 4912-4921
35. Inglis, A.S., Nicholls, P.W., and Roxburgh, C.M. (1971) Aust. J. Biol. Sci. 24, 1247-1250
36. Edman, P. (1970) in Protein Sequence Determination (Needleman, S.B., Ed) pp.211-255, Springer-Verlag, New York
37. Pisano, J.J., and Bronzert, T.J. (1969) J. Biol. Chem. 244, 5597-5607
38. Yamada, S., and Itano, H.A. (1966) Biochim. Biophys. Acta 130, 538-540
39. Peterson, J.D., Nehrlich, S., Oyer, P.E., and Steiner, D.F. (1972) J. Biol. Chem. 247, 4864-4871
40. Lauer, J-P., Ross, G.T., Birken, S., and Canfield, R.E. (1974) J. Clin. Endocr. Metab. 39, 1155-1158
41. Chen, H-C., Hodgen, G.D., Matsuura, S., Lin, L.J., Gross, E., Reichert, L.E., Jr., Birken, S., Canfield, R.E., and Ross, G.T. (1976) Proc. Natl. Acad. Sci. 73, 2885-2889

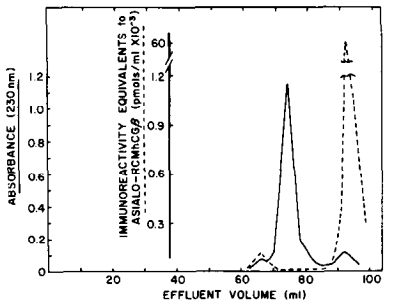

FIGURE S.1 - Separation of products of tryptic digest of asialo, hCG β (3mg). Gel filtration of tryptic peptides on Sephadex G-100 superfine (1.2 x 95 cm), eluant, 0.08M ammonium acetate, pH 5.9, flow rate 5 ml/hr; fraction size, 1 ml; temperature, 23°. Absorbance at 230 nm (——); RIA to COOH-terminal region (– – –). Amino acid analysis of the material containing the major immunoactivity indicated that it contained residues 123-145 which was confirmed by Edman degradation: ASP = 1.0(1), Thr = 0.9(1); Ser = 3.1(4); Glu = 0.8(1); Pro = 9.0(9), Gly = 1.0(1); Ala = 1.0(1); Ile = 0.7(1); Leu = 2.5(3).

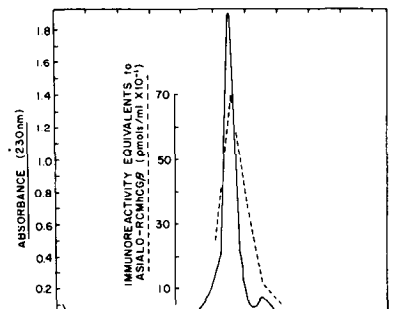

FIGURE S.2 - Separation of products of chymotryptic digest of asialo hCG β (3mg). Chromatography of products of chymotryptic digest on G-75 superfine (1.2 x 95cm), eluant, 0.08M ammonium acetate, pH 5.9, flow rate 8 ml/hr; fraction size, 1 ml; temp 23°. Absorbance at 230 nm (——); RIA to COOH-terminal region (– – –).

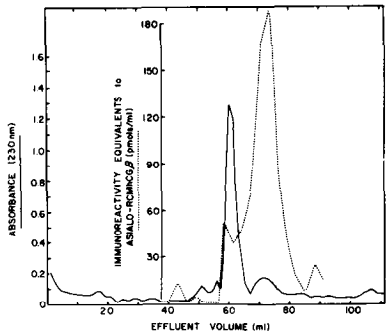

FIGURE S.3 - Separation of products of thermolysin digest of native hCG (3mg). Chromatography of thermolytic digest peptides on G-75 superfine (1.2 x 95cm), eluant, 0.08M ammonium acetate, pH 5.9, flow rate 8 ml/hr; fraction size, 1 ml, temp 23°. Absorbance at 230 nm (——); RIA to COOH-terminal region (– – –). The fractions containing the major immunoreactivity were pooled, lyophilized, and subjected to amino acid analysis. The resultant analysis indicated the product contained residues 115-141, shown in parenthesis. Asp = 2.1(2); Thr = 1.0(1), Ser = 7.1(8); Glu = 1.4(1); Pro = 8.3(8); Gly = 1.0(1); Ala = 1.0(1); Leu = 2.5(2); Phe = 1.5(1); Lys = 1.0(1); Arg = 0.8(1). Manual Edman degradation of this peptide confirmed the NH_2-terminal Phe-Gln-Asp sequence.

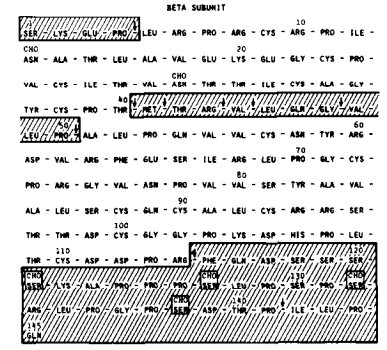

FIGURE S.4 - Probable sites of cleavage of asialo hCG β. The sites of cleavage Thermolysin under the conditions described in the Methods section are shown by arrows. Cross hatched areas denote portions removed. Carbohydrate substituted serine residues are shown in boxes. The sites of cleavage by thermolysin were deduced by the NH_2-terminal sequences present in the mixture β Th-2 (see Tables S.IIA, S.IIB) and by the isolation of several small peptides: HV-1: 142-145; HV-2: 44-47; HV-3: 45-47; HV-4: 47-50; HV-5: 41-44; HV-6: Free Met (see Table III).

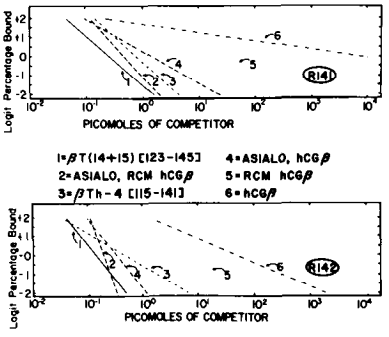

FIGURE S.5 - Characterization of antisera produced to a conjugated immunogen of βT(14+15) to ovalbumin. The tracer used is ^{125}I-labelled-asialo, RCM hCG β. Competitors are detailed on the figure. The ordinate is a logit plot of percentage of tracer bound, abscissa represents the dose in picomoles of each competitor added to each RIA tube.

TABLE S.I

Amino Acid Analyses of sTh-1 and sTh-2[a]

Amino Acid	sTh-1[b]	sTh-2[c]
Aspartic Acid	11.0(11)	9.0(9)
Threonine	10.2(10)	7.8(9)
Serine	9.0(13)	3.7(5)
Glutamic Acid	10.4(9)	5.5(7)
Proline	16.5(22)	10.4(13)
Glycine	7.1(8)	5.9(7)
Alanine	7.0(8)	7.0(7)
Cysteine	8.3(12)	10.0(12)
Valine	9.0(12)	9.9(12)
Methionine	1.5(1)	0.3(1)
Isoleucine	3.7(5)	4.1(4)
Leucine	10.6(12)	9.6(9)
Tyrosine	2.45(3)	2.6(3)
Phenylalanine	1.37(2)	1.0(1)
Histidine	2.75(1)	1.0(1)
Lysine	5.32(4)	2.5(3)
Arginine	8.02(12)	10.1(11)

[a]The significant differences between these two analyses are in serine and proline content. These data suggest that the COOH-terminal region has been cleaved from sTh-2 and not from sTh-1.

[b]Aspartic acid taken as 11.0 residues. Numbers in parentheses are based on structure of intact α subunit.

[c]Aspartic acid taken as 9.0 residues. Numbers in parentheses are residues in 1-114. Many amino acids are lower than predicted, due to partial or complete loss of peptides 1-4 and 41-50.

TABLE S.IIA

Results of Quantitative Edman Degradation of Peptides Present in sTh-2 (Yield in Nanomoles)

Step No.	Asp	Thr	Ser	Glu	Pro	Gly	Alac	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg
1	7	-[d]	-	5	12	7	237[e]	92	-	175	-	-	-	-	-
2	6	7	-	7	5	5	9	-	-	338	5	-	-	75	107
3	9	7	-	93	298	-	7	-	-	7	-	-	-	-	8
4	12	-	-	102	143	5	88	-	5	9	6	-	-	-	55
5	7	-	-	45	61	7	9	50	-	138	-	-	-	-	27
6	7	-	-	23	84	9	6	80	5	33	6	-	-	-	81
7	7	-	-	61	113	8	6	64	-	16	-	-	-	-	58
8	31	-	-	20	68	7	5	69	38	8	-	-	-	-	32
9	32	-	-	8	36	8	6	62	22	6	24	-	-	-	17
10	27	-	-	5	27	7	43	42	23	5	25	-	-	-	29
11	68	21	-	7	43	10	42	33	20	7	20	-	-	-	46
12	43	20	-	-	24	8	21	-	16	31	27	-	-	-	21

[a]As α-amino butyric acid.

[b]Appears as alanine.

[c]Ala represents both alanine and serine.

[d]Less than 5 nanomoles

[e]Underlined numbers represents amino terminal amino acid expected from the NH_2-terminals present according to the scheme outlined in Table S.IIB.

TABLE S.IIB

Summary of Probable NH_2-Terminal Sequences Present in sTh-2*

Edman cycle number	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	⑪	⑫
	1	2	3	4	5	6	7	8	9	10	11	12
A	Ser-	Lys-	Glu-	Pro-	Leu-	Arg-	Pro-	Arg-	Cys-	Arg-	Pro-	Ile
	5	6	7	8	9	10	11	12	13	14	15	16
B	Leu-	Arg-	Pro-	Arg-	Cys-	Arg-	Pro-	Ile-	Asn-	Ala-	Thr-	Leu
	48	49	50	51	52	53	54	55	56	57	58	59
C	Val-	Leu-	Pro-	Ala-	Leu-	Pro-	Gln-	Val-	Val-	Cys-	Asn-	Tyr
	51	52	53	54	55	56	57	58	59	60	61	62
D	Ala-	Leu-	Pro-	Gln-	Val-	Val-	Cys-	Asn-	Tyr-	Arg-	Asp-	Val

*This table shows the probable NH_2-terminal sequence content of the sTh-2 "core" fragment as deduced from the data tabulated in Table S.IIA and the known sequence of the hCG α subunit. sTh-2 appears to be a heterogeneous group of chains held together by disulfide bonds. Several peptides have been excised from the mid-region (41-50) by thermolysin. The presence of the above four sequences was deduced by examination of the quantitative yields from Edman degradation (Table S.IIA) and the isolation of some of the peptides cleaved from within this "core" fragment of hCG β. Sequence A is the structure of the NH_2-terminus of the intact beta subunit. Sequence B is evident from the data of Table S.IIA with the arginine at step 4, the isoleucine at step 8, and threonine at step 11. A peptide consisting of residues 1-4 was present in sTh-3. Sequence C was substantiated by isolation of peptide HV-2 (see Fig. S.4, Table S.III) and by the presence of valine at step 1 and alanine at step 4. Sequence D is supported by the isolation of peptide HV-4 (see Fig. S.4, Table S.III) and by the finding of glutamic acid at step 4, aspartic acid at step 8, and tyrosine at step 9.

TABLE S.III

Amino Acid Analyses of Small Peptides[a]

Amino Acid	HV-1[b]	HV-2[b]	HV-3[b]	HV-4[b]	HV-5[b]	HV-6
Asp	-	0.1	0.1	-	0.5	-
Thr	-	-	-	-	0.9(1)	0.4
Ser	-	0.1	-	-	0.2	-
Glu	1.0(1)	1.0(1)	0.7(1)	-	0.2	-
Pro	1.0(1)	-	0.3	1.1(1)	0.2	-
Gly	-	1.1(1)	0.7(1)	-	-	-
Ala	-	-	-	-	-	-
Cys	-	-	-	-	-	-
Val	-	1.0(1)	0.2	1.1(1)	0.9(1)	-
Met	-	-	-	-	1.0(1)	1.0(1)
Ile	0.9(1)	0.1	-	-	-	-
Leu	1.0(1)	1.0(1)	1.0(1)	1.2(1)	-	-
Tyr	-	-	-	-	-	-
Phe	-	-	-	-	-	-
His	-	-	-	-	-	-
Lys	-	-	-	-	0.3	-
Arg	-	-	-	-	1.0(1)	0.2
Rf[c]	0.35	0.41	0.44	0.47	0.76	0.81

[a]Given as relative number of ratios. Amino acid analyses are uncorrected 24 hr hydrolyses. Less than 0.1 residues are not shown.

[b]These peptides were subjected to manual Edman degradation and identified as follows:

HV-1: Ile-Leu-Pro-Gln: residues 142-145
HV-2: Val-Leu-Gln-Gly: residues 44-47
HV-3: Leu-Gln-Gly : residues 45-47
HV-4: Val-(Leu, Pro) : residues 47-50
HV-5: Met-Thr-X-Val : residues 41-44
HV-6: (Assumed to be mostly free Met)

[c]Rf is relative to arginine migration.

TABLE S.IV

Quantitative Edman Degradation of 540 Nanomoles of sTh-4[a] (Nanomoles of amino acids recovered after hydrolysis of PTH-amino acids)

Step No.	115[b] Phe	116 Gln	117 Asp	118 Ser	119 Ser	120 Ser	121 CHO Ser	122 Lys	123 Ala	124 Pro	125 Pro	126 Pro	127 CHO Ser	128 Leu	129 Pro	130 Ser	131 Pro
1	(456)	6															
2		(378)	10														
3		13	(386)														
4			10	(34)													
5			5		(56)												
6						(36)											
7							(22)										
8								(122)	14								
9									(176)								
10									18	(172)							
11									10		(172)						
12									?			(158)					
13												16	(14)				
14												13		(84)			

(Yield nanomoles)

[a]These yields are corrected for loss upon hydrolysis of PTH-amino acids. Yields under 5 nanomoles are omitted. Serines are identified as alanine, threonine as α-amino butyric acid, and isoleucine as itself and its "allo" form. The major amino acid found after each step of Edman degradation is circled.

[b]Residue number in sequence of hCG β.

TABLE S.V

Quantitative Edman Degradation of 500 Nanomoles of sT(14+15) (Nanomoles of amino acids recovered after hydrolysis of PTH-amino acids)

Step No.	123 Ala	124 Pro	125 Pro	126 Pro	CHO Ser	128 Leu	129 Pro	130 Ser	131 Pro	CHO Ser	133 Arg	134 Leu	135 Pro	136 Gly	137 Pro
1	(422)														
2		(434)													
3			(530)												
4				(415)											
5				5											
6						(362)									
7						13	(288)								
8							41	(54)							
9								7	(288)						
10									25						
11											*				
12												(160)			
13												18	(140)		
14												23	6	(56)	

(Yield nanomoles)

*Determined by phenanthrenequinone paper spot test (38). Also see footnotes of table S.IV.

TABLE S.VI

Quantitative Edman Degradation of 250 Nanomoles of sT15 (Nanomoles of amino acids recovered after hydrolysis of PTH-amino acids)

Step No.	134 Leu	135 Pro	136 Gly	137 Pro	CHO Ser	139 Asp	140 Thr	141 Pro	142 Ile
1	(216)								
2		(214)							
3			(146)						
4				(142)					
5									
6						(60)			
7						7	(32)		
8								(12)	
9									(12)

(Yield nanomoles)

Quantitative Edman Degradation of 320 Nanomoles of sHV-1 (Nanomoles of amino acids recovered after hydrolysis of PTH-amino acids)

Step No.	142 Ile	143 Leu	144 Pro	145 Gln
1	(244)			
2		(185)		
3			(174)	
4				(152)*

(Yield nanomoles)

*Identified as Gln by TLC of the PTH-amino acid. Also, see footnotes of table S.IV.