Two Contiguous Thrombin Fragments of Human Somatotropin Form a Functionally Active Recombinant, but the Two Homologous Fragments from Sheep Hormone Do Not†

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ABSTRACT: Two thrombin fragments of reduced–carbamidomethylated human somatotropin representing the full primary structure of the native hormone (residues 1–134 and 135–191) have been found to form a recombinant molecule with properties similar to those of reduced–carbamidomethylated human somatotropin as shown by circular dichroism spectroscopy, two receptor-binding assays, and radioimmunoassay. In contrast, the homologous thrombin fragments of reduced–carbamidomethylated sheep hormone (residues 1–133 and 134–191) do not undergo recombination. Furthermore, neither the reduced–alkylated nor the reduced and nonalkylated C-terminal thrombin fragment of sheep hormone is able to interact with the reduced–carbamidomethylated N-terminal thrombin fragment of human hormone, under conditions which favor the recombination of the two human somatotropin fragments.

It is now generally accepted that the biologically active conformation of a protein is largely determined by noncovalent intramolecular forces originating from its amino acid sequence. Richards & Vithayathil (1959) discovered that the two enzymatically inactive, 20 and 104 amino acid residue subtilisin fragments of bovine pancreatic ribonuclease A noncovalently interact each other to give a fully active complex conformationally indistinguishable from intact enzyme. During the last two decades, the biological activity and intact conformation of several other proteins, such as staphylococcal nuclease (Taniuchi et al., 1967), human somatotropin (HGH)[1] (Li & Bewley, 1976), cytochrome *c* (Harris & Offord, 1977), bacillus amyloliquefacines ribonuclease (Hartley, 1977), and sheep prolactin (Birk & Li, 1978), could be restored by complementation of their biologically inactive or slightly active fragments obtained by limited proteolytic cleavages.

In the case of HGH the two natural fragments (residues 1–134 and 141–191) of the complementing system were derived by plasmin digestion and reduction–carbamidomethylation of the hormone (Li & Gráf, 1974). The structural requirements of this recombination reaction have been extensively studied by complementing the large N-terminal plasmin fragment of HGH (residues 1–134) with different synthetic analogues of the C-terminal plasmin fragment (Li et al., 1977, 1978; Li & Blake, 1979), and it has been concluded that even considerable structural changes of the C-terminal fragment, like its shortening to residues 150–191 (Li & Blake, 1979), cause no significant change in the complementation reaction. On the other hand, no recombinant molecule containing the full primary structure of native HGH has been prepared so far. The restrictive cleavage of HGH by thrombin at Arg^{134}–Thr^{135} (see Figure 1) as first observed by Gráf et al. (1976) and recently confirmed by Mills et al. (1980) offered itself to prepare the two contiguous HGH fragments, residues 1–134 and 135–191, for recombination studies. In addition, thrombin has been found to split selectively the homologous peptide bond, Arg^{133}–Ala^{134} of SGH (Gráf et al., 1976), suggesting the extension of our fragment complementation attempts to another species homologue of somatotropin. Results of these investigations are described herein.[2]

Materials and Methods

Preparation of Thrombin Fragments from HGH and SGH. HGH and SGH were prepared as described previously [Li et al. (1962) and Papkoff & Li (1958), respectively]. Totally reduced and carbamidomethylated HGH was prepared according to Bewley et al. (1969). Bovine thrombin (428 NIH units/mg of protein) was obtained from Sigma Chemical Co. HGH was digested with thrombin in 0.05 M NH_4HCO_3 of pH 7.8 with an enzyme to substrate ratio of 1:10 (w/w) at 37 °C for 35 h. For the digestion of SGH, a thrombin to hormone ratio of 1:50 (w/w) was used, and the incubation was performed in the same buffer at 37 °C for 8 h. The thrombin digests were lyophilized, and the reduction of the disulfide bonds was carried out with a 20-fold molar excess of dithiothreitol over the cystine content, in 0.05 M NH_4HCO_3 of pH 8.3 containing 8 M urea, under N_2 for 1 h. Alkylation was carried out under the same conditions by adding either α-iodoacetamide or sodium α-iodoacetate (both preparations were obtained from Calbiochem and recrystallized before use) in 20-fold molar excess to dithiothreitol. Excess reagents and urea were removed from the mixtures by chromatography on Sephadex G-10 in 0.05 M NH_4HCO_3 of pH 8.7. After lyophilization the reduced–alkylated protein mixtures were separated on a Sephadex G-75 column (2.5 × 120 cm) in 10% acetic acid. In one experiment, the dithiothreitol-treated thrombin digest of SGH was directly applied to the same

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‖ Recipient of a scholarship from the Northern California Chapter of the Achievement Rewards for College Scientists Foundation.

[1] Abbreviations used: HGH, human somatotropin; SGH, sheep somatotropin; BGH, bovine somatotropin; HCS, human choriomammotropin; $NaDodSO_4$, sodium dodecyl sulfate; Cys(Cam), (carbamidomethyl)cysteine; CD, circular dichroism.

[2] A preliminary account of some portions of our recombination studies on the two contiguous HGH fragments has been reported (Gráf et al., 1981).

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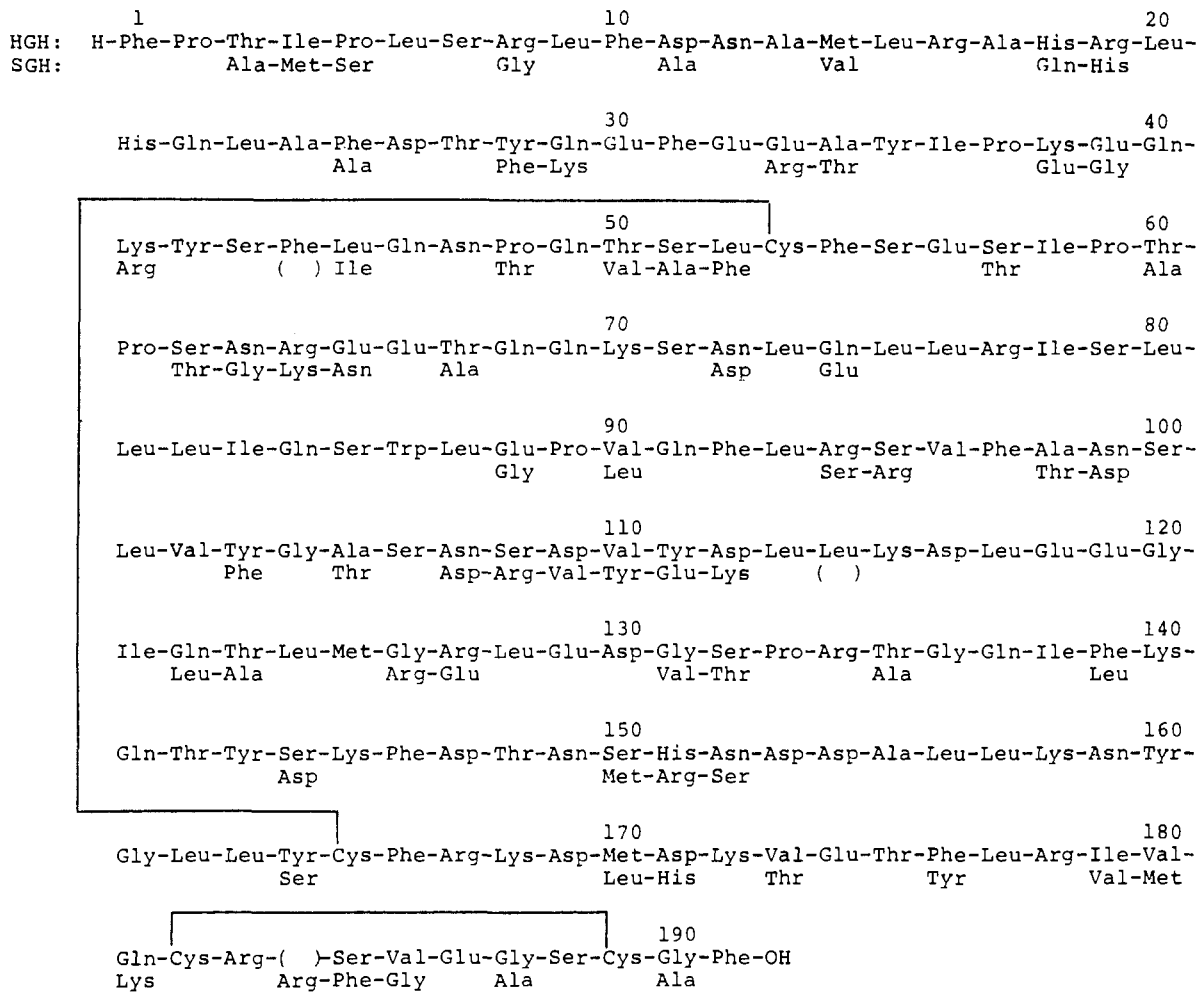

FIGURE 1: Primary structures of HGH (Li, 1972) and SGH (Li et al., 1973).

Sephadex G-75 column that was, however, deoxygenated by passing through 20 mg of dithiothreitol before chromatography using a 10% acetic acid eluant purged with N_2.

Complementation Experiments. Three different procedures were applied for the complementation of the thrombin fragments of HGH and SGH.

(1) This method is essentially the same routinely used in this laboratory (Li & Bewley, 1976; Li & Blake, 1979). Briefly, 5 mg of the N-terminal fragment was dissolved in 1.8 mL of 0.2 M NH_4HCO_3 of pH 8.4 containing 2% 1-butanol. The C-terminal growth hormone fragment (2 mg) was dissolved in 0.2 mL of 0.05 M NH_4OH, and the two solutions were mixed to give a turbid solution that was stored at 2–4 °C for 6 days. After removal of the insoluble material, the clear supernatant was chromatographed on a Sephadex G-100 column (1.4 × 60 cm) in 0.01 M NH_4HCO_3 of pH 8.4.

(2) Five milligrams of the N-terminal and 2 mg of the C-terminal growth hormone fragments were dissolved in 2 mL of 2% acetic acid containing 2% 1-butanol to give a clear solution of pH 3.5. This solution was stored at 2–4 °C for 6 days and then directly applied to a Sephadex G-100 column of identical size with that used in procedure 1 but equilibrated with 0.05 M acetic acid instead of 0.01 M NH_4HCO_3. In an attempt to complement the reduced–carbamidomethylated N-terminal thrombin fragment of HGH with the reduced but nonalkylated C-terminal fragment of SGH, the same procedure was used, except that the complementation mixture also contained 1 mg of dithiothreitol to keep the latter fragment in reduced form. In this experiment, the separation of the reaction mixture was carried out on the same Sephadex G-100 column in 0.05 M acetic acid deoxygenated as described above. $NaDodSO_4$ gel electrophoresis, in the absence of reducing agents, indicated that the nonalkylated C-terminal fragment of SGH did not undergo polymerization through intermolecular disulfides during the separation of the complementation mixture.

(3) The same amounts of peptides as in procedures 1 and 2 were dissolved as in procedure 2. Subsequently, the pH of the acid solution was adjusted to 8.5 by slowly adding concentrated NH_4OH, with constant stirring. Precipitate was formed during this procedure. The mixture was stored as described above, and after the removal of the insoluble material, the clear supernatant was purified by chromatography on the same column used for procedure 1.

Peptide Analytical Techniques. Sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis according to Swank & Munkres (1971) was used to check the extent of thrombin digestion of growth hormones and also for the identification of the isolated fragments and recombinants. When disulfide bond containing substances were run on the $NaDodSO_4$ gel, the samples had been reduced in the presence of 2% dithiothreitol (pH 7) at 37 °C for 1 h prior to application to the gel; reduced–alkylated derivatives were applied to the gel without reducing agents.

Digestion with carboxypeptidases A and B (Worthington) was performed in 0.1 M $NaHCO_3$ with an enzyme to substrate ratio of 1:10 (w/w) at 37 °C for 6 h. Amino acid compositions of the carboxypeptidase and acid (6 M HCl; 110 °C; 22 h) hydrolysates of the peptides were determined in an automatic amino acid analyzer (Model 119A, Beckman Instruments) by

Table I: Amino Acid Compositions[a] of the Thrombin Fragments of Reduced-Carbamidomethylated HGH (HN and HC) and SGH (SN and SC; see Figures 2 and 3)

amino acid	HN	HGH-(1-134)[b]	HC	HGH-(135-191)[b]	SN	SGH-(1-133)[c]	SC	SGH-(134-191)[c]
Cys(Cam)	0.8		2.8		1.1		2.6	
Asp	12.0	12	8.2	8	9.5	9	6.8	7
Thr	5.5	6	4.0	4	8.4	8	3.7	4
Ser	12.3	14	3.7	4	9.5	10	2.8	3
Glu	21.5	22	5.5	5	20.0	20	4.3	4
Pro	7.6	8			6.9	6	0.1	
$^1/_2$-Cys		1		3		1		3
Gly	4.2	4	4.2	4	6.4	6	3.1	3
Ala	5.9	6	1.1	1	11.7	12	3.2	3
Val	4.1	4	3.1	3	6.4	6	1.1	1
Met	1.9	2	1.0	1	2.1	2	1.8	2
Ile	5.4	6	1.8	2	5.5	6	1.2	1
Leu	20.4	21	5.4	5	19.4	20	6.8	7
Tyr	4.7	5	3.0	3	3.0	3	2.7	3
Phe	8.0	8	5.0	5	9.4	10	2.8	3
His	1.9	2	0.9	1	1.9	2	0.9	1
Lys	4.0	4	4.9	5	6.2	6	5.0	5
Arg	8.0	8	3.1	3	8.9	9	4.1	4

[a] Molar ratios. [b] Theoretical values taken from the HGH sequence (Li, 1972). [c] Theoretical values taken from the SGH sequence (Li et al., 1973).

the procedure of Spackman et al. (1958). N-Terminal residue analysis was performed by the dansyl method (Gray, 1967; Woods & Wang, 1967).

Circular Dichroism Measurements. Circular dichroism (CD) spectra were taken with a Cary 60 spectropolarimeter, equipped with a Model 6002 circular dichroism attachment. Sample concentrations ranged from 0.06 to 0.80 mg/mL, the path length was 0.1 or 1.0 cm, the temperature was maintained at 27 °C, and the dynode voltage did not exceed 600 V. All samples were dissolved in 0.1 M Tris-HCl bufer of pH 8.2. Molecular weights and mean residue weights of the peptides were determined from their respective sequences. Spectra are reported in terms of molar ellipticity in the near-UV region (above 250 nm) and mean residue ellipticity in the far-UV region (below 250 nm). α-Helix contents were estimated from the parameters reported by Bewley et al. (1969).

Protein concentration was determined spectrophotometrically with a Perkin-Elmer Model 552 spectrophotometer. A correction for light scattering was made according to the method of Leach & Scheraga (1960) from optical density values between 360 and 320 nm. An absorptivity of 0.920 for Cys(CAM)-HGH and recombinant HGH was based on the value for native HGH (Bewley et al., 1969) with a correction for loss of disulfide described by Wetlaufer (1962). Absorptivity values for the HGH fragments were estimated according to the method used by Bewley & Li (1978).

Receptor-Binding Assays and Radioimmunoassay. In receptor-binding assays membranes from late pregnant rabbit livers (Tsushima & Friesen, 1973) and mammary glands were prepared (Shin et al., 1973). Iodinated HGH and sheep prolactin were prepared by the lactoperoxidase method (Thorell & Johansson, 1971). The assays were performed at 4 °C for 20 h (HGH radioreceptor assay) or 48 h (prolactin radioreceptor assay) in a final volume of 0.5 mL containing 25 mM Tris-HCl buffer (55 mM Ca^{2+}; 0.4% BSA; pH 7.6), 50 000 cpm of iodinated hormone, 50 μg of liver membrane or 150 μg of mammary gland membrane protein, and the appropriate amounts of the test peptides, in triplicate. At the end of incubation, 3 mL of ice-cold BSA buffer was added to each tube which were centrifuged at 3000 rpm for 30 min at 4 °C. The supernatants were decanted, the tubes drained upside down, and the radioactivity was counted in a Beckman γ counter.

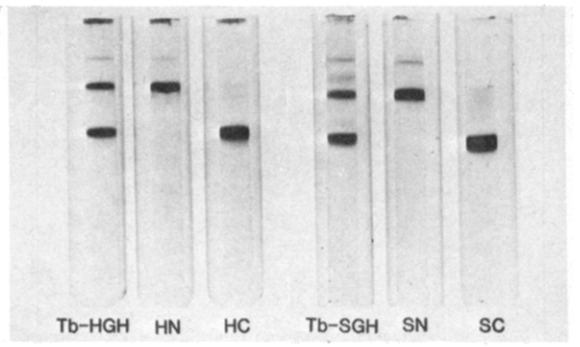

FIGURE 2: $NaDodSO_4$ gel electrophoresis of thrombin-digested HGH (Tb-HGH) and SGH (Tb-SGH) and the separated (see Figure 3) fragments of reduced-carbamidomethylated Tb-HGH (HN and HC) and Tb-SGH (SN and SC).

For radioimmunoassay, the double-antibody procedure (Schalch & Reichlin, 1966) was applied by using rabbit antiserum against HGH in a final dilution of 1:50 000. Iodination of HGH was carried out by the lactoperoxidase method. (Thorell & Johansson, 1971).

Results

Isolation and Identification of the Thrombin Fragments of HGH and SGH. $NaDodSO_4$ gel electrophoresis as performed in the presence of dithiothreitol resolved both thrombin-modified HGH and SGH into two electrophoretic components with molecular weights of about 15 000 and 7000 (Figure 2). After reduction and carbamidomethylation of the thrombin-treated hormones, these fragments were separated by chromatography on Sephadex G-75 in 10% acetic acid (Figure 3). They were shown to be homogeneous by $NaDodSO_4$ gel electrophoresis (Figure 2) and other peptide analytical methods. The dansyl procedure revealed Phe as the N-terminal residue for both HN and SN and Thr and Ala for HC and SC, respectively. Carboxypeptidase A was able to digest neither HN nor SN, whereas carboxypeptidase B generated some Arg from both fragments. By carboxypeptidase A digestion -Gly-Phe-COOH and -Ala-Phe-COOH C-terminal sequences were identified for HC and SC, respectively. These data together with the amino acid compositions of the four fragments (Table I) clearly indicate that the large thrombin

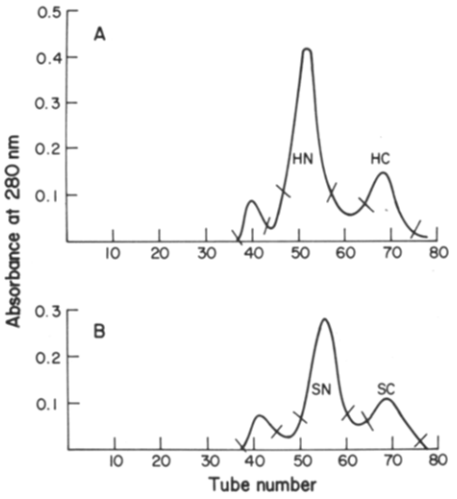

FIGURE 3: Chromatography of the reduced–carbamidomethylated thrombin digests of HGH (A) and SGH (B) on a Sephadex G-75 column in 10% acetic acid. For (A) and (B), 50- and 60-mg samples were applied to the column, respectively.

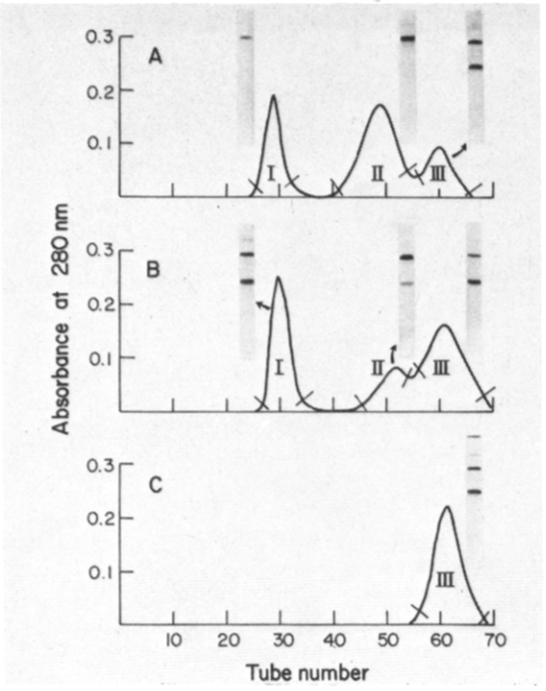

FIGURE 4: Chromatography of mixtures of the two HGH fragments on Sephadex G-100 columns in either 0.01 M NH_4HCO_3 of pH 8.4 (A; C) or 0.05 M acetic acid (B). For the preparation of the complementation mixtures, see Materials and Methods. $NaDodSO_4$ gel electrophoretic patterns of individual peaks are also shown.

fragments of HGH and SGH represent the 134- and 133-residue N-terminal sequence portions of HGH and SGH, respectively, while HC and SC are the homologous C-terminal fragments of the hormones containing residues 135–191 and 134–191, respectively (Figure 1). Thus, our data confirmed the previous report (Gräf et al., 1976) on the selective cleavage of HGH and SGH by thrombin at a single homologous peptide bond, Arg^{134}–Thr^{135} and Arg^{133}–Ala^{134} (see Figure 1), respectively.

Preparation of Noncovalent Recombinants from the HGH Fragments. The complementation mixture prepared under alkaline conditions (procedure 1 under Materials and Methods) was resolved into three peaks (Figure 4A). According to the examination of peaks I and II by amino acid analysis (data not shown) and $NaDodSO_4$ gel electrophoresis (Figure 4A),

Table II: Amino Acid Compositions[a] of HGH and a Recombinant[b] of the Two Thrombin Fragments of Reduced–Carbamidomethylated HGH

amino acid	HGH	recombinant	theoretical
Cys(Cam)		3.9	
Asp	19.7	20.5	20
Thr	9.8	10.3	10
Ser	16.9	17.3	18
Glu	26.9	26.6	27
Pro	7.9	7.8	8
$^1/_2$-Cys	3.9		4
Gly	8.5	8.6	8
Ala	7.2	7.4	7
Val	7.0	7.2	7
Met	3.0	3.1	3
Ile	7.6	7.5	8
Leu	26.2	26.3	26
Tyr	7.8	7.9	8
Phe	13.0	13.0	13
His	3.1	3.0	3
Lys	9.3	9.4	9
Arg	11.4	11.1	11

[a] Molar ratios. [b] Peak III in Figure 4C.

both were composed of different aggregated forms of the N-terminal thrombin fragment of HGH. $NaDodSO_4$ gel electrophoresis showed peak III to be a noncovalent recombinant of the N- and C-terminal thrombin fragments of HGH. Twenty-five percent of the protein of the incubation mixture appeared in this latter peak.

Chromatography of the peptide mixture stored under acid conditions (procedure 2 under Materials and Methods) gave a similar elution profile, though the relative ratio and the fragment composition of the peaks were somewhat different (Figure 4B). $NaDodSO_4$ gel electrophoresis revealed that peak I contained both fragments, peak II was mostly composed of the N-terminal fragment, and peak III had practically the same electrophoretic pattern as that of peak III in Figure 4A. This latter peak represented 36% protein of the fragment mixture.

The supernatant fraction of the peptide mixture prepared according to procedure 3 (Materials and Methods) eluted from the Sephadex G-100 column as a single peak at the position of peak III in Figure 4A,B, with a yield of 46% (Figure 4C). The fragment composition of this peak was also identical with those of peak III in Figure 4A,B. It should be noted that peak III in all three experiments had the same retention volume (V_e/V_0 = 2.01–2.03) as that of native HGH.

The amino acid compositions of peak III from the three separations (Figure 4) were about the same and indistinguishable from that of native HGH, except that the former ones contained (carbamidomethyl)cysteine [Cys(Cam)] residues instead of cystines. Table II shows the amino acid composition of peak III of Figure 4C.

Attempts To Complement the Thrombin Fragments of SGH with Each Other and with HGH Fragments. The preparation of noncovalent recombinants from the reduced–carbamidomethylated SGH fragments and also from different mixtures of SGH and HGH fragments was attempted. Complementation mixtures were prepared by procedures 1 and 2 as described under Materials and Methods. Since the results with both methods were essentially the same, only complementation experiments performed under acid conditions (procedure 2) are demonstrated (Figure 5). Figure 5A shows the chromatography profile of a mixture of the reduced–carbamidomethylated N-terminal and C-terminal SGH fragments. The $NaDodSO_4$ gel electrophoretic patterns of the

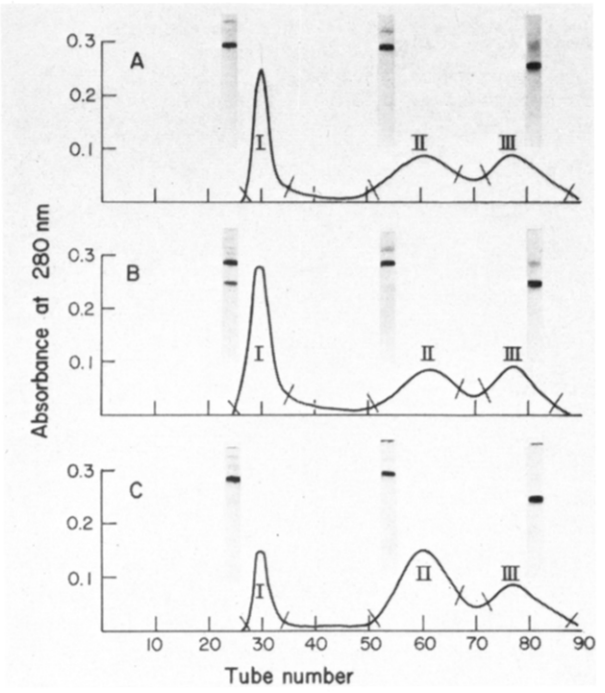

FIGURE 5: Separation of mixtures of the N- and C-terminal SGH fragments (A), the N-terminal SGH fragment and the C-terminal HGH fragment (B), and the N-terminal HGH fragment and the C-terminal SGH fragment (C) on a Sephadex G-100 column in 0.05 M acetic acid. $NaDodSO_4$ gel electrophoretic patterns of each peak are included.

three individual peaks revealed that they contained the aggregated forms of either the N-terminal thrombin fragment of SGH (peaks I and II) or the C-terminal thrombin fragment of SGH (peak III). Thus, the two fragments did not undergo noncovalent reconstitution to form a chromatographically distinct peak, like the homologous HGH fragments did in the analogous experiment (Figure 4B). Attempts to produce hybrid recombinants from the N-terminal fragment of reduced–carbamidomethylated SGH and the C-terminal fragment of reduced–carbamidomethylated HGH (Figure 5B) as well as from the N-terminal fragment of reduced–carbamidomethylated HGH and the reduced–carbamidomethylated C-terminal SGH fragment also failed (Figure 5C). Furthermore, neither the reduced–carbamidomethylated nor the reduced and nonalkylated C-terminal thrombin fragment of SGH was able to interact with the N-terminal thrombin fragment of reduced–carbamidomethylated HGH, as examined in experiments analogous to those demonstrated in Figure 5 (data not shown).

CD Spectra of the Thrombin Fragments of HGH and Their Recombinant. The cleavage of HGH with thrombin resulted in slight changes in the CD spectrum between 258 and 280 nm but caused no change in the far-UV region, with both species showing an α-helix content of 55 ± 5% (Figure 6). The isolated reduced–carbamidomethylated thrombin fragments gave significantly different CD spectra from that of HGH and thrombin-nicked HGH. The N-terminal fragment had an α-helix content of 45 ± 5%, showed a loss of positive dichroism near 290 nm, and was shifted toward positive ellipticity values between 250 and 285 nm. In addition, the fine structure between 275 and 285 nm was lost. CD spectroscopy gave no evidence for rigid secondary or tertiary structure in the C-terminal fragment (Figure 6). Recombination of the fragments restored the far-UV spectrum to its original form and α-helix content of 55 ± 5%. The near-UV region differed from thrombin-nicked HGH in the intensity of bands and

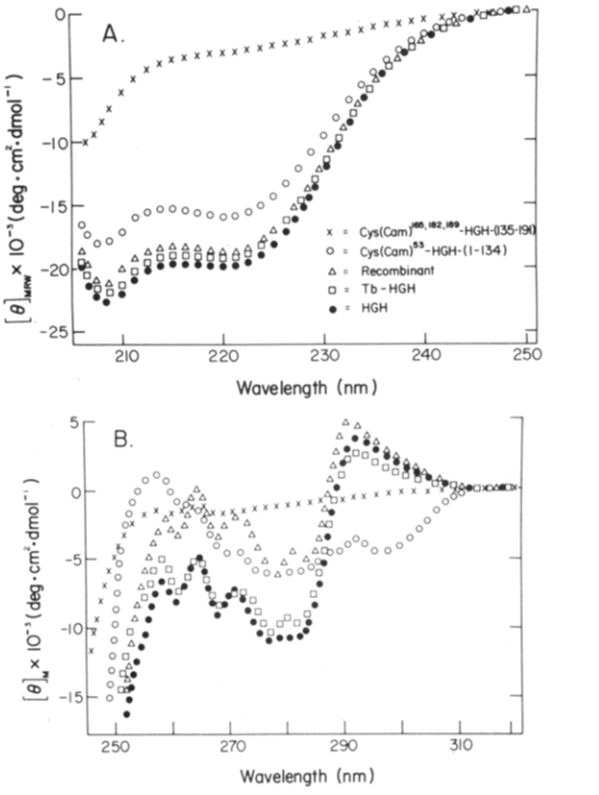

FIGURE 6: Circular dichroism spectra of HGH (●), thrombin-nicked HGH [Tb-HGH (□)], the N-terminal [HN (○)] and C-terminal thrombin fragments of reduced–carbamidomethylated HGH [HC (×)], and the recombinant from the two latter fragments [Recombinant (△)] in the region of amide bond absorption (A) and in the region of side-chain absorption (B).

Table III: Receptor-Binding Activity on Rabbit Liver Membrane of Reduced–Carbamidomethylated Thrombin Fragments of HGH and SGH and Recombinants Prepared from the HGH Fragments

peptide	ID_{50} [a]	relative potency [b]
HGH	0.19 ± 0.01	100
SGH	1.36 ± 0.04	14.00 (12.5–15.6)
Cys(Cam)^{53,165,182,189}-HGH	0.64 ± 0.02	29.74 (25.6–34.6)
Cys(Cam)^{53,164,181,189}-SGH	52.50 ± 2.54	0.36 (0.3–0.5)
Cys(Cam)^{53}-HGH-(1–134)	12.58 ± 0.46	1.42 (1.3–1.6)
Cys(Cam)^{165,182,189}-HGH-(135–191)	106.00 ± 3.74	0.17 (0.1–0.2)
Cys(Cam)^{53}-SGH-(1–133)	>1000	<0.02
Cys(Cam)^{164,181,189}-SGH-(134–191)	>2000	<0.01
recombinant A [c]	0.84 ± 0.03	21.58 (19.4–24.0)
recombinant B [d]	0.85 ± 0.03	21.33 (18.7–24.2)
recombinant C [e]	0.80 ± 0.03	22.21 (19.1–24.8)

[a] Amount of the peptide in picomoles causing 50% inhibition of binding ± standard error. [b] 95% confidence limit in parentheses. [c] Prepared by procedure 1. [d] Prepared by procedure 2. [e] Prepared by procedure 3.

showed 1–2-nm red shifts in maxima at 258 and 281 nm (Figure 6).

Receptor-Binding and Immunological Activities of the Thrombin Fragments and Their Recombinants. Receptor-binding assay on rabbit liver membrane showed that the thrombin fragments of HGH, when tested separately, had a

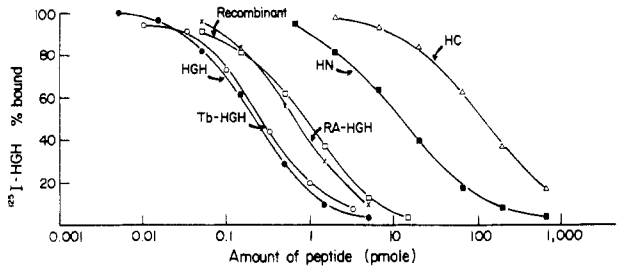

FIGURE 7: Displacement curves in the liver membrane system, plotted as percent of initial [^{125}I]HGH binding as a function of the amount (picomoles) of HGH, thrombin-digested HGH (Tb-HGH), reduced–carbamidomethylated HGH, the two thrombin fragments of HGH, and their recombinant (peak III of Figure 4C).

Table IV: Receptor-Binding Activity on Rabbit Mammary Gland Membranes of Thrombin Fragments of Reduced–Carbamidomethylated HGH and Their Recombinants

peptide	ID_{50} [a]	relative potency [b]
HGH	0.16 ± 0.01	100
SGH	79.90 ± 10.79	0.21 (0.2–0.4)
Cys(Cam)^{53,165,182,189}-HGH	0.26 ± 0.02	62.74 (54.0–73.0)
Cys(Cam)^{53}-HGH-(1–134)	33.31 ± 1.94	0.49 (0.4–0.6)
Cys(Cam)^{165,182,189}-HGH-(135–191)	>1000	<0.02
recombinant A [c]	0.30 ± 0.02	52.23 (47.3–57.7)
recombinant B [d]	0.30 ± 0.02	51.17 (45.6–57.4)
recombinant C [e]	0.30 ± 0.02	49.19 (42.1–57.5)

[a] Amount of peptide in picomoles causing 50% inhibition of binding ± standard error. [b] 95% confidence limit in parentheses. [c] Prepared by procedure 1. [d] Prepared by procedure 2. [e] Prepared by procedure 3.

slight activity (Figure 7; Table III). The binding affinities of the three recombinants of these fragments prepared by the three different procedures were practically identical with and closely similar to that of fully reduced and carbamidomethylated HGH (Table III). This latter derivative, however, was significantly less active than native HGH. The digestion of native HGH with thrombin in itself did not affect the receptor-binding affinity of the hormone (Figure 7). Interestingly and inconsistently with a previous report (Tsushima & Friesen, 1973) SGH exhibited much lower binding affinity to the liver membranes than HGH (Table III). Reduction and carbamidomethylation of SGH in the presence of 8 M urea led to an ~50-fold decrease of its receptor-binding activity, and the two thrombin fragments of this latter derivative were practically inactive.

Results of the receptor-binding assay on mammary gland membranes are in fair agreement with those of the liver membrane assay (Table IV). Reduction and carbamidomethylation of HGH also resulted in a decrease of the binding activity, though a less moderate one than in the liver membranes. The isolated thrombin fragments had a very low affinity for the mammary gland membranes, but their noncovalent interaction restored the full activity of the reduced–carbamidomethylated HGH. As expected, SGH was almost 3 orders of magnitude less active than HGH in this binding assay (Table IV).

In the HGH radioimmunoassay system, thrombin-nicked HGH was practically equipotent with intact HGH (Table V). The isolated thrombin fragments had only a slight cross-reaction with the HGH antibody, whereas the recombinant of latter fragments displayed 22.5% potency as compared with HGH (Table V). The reduction–carbamidomethylation of HGH itself appeared to decrease the immunoreactivity to 58.5% (Table V).

Table V: Immunoreactivity of Thrombin-Nicked HGH (Tb-HGH), Reduced–Carbamidomethylated HGH, the Thrombin Fragments of the Latter Derivative, and a Recombinant from Thrombin Fragments

peptide	IC_{50} [a]	relative potency [b]
HGH	0.0130 ± 0.0005	100
Tb-HGH	0.0106 ± 0.0004	121.4 (106.1–138.9)
Cys(Cam)^{53,165,182,189}-HGH	0.0164 ± 0.0005	58.5 (48.3–68.7)
Cys(Cam)^{53}-HGH-(1–134)	0.9942 ± 0.0729	0.9 (0.6–1.5)
Cys(Cam)^{165,182,189}-HGH (135–191)	62.0400 ± 3.023	0.02
recombinant C [c]	0.0575 ± 0.0025	22.5 (19.2–26.3)

[a] Amount of peptide in picomoles resulting in 50% inhibition ± standard error. [b] 95% confidence limit in parentheses. [c] Prepared by procedure 3.

Discussion

Plasmin and thrombin were shown to split the same Arg^{134}–Thr^{135} peptide bond of HGH, thus allowing the isolation of the same 134-residue N-terminal fragment from either digest after reduction and carbamidomethylation of the Cys^{53}–Cys^{165} disulfide bond of the hormone (Li & Gråf, 1974; Gråf et al., 1976). In the present study this N-terminal fragment has been shown to interact noncovalently with the 57-residue thrombin fragment of HGH, just like with the 51-residue C-terminal plasmin fragment of HGH (Li & Bewley, 1976) and several 42–57-residue synthetic analogues of this plasmin fragment (Li et al., 1977, 1981; Li & Blake, 1979). It may be noted, however, that the recombinant reported in this paper is the first one which contains the full primary structure of HGH with carbamidomethyl groups on the four cysteine residues.

It is worth mentioning that the yields of the recombinants recovered from the fragment mixtures significantly depend on the preparative procedure applied. The yield of the biologically active recombinant was only 25% when the fragments were mixed and stored under alkaline conditions (Figure 4A), and 36% (Figure 4B) and 46% (Figure 4C) if the fragments were dissolved in dilute acetic acid and stored in acid and alkaline conditions, respectively. Though these differences in the yields may not seem to be very significant, they will certainly initiate further studies to develop the opitimal conditions for the recombination reaction.

Our CD studies have shown that the recombination of the two contiguous thrombin fragments of reduced–carbamidomethylated HGH results in complete restoration of the α helix to the value observed for native HGH (Figure 6). The shift in the near-UV region between the recombinant and the native HGH (Figure 6) is similar in position and magnitude to the shift caused by reduction and alkylation of the disulfide bonds in BGH (Bewley, 1977b) and in plasmin-modified HGH (Bewley, 1977a). The receptor-binding assay data have indicated that both the reduced–carbamidomethylated HGH and the thrombin fragment recombinant exhibit somewhat less binding affinities to both the liver membrane and mammary gland membrane receptors than native HGH (Tables III and IV and Figure 7). Recently, Li et al. (1981) have reported

similar observations with natural and semisynthetic recombinants from plasmin fragments of HGH. In addition, our radioimmunoassay system clearly differentiated between HGH, reduced–carbamidomethylated HGH, and the thrombin fragment recombinant (Table V). Thus, it is apparent from both the CD and binding data of this study that although the general conformational features of HGH, reduced–carbamidomethylated HGH, and the recombinant are very similar, there are several minor conformational differences among these molecules.

Classic reduction–oxidation studies on intact bovine pancreatic ribonuclease A (Anfinsen, 1967) and subsequently several disulfide bridge containing proteins including HGH (Bewley & Li, 1970) have shown that the temporarily disrupted disulfide bonds are properly reformed under oxidizing conditions. Thus, noncovalent intramolecular interactions arising from the native amino acid sequence appear to direct the correct pairing of the disulfide bonds, and then these latter may just stabilize the native conformation of the protein (Anfinsen, 1956, 1967; Bewley & Li, 1970; Andria & Taniuchi, 1978). This view has been strongly supported by the observation that the reduction–carbamidomethylation of the two disulfide bonds in HGH had a relatively small effect on the secondary–tertiary structure and biological properties of this hormone (Bewley et al., 1969; this study) and also by the restoration of a conformation and biological activities closely similar to those of native HGH by complementation of its reduced–carbamidomethylated plasmin (Li & Bewley, 1976) and thrombin fragments as described herein.

One of the most intriguing observations in our recent study is, however, that the homologous thrombin fragments of SGH were not able to interact each other noncovalently (Figure 5). There is evidence that the Cys^{53}–Cys^{164} disulfide bond of SGH (homologous to Cys^{53}–Cys^{165} in the HGH structure; see Figure 1) is extremely resistant to the reducing conditions which result in the scission of the corresponding disulfide bond in HGH (Gráf et al., 1975) and also that the reduction–alkylation of this particular disulfide bond of SGH under denaturing conditions leads to the complete loss of both the native conformation and the biological activity of the hormone (unpublished data). Thus, this particular disulfide bridge, just like Cys^{58}–Cys^{174} in the prolactin structure (Doneen et al., 1979), appears to be more intimately involved in the maintenance of the biologically active conformation than the homologous disulfide bond of HGH. Our failure to recombine the two thrombin fragments of reduced–carbamidomethylated SGH may also be explained by this curiously essential role of Cys^{53}–Cys^{164} in maintaining the biologically important feature of the SGH conformation.

More surprisingly, we have also failed to complement the reduced–carbamidomethylated 134-residue thrombin fragment of HGH with the differently modified C-terminal thrombin fragment (residues 134–191) of SGH. The lack of apparent recombination between the HGH and SGH fragments cannot be due to the presence of the blocking groups on Cys^{164}, Cys^{181}, and Cys^{189} of the SGH fragment, since even the free cysteine-containing derivative was unable to interact with the N-terminal HGH fragment. In addition, the substitution of the same cysteines with alanines in some synthetic analogues of the coresponding HGH fragment did not seem to affect the complementation reaction between the two protions of HGH (Li et al., 1978; Li & Blake, 1979). It is also known from the previous complementation studies that neither the omission of the first 15 N-terminal residues from HGH-(135–191) (Li & Blake, 1979) or the last 4 C-terminal residues from HGH-(140–191) (Li et al., 1981) nor substitutions at positions 153 and 179 of the HGH-(141–191) segment [HCS-(141–191) (Li, 1978)] nor the replacement of Met^{170} with Nle in several synthetic analogues (Li et al., 1978, 1981; Li & Blake, 1979) prevented the recombination of these synthetic and natural analogues of the C-terminal plasmin fragment of HGH with natural HGH-(1–134).

Evidently, the C-terminal thrombin fragment of SGH (residues 134–191) is much more different from the homologous HGH fragment than any of the natural and synthetic C-terminal fragment analogues used for complementation experiments so far. Out of the nineteen species differences between the two C-terminal thrombin fragments of HGH and SGH, eight are found within residues 179–191 of the HGH sequence (Figure 1). In this context, it is interesting to note that a recent attempt to complement the natural N-terminal 134-residue fragment of HGH with a synthetic analogue representing residues 140–182 of the HGH structure has also failed (Li et al., 1981). In view of this observation, one is tempted to speculate that the lack of noncovalent interaction between the HGH-(1–134) and SGH-(134–191) fragments may be due, at least in part, to the significant sequence differences (including a one-residue insertion in the SGH structure) of the two species' homologues within residues 179–187 of HGH (Figure 1).

Acknowledgments

We thank Katherine Hines and Jonathan Roeder for the excellent technical assistance.

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Activation of Intrinsic Blood Coagulation by Ellagic Acid: Insoluble Ellagic Acid–Metal Ion Complexes Are the Activating Species†

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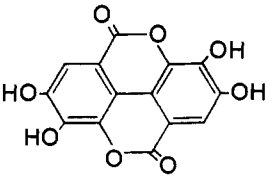
ABSTRACT: The species of ellagic acid responsible for initiating intrinsic blood coagulation has been characterized. Ellagic acid is soluble at a level of 30 ± 10 μM in pH 7.4 Tris–NaCl buffer at 22 °C. Dilution of soluble ellagic acid resulted in enhanced procoagulant and kallikrein-generating activity in normal human plasma and the appearance of a new absorbance spectrum. These effects were prevented by 1 mM EDTA, and the new species could be removed by centrifugation. Addition of stoichiometric Cu^{2+} to Millipore-filtered soluble ellagic acid generated an absorbance spectrum similar to that caused by dilution in the absence of EDTA, as well as procoagulant and kallikrein-generating activities. Although no turbidity was visible and the spectral properties did not indicate extensive light scattering, centrifugation resulted in loss of the absorbance spectrum and kallikrein-generating activity. Titration of ellagic acid with Cu^{2+} showed that the absorbance spectral change was correlated with the formation of insoluble complexes and increased procoagulant activity. Zn^{2+}, Co^{2+}, and Fe^{3+} caused similar spectral changes and the formation of insoluble species capable of inducing prekallikrein activation. We conclude that the procoagulant and kallikrein-generating activity of ellagic acid solutions can be ascribed to slowly settling insoluble aggregates of ellagic acid–metal ion complexes, which are formed with adventitious metal ions present in the diluting buffer. Formation of these aggregates, and the development of biological activity, could be prevented but not reversed by 1 mM EDTA. The activity of these complexes and Cu^{2+}–ellagic acid complexes was dependent on the presence of each of the four proteins of the contact activation system. Although soluble ellagic acid may bind to factor XII, this interaction does not initiate blood coagulation or prekallikrein activation, since these activities were only associated with the insoluble species.

Initiation of the intrinsic blood coagulation system occurs when normal plasma contacts a negatively charged surface. The surface-dependent conversion of factor XII to an active serine protease is the first event in the cascade of reactions leading to the formation of an insoluble fibrin clot. The participation of prekallikrein and high molecular weight kininogen in contact activation results in the generation of fibrinolytic activity and the formation of kinins (Griffin & Cochrane, 1979; Ulevitch & Cochrane, 1977).

Activation of the contact system can be achieved with a variety of negatively charged insoluble substances. The action of these substances involves the specific adsorption of factor XII and the other proteins of the contact activation system, leading to an acceleration of the proteolytic activation of factor XII, prekallikrein, and factor XI (Wiggins et al., 1977; Griffin, 1978; Griffin & Cochrane, 1976; Meier et al., 1977; Revak et al., 1977). Although most known activators are insoluble, activation has been reported to occur in solution by the polyanions heparin (Moskowitz et al., 1970), dextran sulfate (Kluft, 1978), and carrageenans (Schwartz & Kellermeyer, 1969) as well as by soluble ellagic acid (Ratnoff & Crum, 1964). Unique among these substances, ellagic acid was of particular interest since this compound has a low molecular weight and the following chemical structure:



Studies of the mechanism of human factor XII activation

† From the Division of Biochemical Research, Department of Pathology, Henry Ford Hospital, Detroit, Michigan 48202. *Received February 17, 1981.* This work was supported by National Institutes of Health Grant HL25670-01 from the Heart, Lung and Blood Institute. P.E.B. was supported by a research fellowship from the Michigan Heart Association and National Institutes of Health National Research Service Award 2F32 HL05154-03 from the Heart, Lung and Blood Institute.

0006-2960/81/0420-7258$01.25/0