Recombination of the Biologically Active Peptides from a Tryptic Digest of Bovine Growth Hormone†

Kenji Hara,‡ Chen-Jung Hsu Chen,§ and Martin Sonenberg*

ABSTRACT: Two bovine growth hormone peptides containing residues 96 to 133 in one and a larger peptide corresponding to residues 1 to 95 and 151 to 191 linked by a disulfide bond have been recombined. Recombination was performed in 1.0 N acetic acid without and with 8 M urea and the recombined peptides were purified by gel filtration. Evidence of recombination of the peptides has been established by gel filtration, polyacrylamide gel disc electrophoresis, amino acid composition, ultraviolet absorption, circular dichroism, and intrinsic fluorescence. The molar growth promoting activity of the recombined peptides measured by tibial width assay in hypophysectomized rats was 10% that of the native hormone and greater than the sum (1%) of the growth promoting activities of the individual peptides. These studies suggest that some contribution of the larger peptide is necessary for more complete expression of the growth promoting activity of the bovine growth peptide corresponding to residues 96 to 133.

From tryptic digests of bGH (TbGH)[1] we have isolated a single component, TbGH-d, as demonstrated by analytical disc electrophoresis and sedimentation equilibrium (Sonenberg et al., 1968). We have reported (Yamasaki et al., 1970) fractionation of TbGH-d by gel filtration in 50% acetic acid and have isolated two components, bGH 1-95 and 151-191, a peptide of 16 000 molecular weight, and bGH 96-133, a peptide of 5000 molecular weight (Figure 1).

bGH 1-95 and 151-191 and bGH 96-133 are active in the weight gain and tibial width test in hypophysectomized rats, but the level of activity was much less than that of native bGH from which these peptides were produced (Yamasaki et al., 1970; Hara and Sonenberg, 1977). bGH 1-95 and 151-191 was much less active in growth promoting assays than bGH 96-133 (Yamasaki et al., 1970; Hara and Sonenberg, 1977). Both peptides were also active *in vitro* in stimulating glucose uptake and conversion to carbon dioxide, lipolysis, glycerol release, and histidine incorporation into protein in isolated adipose tissue of hypophysectomized rats (Swislocki et al., 1970). In addition, bGH 96-133 was found to have metabolic activity in humans similar to hGH (Sonenberg et al., 1972, 1973; Levine et al., 1973).

The amino acid sequence of bGH 96-133 has been determined (Yamasaki et al., 1972, 1975). It consists of 38 amino acid residues and is homologous with region 96-133 of the bGH molecule (Wallis, 1973; Santomé et al., 1973; Gráf and Li, 1974a). Because of the good homology in the amino acid sequence between bGH 96-133 and a corresponding peptide occurring in hGH, human chorionic somatomammotropin, and ovine growth hormone, bGH 96-133 may represent some of the amino acid sequence responsible for its metabolic activities (Swislocki et al., 1970; Yamasaki et al., 1972, 1975). TbGH-d, the parent component from which bGH 1-95 and 151-191 and bGH 96-133 were derived, retained full activity compared with bGH (Yamasaki et al., 1970; Sonenberg et al., 1972).

bGH 96-133 may have reduced growth promoting activity because of a secondary structure and tertiary structure significantly different from the native hormone from which it was derived. This could result in impaired affinity for the target cell receptor for growth hormone and changes in its ability to stimulate a biological response. These considerations form the basis of separate studies (submitted). The possibility remained that the low growth promoting potency of the smaller peptide (bGH 96-133) was the consequence of the loss of some contribution of the larger peptide (bGH 1-95 and 151-191) to the size and conformation of the smaller peptide.

In order to evaluate the contribution of the larger peptide to the growth promoting activity of the smaller peptide, an attempt has been made to recombine the biologically active smaller peptide with the larger peptide. In this paper, only growth promoting activity is considered. Metabolic and immunologic activities have previously been reported (Swislocki et al., 1970; Sonenberg et al., 1973). Preliminary reports of other recombination studies from this laboratory have appeared (Sonenberg and Beychok, 1971; Sonenberg et al., 1972). Recombination of fragments of hGH with nearly full restoration of biological activity has also been reported (Li and Bewley, 1976; Li et al., 1976, 1977).

Experimental Procedures

Materials. bGH was prepared by the methods previously reported (Dellacha and Sonenberg, 1964; Free and Sonenberg, 1966). Trypsin and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp., Freehold, N.J. Urea (UltraPure grade, no copper, iron, lead, or cyanide) was a product of Schwarz/Mann, Orangeburg, N.Y. 1-Dimethylamino-5-naphthalenesulfonyl chloride (Dns) (10% solution

† From the Memorial Sloan-Kettering Cancer Center and Cornell University, Graduate School of Medical Sciences, New York, New York 10021. *Received April 18, 1977.* Supported in part by Grants CA-08748 of the National Cancer Institute and BC-147 of the American Cancer Society.

‡ Present address: Kao Tokyo Research Institute, 2-1-3, Bunka, Sumida-ku, Tokyo, 131, Japan.

§ Present address: Department of Pathology, Yale University School of Medicine, New Haven, Conn. 06510.

[1] Abbreviations used: bGH, bovine pituitary growth hormone; TbGH, tryptic digest of bovine growth hormone; TbGH-d, homogeneous component of a tryptic digest of bovine growth hormone prepared by Yamasaki et al. (1970); TbGH-d (HoAC), TbGH-d exposed to 50% acetic acid; TbGH-x, homogeneous component of tryptic digest of bovine growth hormone prepared by the methods of Yamasaki et al. (1970) for this study; bGH 1-95 and 151-191, peptide of bovine growth hormone corresponding to residues 1 to 95 and 151 to 191 linked by a disulfide bond; bGH 96-133, peptide of bovine growth hormone corresponding to residues 96 to 133; bGH 134-150, peptide of bovine growth hormone corresponding to residues 134 to 150; hGH, human pituitary growth hormone; oGH, ovine growth hormone; $NaDodSO_4$, sodium dodecyl sulfate; dansyl, Dns, 1-dimethylamino-5-naphthalenesulfonyl; CD, circular dichroism.

0006-2960/78/0417-0550$01.00/0

in acetone) was obtained from Pierce Chemical Co., Rockford, Ill. Polyamide layer sheets for the separation of Dns-amino acids were obtained from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y. Other chemicals used in this study were reagent grade.

Preparation and Fractionation of Tryptic Digests of Bovine Growth Hormone. Tryptic digests of bGH were prepared by the methods previously reported (Sonenberg et al., 1965, 1968; Nadler et al., 1968; Yamasaki et al., 1970). The single component of the tryptic digest of bGH (TbGH-x) was isolated from the tryptic digests of bGH by ion-exchange chromatography on a DEAE-cellulose column by the methods of Yamasaki et al. (1970). TbGH-x, with which subsequent studies were done, appeared in fractions eluted with 0.03 and 0.05 M ammonium bicarbonate buffer. It had the same electrophoretic mobility previously designated the *d* component of the tryptic digests of bGH (TbGH-d). For further purification of TbGH-x, the same chromatographic system was employed. The component which was homogeneous by analytical disc electrophoresis was used for the isolation of the individual peptides.

Isolation and Characterization of the Biologically Active Peptides. The biologically active peptides were prepared from a single component, TbGH-x, of tryptic digests of bGH, dissociated by 50% acetic acid as previously described (Yamasaki et al., 1970). In addition, gel filtration of the single component (TbGH-x) of TbGH was carried out on a Sephadex G-75 column with 0.1 M carbonate buffer (pH 9.5) containing 8 M urea. The proteins were identified in four fractions. From 300 mg of TbGH-x, the yield was 70 mg of the most rapidly eluted, 120 mg of bGH 1-95 and 151-191, 55 mg of bGH 96-133 and 20 mg of bGH 134-150. The peptide fractions were pooled and applied on a Sephadex G-25 or Bio-Gel P-2 column to remove urea with 0.01 M ammonium bicarbonate buffer (pH 8.4) and lyophilized. The three most rapidly eluted fractions were further purified by repeating the gel filtration in 0.1 M carbonate buffer (pH 9.5) containing 8 M urea.

The purity of these peptides was established by disc electrophoresis, amino-terminal amino acids, amino acid composition, and spectrofluorescence. From fluorescence spectra of bGH 96-133, only tyrosine fluorescence was noted whereas bGH 1-95 and 151-191 had evidence of tryptophan fluorescence. The molecular weight of bGH 96-133 was determined to be 5000 by using 10% cross-linked polyacrylamide gel Na-DodSO_4 disc electrophoresis. From amino acid analysis, ultraviolet absorption, fluorescence, and circular dichroism spectra, it was concluded that the 5000 molecular weight peptide originated in residues 96-133 (Figure 1) of the bGH molecule (Wallis, 1973; Santomé et al., 1973; Gráf and Li, 1974a). The largest peptide consisted of the amino-terminal portion (1-95 of the sequence of bGH) and carboxyl-terminal position (151-191 of the sequence of bGH) which are linked by a disulfide bond (Figure 1).

The smallest peptide was separated and urea was removed by gel filtration through Bio-Gel P-2. The purified peptide was subjected to paper chromatography in the 1-butanol-pyridine-acetic acid-water (15:10:3:12, v/v) system and one homogeneous peptide (R_f 0.29) was obtained and consisted of residues 134-150 (Figure 1) of the bGH molecule (Wallis, 1973; Santomé et al., 1973; Gráf and Li, 1974a). Only limited studies were performed with this peptide.

Recombination of Peptides. For the recombination of peptides derived from tryptic digests of bGH, 32 mg of bGH 1-95 and 151-191 and 10 mg of bGH 96-133 were dissolved separately to make a 1.0% protein solution in 1.0 N acetic acid. These were mixed and stirred continuously for 5 h at room temperature. The reaction mixture was freeze dried directly. To purify the recombined complex, the lyophilized reaction mixture was applied to a Sephadex G-75 column and eluted with 1.0 N acetic acid.

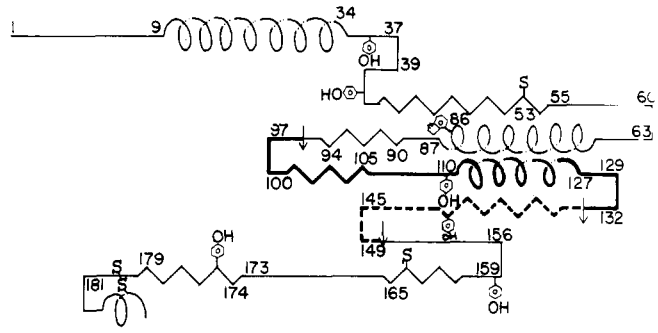


FIGURE 1: Schematic representation of the components of TbGH-x. bGH 1-95 and 151-191 (—), bGH 96-133 (—), and bGH 134-150 (- - -) modified from Chen and Sonenberg (1977).

Recombination in urea solution was performed as follows; 32 mg of bGH 1-95 and 151-191 and 10 mg of bGH 96-133 were dissolved separately in 3.2 mL and 1.0 mL of 1.0 N acetic acid solution containing 8 M urea. Both peptide solutions were mixed and stirred constantly for 5 h at room temperature. The reaction mixture was transferred into a Visking tube and dialyzed against 1.0 N acetic acid solution containing 8 M urea for 24 h. The outer solution was then changed to 1.0 N acetic acid solution containing sequentially 6 M urea, 4 M urea, 2 M urea, and no urea. The recombined complex was recovered by freeze-drying.

Purification of Recombined Peptides. The lyophilized crude products of recombined peptides were applied on a column of Sephadex G-75 equilibrated with 1.0 N acetic acid. The column was developed with the same solution. The pooled fractions were lyophilized directly. For characterization of the recombined peptides, fractions were further purified by repeating the gel filtration in 1.0 N acetic acid.

Disc Electrophoresis with and without Sodium Dodecyl Sulfate. Disc electrophoresis in polyacrylamide gels was carried out by the methods of Ornstein (1964), Davis (1964), and Reisfeld et al. (1962). Electrophoresis was performed at pH 9.5 and/or pH 4.3 in 7.5% polyacrylamide gel. Protein components were detected by staining with Amido Black 10B, followed by removing unbound dye with several changes of 7% acetic acid.

Polyacrylamide gel disc electrophoresis in Na-DodSO_4 was carried out by the methods of Shapiro et al. (1967), and Weber and Osborn (1969). The marker proteins (bacitracin, cytochrome *c*, chymotrypsinogen A, ovalbumin, albumin) for the determination of molecular weight were purchased from Schwarz/Mann, Orangeburg, N.Y.

Amino Acid Analysis and Amino-Terminal Amino Acid Analysis. Protein or peptide samples were hydrolyzed in vacuo with 6.0 N HCl at 110 °C for 24 h. Hydrolysates were analyzed on an automatic amino acid analyzer Beckman Model 121 according to Spackman et al. (1958). Amino-terminal amino acid analysis was performed by the method of Gray and Hartley (1963). An aliquot of protein or peptide sample was reacted with dansyl chloride. The resulting Dns-peptide was then hydrolyzed in 6.0 N HCl at 106 °C for 16 h in vacuo. Dns-amino acids were separated and identified by two-dimensional polyamide layer chromatography (Woods and Wang, 1967). Development in a covered jar by ascending solvent systems was employed: (I) water–90% formic acid

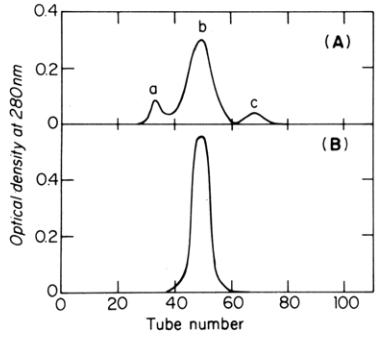


FIGURE 2: Gel filtration of (A) recombined peptides, bGH 1–95 and 151–191 and bGH 96–133 (30 mg) and (B) bGH (30 mg) on Sephadex G-75 column (2.5 × 43.0 cm) in 1.0 N acetic acid; fraction volume, 2.1 mL.

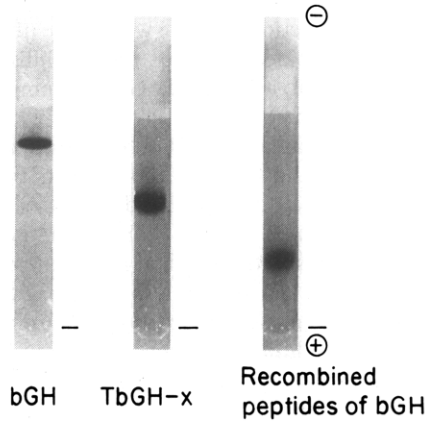


FIGURE 3: Polyacrylamide gel disc electrophoresis of bGH, TbGH-x, and recombined bGH 1–95 and 151–191 and bGH 96–133.

(200:3, v/v); (II) benzene–glacial acetic acid (9:1, v/v) (Woods and Wang, 1969).

Ultraviolet Absorption Spectra. Ultraviolet absorption spectra were measured with a Cary Model 11 recording spectrophotometer. Cylindrical quartz tandem cells (10-mm path length) were used so that both peptide solutions were in the light path, either separated by a quartz partition, or mixed in the same compartment. The protein concentrations were 0.3 mg/mL for bGH 96–133, 0.96 mg/mL for bGH 1–95 and 151–191, and 1.26 mg/mL for separated and mixed peptides in 0.1 N acetic acid solution. Ultraviolet absorption spectra were also measured in 0.1 M sodium carbonate buffer (pH 9.5) to compare with bGH.

Circular Dichroism Spectra. Circular dichroism determinations were made in the far ultraviolet range (250–195 nm) with a Cary Model 60 recording spectropolarimeter with circular dichroism attachment 6002. Cylindrical quartz tandem cells (10-mm path length) were used so that solutions of bGH 1–95 and 151–191 and bGH 96–133 were both in the light path, either separated by a quartz partition, or mixed in the same compartment. Aliquots of the protein solutions were taken for Lowry-Folin test (Lowry et al., 1951) to determine the protein concentration for each experiment.

Fluorescence Spectra of the Recombined Peptides. Fluorescence measurements were made on a Cary 50-026-900 differential spectrofluorimeter equipped with a Rhodamine B quantum detector located within the sample compartment and employing front surface illumination, where the exciting and emitting beams subtend an angle of 23°. Other details with respect to the spectrofluorimeter were as described elsewhere (Sonenberg, 1971). Measurements were made with the excitation monochromator set at 270 nm at 25 °C. The concentration of peptides was 0.1% in 0.1 M sodium carbonate buffer (pH 9.5).

TABLE I: Amino Acid Composition of Recombined Peptides.[a,b]

Amino acid	bGH 1–95 and 151–191[c] + bGH 96–133	bGH 1–95 and 96–133[d] + bGH 96–133	1–133[e] + 151–191
Asp	14.1	13.8	13
Thr	9.7	9.3	10
Ser	10.4	10.3	13
Glu	22.0	22.0	22
Pro	6.6	6.3	6
Gly	9.1	9.3	9
Ala	12.1	11.4	14
1/2-Cystine	3.3	3.4	4
Val	6.4	7.3	6
Met	2.4	2.9	3
Ile	6.1	5.6	6
Leu	23.1	23.3	26
Tyr	4.5	5.0	5
Phe	9.9	11.9	12
Lys	8.8	9.5	9
His	2.4	3.4	3
Arg	12.6	14.2	12
Trp	*f*	*f*	1

[a] Composition in molar ratio. Thr and Ser values are uncorrected. [b] Different lots of peptides have been employed in each recombination. [c] Recombined in 1.0 N acetic acid containing 8 M urea. [d] Recombined in 1.0 N acetic acid. [e] Residue positions in the bGH structure (Wallis, 1973; Santomé et al., 1973; Gráf and Li, 1974a,b). [f] Not determined.

Biological Response. The growth responses of the individual and the recombined peptides were determined in hypophysectomized rats by the 4 day tibial width response test (Geschwind and Li, 1955). Five rats per dose were employed.

Results

Purification of Recombined Peptides. The lyophilized products obtained by recombination in 1.0 N acetic acid were purified by Sephadex G-75 column chromatography from peptides bGH 1–95 and 151–191 and bGH 96–133 previously equilibrated with 1.0 N acetic acid and eluted with the same solution (Figure 2A). One main fraction (fraction b) and two minor fractions were obtained. Fraction a was eluted at the same volume as blue dextran. It was found to contain aggregated material and was not investigated further. Similarly, the other minor fraction, fraction c, was not further characterized. bGH on a calibrated column under the same conditions was eluted at the same volume as fraction b (Figure 1B). The main fraction b was purified further by rechromatography on Sephadex G-75 in 1.0 N acetic acid and a single peak was noted.

Analytical electrophoresis on polyacrylamide gel indicated that the recombined complex was homogeneous. The recombined complex had a greater cathodal mobility than bGH and TbGH-x (Figure 3).

Amino Acid Composition. Comparison of the amino acid compositions reveals almost identical compositions between the recombined peptides of bGH and bGH without residues 134–150 (Table I).

Spectroscopic Characterization of Recombined Peptides. Typical absorption spectra (not shown) of recombined peptides as established above were compared with that of peptides bGH 1–95 and 151–191 and bGH 96–133. The recombined peptides

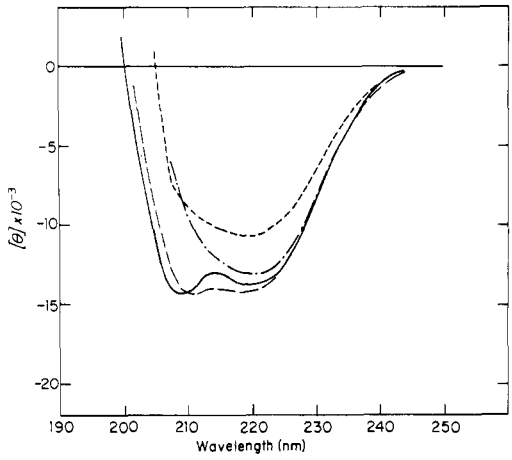


FIGURE 4: Circular dichroism spectra of recombined bGH 1-95 and 151-191 and bGH 96-133 (— — —), unmixed equimolar concentrations of bGH 1-95 and 151-191 and bGH 96-133 in separate compartments of a quartz tandem cell (— • —), bGH 1-95 and 151-191 (—), and bGH 96-133 (- - -) in 0.1 N acetic acid; $[\theta]$, deg cm^2 dmol^{-1}.

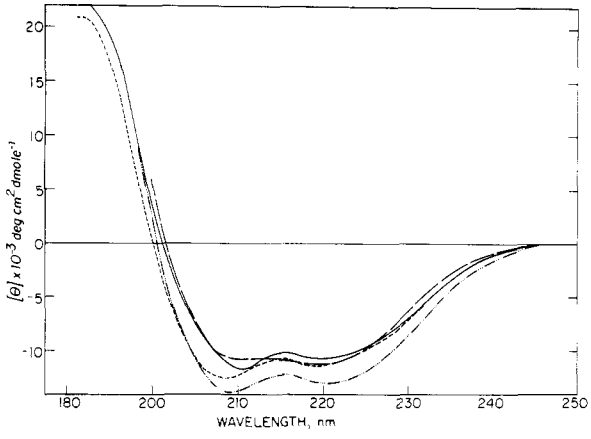


FIGURE 5: Circular dichroism spectra of recombined bGH 1-95 and 151-191 and bGH 96-133 (pH 4). Recombined peptides (0.232 mg/mL) (—). Calculated peptides curve (— — —). TbGH-d treated with HoAC (0.231 mg/mL) (- - -). TbGH-d (0.22 mg/mL) (— • • • —). All solutions were prepared in 0.01 M formate buffer, pH 4. Path length, 0.1 cm. The signal-to-noise ratio at 222 nm was 25:1 and at 208 nm was 18:1.

duplicated the absorption spectrum obtained when the individual peptides were in separate compartments of a quartz tandem cell in 0.1 N acetic acid. Except for some slight discrepancy in the region between 245 and 270 nm, the recombined peptides gave ultraviolet absorption spectra in 0.1 M sodium carbonate buffer, pH 9.5, similar to bGH (not shown).

The circular dichroism spectra between 250 and 200 nm of bGH 1-95 and 151-191 and bGH 96-133 in separate compartments of a quartz tandem cell, and the peptides in 0.1 N acetic acid are shown in Figure 4. The CD spectrum of the recombined complex contains two troughs at approximately 220 and 210 nm with ellipticities, $[\theta]$, of −14 300 and −14 500. There is a crossover at 202 nm. The circular dichroism spectrum of bGH 1-95 and 151-191 contains one trough at approximately 220 nm with ellipticity, $[\theta]$, of −10 600, and that of bGH 96-133 contains two troughs at 222 and 207 nm with ellipticity, $[\theta]$, of −12 400 and −14 000.

The CD spectra of the recombined complex at pH 4 (Figure 5) and pH 9 (Figure 6) are similar to TbGH-d treated with acetic acid and lower in ellipticity than the native TbGH-d spectra. The - - - line is a calculated curve obtained by the

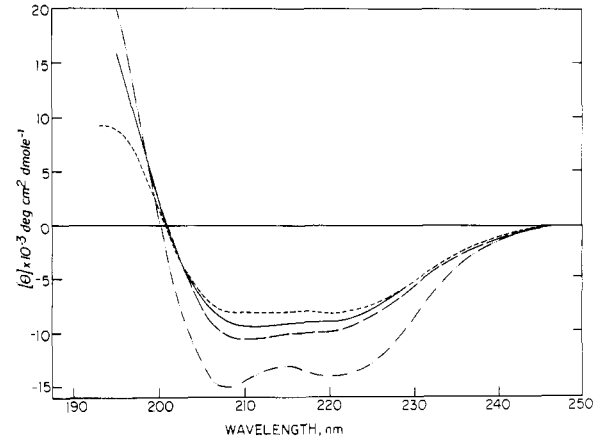


FIGURE 6: Circular dichroism spectra of recombined bGH 1-95 and 151-191 and bGH 96-133 (pH 9). Recombined peptides (0.123 mg/mL) (—). Calculated peptides curve (— — —). TbGH-d treated with HoAC (0.114 mg/mL) (- - -). TbGH-d (0.214 mg/mL) (— • • • —). All solutions were prepared in 0.01 M bicarbonate buffer, pH 9. Path length, 0.1 cm. The signal-to-noise ratio at 222 nm was 25:1 and at 208 was 18:1.

TABLE II: Secondary Structure Analysis of the Recombined Peptides.

Protein	pH	Helix[a]	β[a]
bGH 1-95 and 151-191, bGH 96-133, complex	4	28	17
bGH 1-95 and 151-191 + bGH 96-133 (calcd)	4	29	27
TbGH-d	4	36	8
TbGH-d (HoAC)	4	29	10
bGH 1-95 and 151-191, bGH 96-133, complex	9	22	23
bGH 1-95 and 151-191 + bGH 96-133 (calcd)	9	26	25
TbGH-d	9	40	3
TbGH-d (HoAC)	9	19	18

[a] Secondary structural parameters calculated by the method of Chen et al. (1972).

following formula:

$$[\theta]_{\text{calcd},\lambda} = \frac{136}{174[\theta]_{\text{bGH1-95and151-191},\lambda}} + \frac{38}{174[\theta]_{\text{bGH96-133},\lambda}}$$

where $[\theta]$ is the molar ellipticity of the peptide CD spectrum, and 136, 38, and 174 are the number of amino acids respectively of bGH 1-95 and 151-191, bGH 96-133, and the recombined complex of these two peptides. Assuming no interaction between the two peptides, the calculated CD curve of the peptides is merely the sum of the bGH 1-95 and 151-191 and bGH 96-133 individual spectrum characteristics at the particular wavelength, λ. The calculated curve is different in line shape from the recombined complex spectrum due to the contribution from the bGH 96-133 random coil spectrum. The secondary structural parameter calculation (Table II) shows that TbGH-d contains a large amount of helix structure and almost no β sheet. The peptides of the recombined complex, TbGH-d (HoAC) and bGH 1-95 and 151-191 and bGH 96-133 (calcd) all have comparable amounts of helix and β-sheet structure.

The corresponding fluorescence spectra at pH 9 and pH 4 (not shown) of the recombined peptides have a similar emission maximum at 330-335 nm as that of TbGH-d, but the relative fluorescence intensity is higher. The calculated curve, taking into account the smaller proportion of tryptophan in the

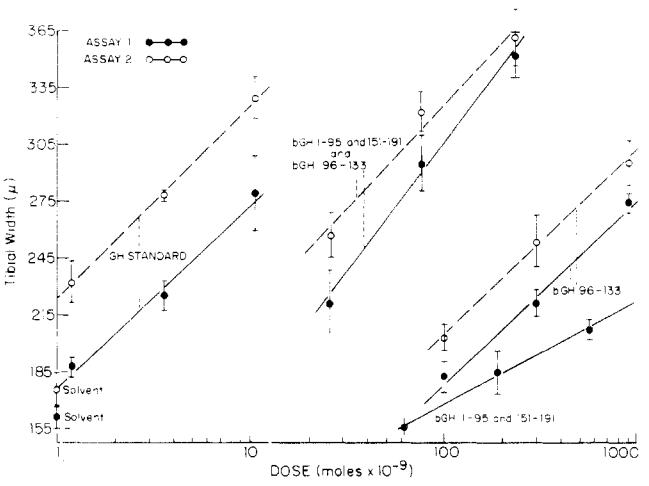


FIGURE 7: Biological response of recombined peptides. The tibial width response, μ, with standard error of the response have been plotted against the nanomolar dose on a log scale.[2]

complex than in bGH 1–95 and 151–191, is different from the recombined complex spectrum indicating a modification of the tryptophan fluorophore in the presence of bGH 96–133.

Biological Response. When bGH 96–133 was assayed at doses (weight) approximately 20 times greater than the native hormone, the International Growth Standard, parallel responses vs. log dose were obtained (Figure 7). Thus, on a weight basis, bGH 96–133 had approximately 5% of the growth promoting potency of bGH; on a molar basis this was about 1% of the growth promoting potency. The larger peptide, bGH 1–95 and 151–191, even at maximal doses produced a response which barely exceeded 200 μ and its slope was distinctly more shallow than the bGH and bGH 96–133 slope of response vs. log dose.

The growth promoting activity of the recombined equimolar amounts of the larger and smaller peptides was significantly greater than the growth promoting activity of bGH 96–133 with bGH 1–95 and 151–191 considered to have insignificant growth promoting activity. When both peptides were recombined in acetic acid, the resultant complex produced a slope of the dose–response curve not significantly different from that of bGH and bGH 96–133. The molar potency estimate for the complex recombined in acetic acid was about 10% of the standard. When both peptides were recombined in urea, the slope of the dose–response curve for the complex was significantly steeper than the slopes of bGH and bGH 96–133. Under the circumstances no potency estimate of this complex could be made.

Discussion

bGH 96–133 retained significant capacity to increase tibial width (Table III; see Supplementary Material Available paragraph at end of paper) and produce weight gain in hypophysectomized rats (Yamasaki et al., 1970), but the biological activity of bGH 96–133 was markedly decreased compared with the parent bGH protein (Table III; Yamasaki et al., 1970). However, the slope in a log dose–response plot was not significantly different for bGH 96–133 compared with bGH (Table III). It should be noted that in the present study and more recent studies (Hara and Sonenberg, 1977) with bGH 96–133, the slope of the log dose–response plot is parallel to that of the International Growth Hormone Standard. This may be related to the larger doses (500 to 4500 μg per rat per 4 days) employed in these studies than the doses (30 to 600 μg per rat per 4 days) employed in earlier studies (Yamasaki et al., 1970; Gráf and Li, 1974b; Li and Gráf, 1974; Gráf et al., 1976). In a sigmoidal dose–response curve, it may be that inadequate doses of bGH 96–133 with low activity were administered in the earlier studies to detect parallelism with native growth hormone. Low biological activity in various small peptides in the region of residues 96 to 133 of growth hormone was also observed in related experiments in other laboratories (Blake and Li, 1973; Chillemi et al., 1972; Wang et al., 1974).

The biological activity of undigested bGH decreased from 1.0 to 0.37 ± 0.06 IU/mg when dissolved in 35% acetic acid for 18 h at 4 °C (Sonenberg et al., 1972). The nature of the structural modification was not identified. The effects of urea on the biological activity of bGH and TbGH-x were tested before use as a dissociating agent with TbGH-x. Bioassay results showed that 8 M urea did not reduce the growth promoting activity of bGH and TbGH-x. Urea, 8 M, was shown to dissociate TbGH-x into three major components. One consisted of a peptide originating in residues 96–133 of the bGH molecule (Wallis, 1973; Santomé et al., 1973; Gráf and Li, 1974a). Another consisted of the amino-terminal position (1–95 of the sequence of bGH) and carboxyl-terminal position (151–191 of the sequence of bGH) which are linked by a disulfide bond. The smallest fraction consisted of residues 134–150 of the bGH molecule. From these results, TbGH-x was formed by the cleavage of arginylvaline, arginylalanine, and arginylserine peptide bonds occurring within the large disulfide loop of bGH.

Previously Yamasaki et al. (1970) showed two homogeneous components, peptide A-I and A-II, were isolated from a single component of a tryptic digest of bGH (TbGH-d). TbGH-d had been obtained as the fraction eluted from a DEAE-cellulose column with 0.05 M ammonium bicarbonate buffer. For the present experiments, TbGH-x appeared in fractions eluted with 0.03 M and/or 0.05 M ammonium bicarbonate buffer. In the present experiments, from the single component of the 0.05 M ammonium bicarbonate fraction (TbGH-x), we prepared three peptides using 8 M urea. Acetic acid, 50%, was also shown to dissociate TbGH-x into three components. These results show that TbGH-x may be different from TbGH-d and is possibly the c or e component of TbGH (Yamasaki et al., 1970), or that the smallest peptide (bGH 134–150) was lost during the purification procedure of TbGH-d (Yamasaki et al., 1970). Previously it was presumed that the 16 000 molecular weight peptide originated in residues 1–95 and 134–191 of the bGH molecule (Wallis, 1973; Santomé et al., 1973; Gráf and Li, 1974a), but the present results show that peptide A-I consisted of the residues 1–95 and 151–191 of the sequence of bGH which are linked by a disulfide bond (Figure 1). Based on the properties of the large and the small peptides under different conditions, there seems to be no obvious difference found in peptides isolated by 50% acetic acid or 8 M urea.

We attempted to recombine the three peptides, bGH 1–95 and 151–191, bGH 96–133 and bGH 134–150. Equimolar amounts of these peptides were incubated in 1.0 M acetic acid. Only bGH 1–95 and 151–191 and bGH 96–133 were recombined and bGH 134–150 behaved as another component. From these results and those of others (Lewis et al., 1973; Singh et al., 1974; Reagan et al., 1975a,b) residues 134–150 of the bGH molecule seem not to be necessary for the biological activity of growth hormone.

[2] The bioassay data with the statistical evaluation were submitted to the scrutiny of the reviewers and will appear in the microfiche version. They may be obtained by interested readers by writing directly to the authors.

The recombination of two peptides was performed in 1.0 N acetic acid because the association of bGH 96–133 was markedly dependent on the concentration of acetic acid employed as solvent. bGH 96–133 was found to exist as a monomeric form in acetic acid solutions, 1.0 N or greater. Decrease in the concentration of acetic acid promoted self-association of bGH 96–133 and dimeric and trimeric forms were observed in 0.1 N acetic acid (Yamasaki and Kangawa, 1973).

The CD spectra of the recombined complex are similar to TbGH-d treated with acetic acid, but have less ellipticity than TbGH-d. The calculated curve of the individual peptides is comparable in ellipticity but slightly different in line shape to the recombined complex. The CD spectrum of the recombined complex (pH 9) is similar to the recombined complex spectrum (pH 9.5) reported by Sonenberg and Beychok (1971). They showed that there is a significant difference in ellipticity between the spectrum of the recombined peptides and the tandem cell spectrum of bGH 1–95 and 151–191 and bGH 96–133 at pH 9.5. The native TbGH-d is more helical and tighter structured than the complex. TbGH-d treated with 50% acetic acid could be looked upon as an intermediate structure in that its secondary structure resembles that of the recombined peptides, whereas its fluorophore characteristic resembles that of native TbGH-d. Fluorescence spectra indicate a conformational change upon recombination since the fluorescence intensity is reduced from the fluorescence predicted from the sum of the intensities of the individual peptides. This induced conformational change must be essential for the more complete expression of the growth promoting biological activity.

The molecular organization seems to reflect biological activity. TbGH-d elicits a higher biological response than both the recombined peptide and TbGH-d (HoAC). The recombined complex is in turn higher than the sum of the effects of the individual peptides. It is noteworthy that the complex of bGH 1–95 and 151–191 and bGH 96–133 produced by recombination in urea was different than that obtained after recombination in acetic acid as indicated by different slopes of their dose–response curves. No spectroscopic comparisons of these two types of recombinations were made.

CD spectroscopy does not predict the absolute determinants of biological activity. bGH contains 63% α helix and 3% β sheet; TbGH-d contains 29% α helix and 29% β sheet (Chen and Sonenberg, 1977), whereas their biological activities are of equivalent magnitude. Thus, molecular disorganization to a certain degree is perhaps allowed in growth hormone without destruction of biological activity.

The present studies have not been able to identify the exact conformations of the individual peptides in their recombined state. The bGH structure predicted based on primary sequence (Chen and Sonenberg, 1977) shows that the bGH 96–133 segment has helical structure, which is probably related to the bGH 96–133 helical state at pH 4 as observed by CD. Therefore, the bGH 96–133 helical conformation is perhaps the active form of the bGH 96–133. When bGH 1–95 and 151–191 and bGH 96–133 were recombined in 1 N acetic acid, bGH 96–133 was already in a helical conformation which could be easily incorporated into the complex with some more conformational adjustments. bGH 1–95 and 151–191 contains a high percentage of β structure which was not seen in bGH indicating that the structure of bGH 1–95 and 151–191 is different from the native bGH 1–95 and 151–191 conformation originally in bGH. The lack of conformational flexibility of this peptide indicates that it is frozen in the high β sheet structure state which is more difficult to revert back to native peptide in bGH. This may explain why the recombined complex is higher in β sheet content, lower in ellipticity and lower in biological activity than native TbGH-d.

Studies of the structure and biological activity of the two individual peptides alone and recombined suggest that these two peptides are both essential for the more complete expression of growth hormone activity.

Acknowledgments

The authors are grateful to Dr. Gary Litman for his assistance in determinations of amino acid composition.

Supplementary Material Available

The biological response of recombined peptides (Table III) (1 page). Ordering information is given on any current masthead page.

References

Blake, J., and Li, C. H. (1973), *Int. J. Peptide Protein Res. 5,* 123.

Chen, C. H., and Sonenberg, M. (1977), *Biochemistry 16,* 2110.

Chen, T. H., Yang, J. T., and Martinez, H. M. (1972), *Biochemistry 11,* 4120.

Chillemi, F., and Pecile, A. (1971), *Experientia 27,* 385.

Chillemi, F., Aiello, A., and Pecile, A. (1972), *Nature (London), New Biol. 238,* 243.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Dellacha, J. M., and Sonenberg, M. (1964), *J. Biol. Chem. 239,* 1515.

Free, C. A., and Sonenberg, M. (1966), *J. Biol. Chem. 241,* 5076.

Geschwind, I. I., and Li, C. H. (1955), in Hypophyseal Growth Hormone, Nature and Actions, Smith, R. W., Gaebler, O. H., and Long, C. N. H., Ed., New York, N.Y., McGraw-Hill, pp 25–58.

Gráf, L., and Li, C. H. (1974a), *Biochem. Biophys. Res. Commun. 56,* 168.

Gráf, L., and Li, C. H. (1974b), *Biochemistry 13,* 5408.

Gráf, L., Barat, E., Borvendég, J., Hermann, I., and Pattny, A. (1976), *Eur. J. Biochem. 64,* 323.

Gray, W. R., and Hartley, B. S. (1963), *Biochem. J. 89,* 59P.

Hara, K., and Sonenberg, M. (1977), *Biochim. Biophys. Acta 492,* 95.

Levine, L. S., Sonenberg, M., and New, M. I. (1973), *J. Clin. Endocrin. Metab. 37,* 607.

Lewis, U. J., Singh, R. N. P., Lindsay, T. T., Seavey, B. K., and Lambert, T. H. (1973), in Advances in Human Growth Hormone Research, Raiti, S., Ed., Washington, D.C., DHEW Publication No. (NIH) 74-612, p 349.

Lewis, U. J., Pence, S. J., Singh, R. N. P., and Van der Laan, W. P. (1975), *Biochem. Biophys. Res. Commun. 67,* 617.

Li, C. H., and Bewley, T. A. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1476.

Li, C. H., and Gráf, L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1197.

Li, C. H., Hayashida, T., Doneen, B. A., and Rao, J. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3463.

Li, C. H., Bewley, T. A., Blake, J., and Hayashida, T. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 1016.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Nadler, A. C., Sonenberg, M., New, M. I., and Free, C. A. (1967), *Metab., Clin. Exp. 16,* 830.

Ornstein, L. (1964), *Ann. N.Y. Acad. Sci. 121,* 321.

Reagan, C. R., Mills, J. B., Kostyo, J. L., and Wilhelmi, A. E.

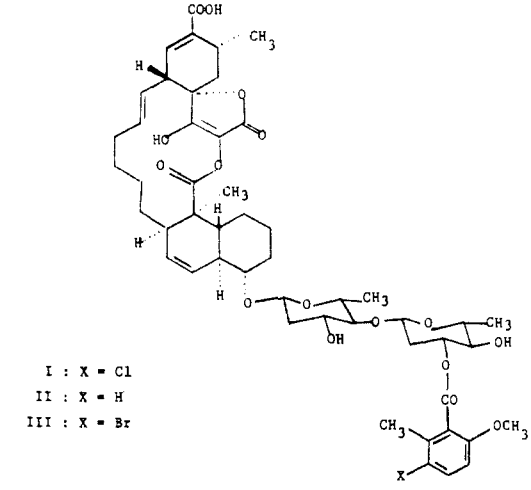
(1975a), *Endocrinology 96,* 625.
Reagan, C. R., Mills, J. B., Kostyo, J. L., and Wilhelmi, A. E. (1975b), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1684.
Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195,* 281.
Santomé, J. A., Dellacha, J. M., Paladini, A. C., Pence, C., Biscoglio, M. J., Daurat, S. T., Poskeno, E., and Wolfenstein, C. E. M. (1973), *Eur. J. Biochem. 37,* 164.
Shapiro, A. L., Vinuela, E., and Maizel, Jr., J. V. (1967), *Biochem. Biophys. Res. Commun. 28,* 815.
Singh, R. N. P., Seavey, B. K., Rice, V. P., Lindsay, T. T., and Lewis, U. J. (1974), *Endocrinology 94,* 883.
Sonenberg, M. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1051.
Sonenberg, M., and Beychok, S. (1971), *Biochim. Biophys. Acta 229,* 88.
Sonenberg, M., Free, C. A., Dellacha, J. M., Bonadonna, G., Haymowitz, A., and Nadler, A. C. (1965), *Metab., Clin. Exp. 14,* 1189.
Sonenberg, M., Kikutani, M., Free, C. A., Nadler, A. C., and Dellacha, J. M. (1968), *Ann. N.Y. Acad. Sci. 148,* 532.
Sonenberg, M., Yamasaki, N., Kikutani, M., Swislocki, N. I., Levine, L., and New, M. I., (1972), *Excerpta Med. Found. Int. Congr. Ser., No. 244,* 75.
Sonenberg, M., Levine, L. S., New, M. I., and Saxena, B. B. (1973), Advances in Human Growth Hormone Research, Washington, D.C., DHEW Publication No. (NIH) 74-612, p 467.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Swislocki, N. I., Sonenberg, M., and Yamasaki, N. (1970), *Endocrinology 87,* 900.
Wallis, M. (1973), *FEBS Lett. 35,* 11.
Wang, S. S., Yang, C. C., Kulesha, I. D., Sonenberg, M., and Merrifield, R. B. (1974), *Int. J. Peptide Protein Res. 6,* 103.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Woods, K. R., and Wang, K.-T. (1967), *Biochim. Biophys. Acta 133,* 369.
Yamasaki, N., and Kangawa, K. (1973), *Agric. Biol. Chem. (Tokyo)* 37, 2637.
Yamasaki, N., Kikutani, M., and Sonenberg, M. (1970), *Biochemistry 9,* 1107.
Yamasaki, N., Kangawa, K., Kobayashi, S., Kikutani, M., and Sonenberg, M. (1972), *J. Biol. Chem. 247,* 3874.
Yamasaki, N., Simanaka, J., and Sonenberg, M. (1975), *J. Biol. Chem. 250,* 2510.

Biosynthesis of the Macrolide Antibiotic Chlorothricin: Basic Building Blocks†

Rainer Holzbach, Hermann Pape,‡ Derek Hook, Ernest F. Kreutzer, Ching-jer Chang, and Heinz G. Floss*,§

ABSTRACT: The biosynthesis of chlorothricin (I), a macrolide antibiotic isolated from *Streptomyces antibioticus* Tü 99, has been studied by feeding experiments with ^{14}C- and ^{3}H-labeled precursors. Acetate and propionate, but not methionine and mevalonate, were incorporated into the macrocyclic aglycone of the antibiotic. Glucose and the various carbon atoms of tyrosine, except the carboxyl carbon, also contributed label to the aglycone. Glucose also seems to be a specific precursor of the 2-deoxyrhamnose moiety, probably via a process involving a hydrogen shift from C-4 to C-6 of the hexose. The substituted 6-methylsalicylic acid moiety seems to be derived from acetate and one *O*-methyl group provided by methionine; shikimic acid is not incorporated.

Chlorothricin (I) is a novel macrolide antibiotic which was first isolated from *Streptomyces antibioticus* strain Tü 99 (Keller-Schierlein et al., 1969a). Its antibiotic activity against gram-positive bacteria on synthetic, but not on complex media, is due to an inhibition of the anaplerotic CO_2 fixation catalyzed by pyruvate carboxylase (Schindler & Zähner, 1972). Further mode of action studies have also shown an interaction between chlorothricin and the membrane phospholipids of *Bacillus subtilis* (Pache & Chapman, 1972). In its binding to pyruvate carboxylase from *B. stearothermophilus,* chlorothricin antagonizes the activating effect of acetyl-coenzyme A on this enzyme (Schindler & Zähner, 1973). Different inhibition



† From the Institut für Biologie, Universität Tübingen, Tübingen, West Germany, and the Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, Indiana 47907. *Received July 1, 1977; revised manuscript received November 10, 1977.* This work was supported by U.S. Public Health Service Research Grant AI 11728 from the National Institute for Allergy and Infectious Diseases.

‡ Present address: Institut für Mikrobiologie, Universität Münster, Münster, West Germany.

§ Address correspondence to this author at Purdue University.

0006-2960/78/0417-0556$01.00/0