Limited Proteolysis of Human Growth Hormone at Low pH: Isolation, Characterization, and Complementation of the Two Biologically Relevant Fragments 1−44 and 45−191†

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ABSTRACT: The limited proteolysis approach was used to analyze the conformational features of human growth hormone (hGH) under acidic solvent conditions (A-state). Pepsin was used as the proteolytic probe because of its poor substrate specificity and its activity at low pH. Limited proteolysis of hGH in its A-state results in a selective cleavage of the Phe44−Leu45 peptide bond, leading to the production of fragments 1−44 and 45−191. The two fragments were isolated in homogeneous form for studying their conformational properties by means of spectroscopic methods. Fragment 1−44 was shown to retain little secondary and tertiary structure at neutral pH, while fragment 45−191 independently folds into a highly helical secondary structure. In particular, we have shown that the two peptic fragments are able to associate into a stable and native-like hGH complex 1−44/45−191. Our proteolysis data indicate that in acid solution hGH adopts a partly folded state characterized by a local unfolding of the first minihelix (residues 38−47) encompassing the Phe44−Leu45 peptide bond. Of interest, hGH has both insulin-like and diabetogenic effects. Two fragments of hGH occur in vivo and exert these two opposite activities, namely, fragment 1−43 showing an insulin-potentiating effect and fragment 44−191 showing a diabetogenic activity. The results of this study suggest that the conformational changes of hGH induced by an acidic pH promote the generation of the two physiologically relevant fragments by proteolytic processing of the hormone. Although pepsin cannot be the enzyme responsible for the in vivo processing of the hormone, we propose that limited proteolysis of hGH at low pH is physiologically relevant, since the hormone is exposed to an acidic environment in the cell. This study reports for the first time the analysis of the conformational features of the two individual functional domains of hGH and of their complex.

Human growth hormone (hGH)[1] belongs to the family of pituitary polypeptide hormones and promotes the growth of body weight and length. However, hGH exerts a plethora of other biological activities and affects the metabolism of proteins, carbohydrates, and lipids (*1*, *2*). The hormone is expressed as a 22 kDa form of 191 amino acid residues (Figure 1), which accounts for about 90% of the circulating hGH, as well as a 20 kDa form of 176 residues characterized by the deletion of the chain segment 32−46 (*3*, *4*). Moreover, multiple forms of hGH do exist in the pituitary, including phosphorylated, glycosylated, acetylated, deamidated, and, in particular, several proteolytically modified forms (*5*−*8*). It has been demonstrated that these various alterations in the covalent structure of the polypeptide chain of hGH strongly influence its diverse biological activities. The hypothesis was advanced that hGH may require proteolytic cleavage to exert its full biological potency. Indeed, the hGH molecule appears to be a prohormone (*6*, *7*), since a variety of hGH fragments, produced in vivo or in vitro by proteolysis of the hormone (9−*18*) or prepared by chemical synthesis (*19*, *20*), have been shown to possess important biological activities. It has been reported that hGH is hydrolyzed by plasmin and thrombin at the level of the chain segment 134−149 and this gives rise to nicked forms of hGH still exhibiting growth-promoting activity (*5*, *21*). There is recent evidence that the N-terminal fragment 1−134 generated by thrombin displays antiangiogenic activity, whereas the native hormone is angiogenic (*22*). Therefore, limited proteolysis of hGH appears to play an important role at the cellular level.

The 191-residue form of hGH is folded in an antiparallel four-helix bundle protein and contains the two disulfide bridges Cys53−Cys165 and Cys182−Cys189 (*23*, *24*). At acidic pH, hGH adopts a conformational state (A-state),

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[1] Abbreviations: hGH, human growth hormone; A-state, acid-induced conformational state of hGH; CD, circular dichroism; ESI, electrospray ionization; E/S, enzyme to substrate ratio; Gdn·HCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; MG, molten globule; PAGE, polyacrylamide gel electrophoresis; kDa, kilodalton; RP, reverse phase; MS, mass spectrometry; UV, ultraviolet; NMR, nuclear magnetic resonance; [*q*], mean residue ellipticity; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; 1−44, N-terminal fragment of hGH; 45−191, C-terminal fragment of hGH cross-linked by two disulfide bridges (Cys53−Cys165 and Cys182−Cys189); 1−44/45−191, noncovalent hGH complex given by an equimolar mixture of fragments 1−44 and 45−191; nicked hGH, the polypeptide chain of 191 residues of hGH with the single peptide bond Phe44−Leu45 cleaved; Tris, tris(hydroxymethyl)aminomethane.

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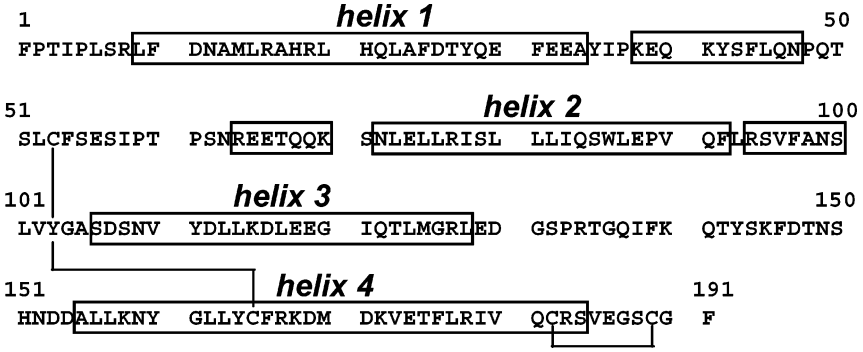

FIGURE 1: Amino acid sequence of hGH. Amino acid residues are given in one letter code, and residues in α-helical segments are boxed. The four major helices of hGH are indicated. The location of the two disulfide bonds Cys53−Cys165 and Cys182−Cys189 is shown by a solid line.

which appears to be quite different from those formed by the homologous porcine and bovine hormones. In fact, whereas these last proteins exhibit a reduction of secondary structure and little or no tertiary structure (*25*−*27*), hGH in acid solution appears to retain a structure quite similar to that of the native state (*28*−*31*). Recently, NMR studies confirmed that at low pH hGH retains a native-like four-helix bundle, whereas regions of unfolding are mainly located at the level of the chain loops connecting the helices (*31*). The molecular features of the A-state of hGH are of clear interest, if one considers that hGH is stored in the pituitary gland in granules at an acidic pH (*32*−*35*) and, after binding to its receptor, is addressed to the broad-specificity proteases of lysosomes (*36*, *37*).

The aim of the present work is to unravel the conformational changes induced by an acidic pH on the native structure of hGH by using the limited proteolysis approach (*38*−*43*). In previous studies, we have demonstrated the utility of using limited proteolysis experiments for analyzing the conformational features of the partly folded or molten globule (MG) states of several model proteins (*40*, *41*). Despite the dynamic nature and conformational heterogeneity of MGs, limited proteolysis experiments under controlled conditions allowed us to identify the sites or regions of the polypeptide chain most prone to proteolysis and thus the sites of high flexibility or local unfolding (*38*−*40*). The results obtained by using proteolytic probes were in agreement with those reached by using classical spectroscopic methods, in particular, NMR and hydrogen/deuterium exchange measurements (*40*, *42*). Here, we show that limited proteolysis of hGH by pepsin at acidic pH occurs at the level of the Phe44−Leu45 peptide bond encompassed by the first minihelix of hGH, thus implying that this chain region is highly flexible or unfolded in the A-state of the hormone. Proteolysis leads to the production of fragments 1−44 and 45−191, which were isolated and studied for their conformational properties in solution at neutral pH. The interest of the fragments herewith investigated resides in the fact that they are very similar to the hGH fragments 1−43 and 44−191 that occur in vivo and display specific activities on carbohydrate metabolism (*14*−*18*). Moreover, here we show that an equimolar mixture of the two fragments can form a nicked hGH (1−44/45−191) with a native-like conformation. The complementing fragments of hGH herewith described can be used as a suitable experimental system for studying aspects of structure, function, and folding of this very important hormone.[2]

MATERIALS AND METHODS

Materials. Recombinant human growth hormone was produced in *Bacillus subtilis* (*44*) and stored as a lyophilized sample at 4 °C. Porcine pepsin was obtained from Sigma (St. Louis, MO). Cyanogen bromide (BrCN), dithiothreitol (DTT), acetonitrile, and trifluoroacetic acid (TFA) were purchased from Fluka (Buchs, Switzerland), and reagents and solvents used for SDS−PAGE were from Bio-Rad (Richmond, CA). All other chemicals were of analytical grade and were obtained from Sigma or Fluka. Water used to prepare buffers was purified by a Millipore Milli-Q Plus system.

Proteolysis Experiments. Proteolysis of hGH by pepsin at pH 2.0 was conducted at 25 °C by dissolving the protein (∼1.3 mg/mL) in 10 mM HCl/0.15 M NaCl, pH 2.0, and by adding the protease at an enzyme/substrate (E/S) ratio of 1/300 (by weight). At intervals aliquots were taken from the reaction mixture, and the proteolysis was quenched by addition of a solution of 3% aqueous ammonia. Limited proteolysis at pH 4.0 was performed on a solution of hGH (0.25 mg/mL) in 10 mM sodium citrate/0.15 M NaCl, pH 4.0, at 4 °C with an E/S ratio of 1/10 (by weight). Samples were concentrated using the Speed-Vac system (Savant) and then analyzed by SDS−PAGE and RP-HPLC.

Electrophoresis and Chromatography. SDS−PAGE was carried out in a vertical slab gel apparatus (Mini-Protean II, Bio-Rad) using the Tricine buffer system (*45*) under reducing as well as nonreducing conditions. The gels were stained with Coomassie brilliant blue R-250. A sample of a partial BrCN cleavage of hGH at the level of the three methionine residues at positions 14, 125, and 170 of the 191-residue chain of the protein was used as the standard for molecular masses. The resulting fragments are made up of residues 15−191 (20.6 kDa), 1−170 (19.8 kDa), 15−170 (18.2 kDa), 1−125 (14.6 kDa), 15−125 (13 kDa), 126−191 (7.6 kDa), 126−170 (5.3 kDa), 1−14 (1.6 kDa), and 171−191 (2.4 kDa). Fragments 15−191 and 1−170 are not well separated from hGH in the SDS−PAGE gel, whereas the small fragments 1−14 and 171−191 are only weakly stained.

Reverse-phase (RP) high-performance liquid chromatography (HPLC) analysis of the limited proteolysis mixture obtained at pH 2.0 was performed on a Vydac C_4 column

[2] A preliminary report of this study was presented at the 5th European Symposium of the Protein Society, March 29−April 2, 2003, Florence, Italy (*75*).

(4.6 × 150 mm) (The Separations Group, Hesperia, CA). Elution was carried out at a flow rate of 0.8 mL/min with a linear gradient of water and acetonitrile containing 0.1% and 0.085% TFA, respectively, from 10% of acetonitrile to 45% in 10 min and from 45% to 60% in 15 min. For the RP-HPLC analysis of the mixture of proteolysis conducted at pH 4.0, a Vydac C_{18} column (4.6 × 150 mm) was used. Elution was at a flow rate of 0.8 mL/min with a linear gradient of water and acetonitrile containing the same percent of TFA as above. The gradient was from 20% of acetonitrile to 50% in 3 min and from 50% to 62% in 24 min. The effluent from the column was monitored by measuring the absorbance at 226 nm.

Analytical gel filtration chromatography was carried out by loading samples of hGH, fragment 1−44, fragment 45−191, and complex 1−44/45−191 onto a Superdex-75 column (type HR 10/30, 1 × 30 cm; Pharmacia) equilibrated and eluted with 10 mM Tris·HCl/0.15 M NaCl, pH 7.5, at a flow rate of 0.4 mL/min. The absorbance of the effluent from the column was recorded at 226 nm. The column was calibrated using a protein mixture kit of low molecular mass (Pharmacia).

N-Terminal Sequencing and Mass Determination. Automated Edman degradation was performed with an Applied Biosystems (Foster City, CA) model 477A protein sequencer, equipped with an on-line analyzer (model 120A) of phenylthiohydantoin derivatives of amino acids. Mass determinations were obtained with a Mariner (System 5220) mass spectrometer (Sciex, Thornill, Ontario, Canada) equipped with an electrospray ionization (ESI) source and a time-of-flight analyzer. Spectra were deconvoluted by the software Data Explorer provided by Applied Biosystems.

Ultraviolet Absorption. Protein and fragment concentrations were determined using a Perkin-Elmer Lambda-20 spectrophotometer (Perkin-Elmer, Forster City, CA). The concentrations of solutions of hGH, fragment 1−44, fragment 45−191, and pepsin were determined from their absorbance at 280 nm according to the method of Gill and von Hippel (*46*). Second-derivative UV absorption spectra of hGH and of the complex 1−44/45−191 in 10 mM Tris·HCl/0.15 M NaCl, pH 7.5, were taken at 25 °C on protein samples obtained after gel filtration chromatography. The average degree of exposure (α) of tyrosine residues to solvent was calculated according to Ragone et al. (*47*). Spectra taken in 6 M Gdn·HCl in Tris buffer, pH 7.5, served as the reference for the full exposure of Tyr residues.

Circular Dichroism. Circular dichroism (CD) spectra were recorded at 25 °C on a Jasco J-710 spectropolarimeter (Tokyo, Japan) equipped with a thermostatically controlled cell holder. The instrument was calibrated with *d*-(+)-10-camphorsulfonic acid. Far- and near-UV CD spectra were recorded at 25 °C at a protein concentration of 0.05−0.1 and 0.3−0.5 mg/mL, respectively, using quartz cuvettes (Hellma, Forest Hills, NY) with a path length of 0.1 cm and of 0.5 cm for the far- and near-UV CD measurements, respectively. The results were expressed as mean residue ellipticity $[\theta]$ (deg·cm^2·dmol^{-1}) calculated from the formula $[\theta] = (\theta_{obs}/10)(MRW/lc)$, where θ_{obs} is the observed ellipticity at a given wavelength, MRW is the mean residue molecular weight (molecular mass of the protein or fragment divided by the number of amino acid residues), l the optical path length in cm, and c the protein concentration in g·mL^{-1}. Quantitative analysis of far-UV CD spectra in terms of helical secondary structure was conducted using the method of Chen et al. (*48*).

Fluorescence. Fluorescence emission spectra were recorded at 25 °C using a Perkin-Elmer model LS-50 spectrofluorometer, utilizing a cuvette of 1 cm path length. The emission spectra were obtained after excitation at 280 or 295 nm and recording the emission from 285 or 300 nm to 450 nm. The spectra of hGH, fragment 1−44, fragment 45−191, and complex 1−44/45−191 were recorded in 10 mM Tris·HCl/0.15 M NaCl, pH 7.5, or in 6 M Gdn·HCl in Tris buffer.

RESULTS

Spectroscopic Characterization of hGH at pH 2.0 and 4.0. The effect of pH on the conformation of hGH has been previously analyzed (*30*, *49*, *50*). It has been reported that in acid solution hGH retains most of its secondary structure, while showing a less rigid tertiary structure. However, it has been shown also that the helical content of hGH at low pH is reduced with respect to that observed at neutral pH (*50*). Here, we have reexamined the conformational properties of hGH under the specific solvent conditions used for the limited proteolysis experiments, i.e., at pH 2.0, 4.0, and 7.5. Far-UV CD spectra of hGH at acidic pH (Figure 2A) show some 8% reduction of the α-helical content at both pH 2.0 and pH 4.0 with respect to that observed at neutral pH. In the near-UV region (Figure 2B) the wavelength positions of the dichroic maxima and minima are retained, while the intensity of the Trp band at 293 nm, in particular at pH 2.0, is strongly reduced, suggesting that in acid solution the single Trp86 appears to experience a more flexible environment (*51*). The intensity of the band at 277−283 nm, attributed to the Tyr residues (*51*, *52*), is retained in acid solution, but the red shift of the minimum is an indication that the environment of Tyr residues is somewhat perturbed. Finally, the fine structure in the 258−270 nm region, associated with Phe residues, suggests that many tertiary interactions are retained in hGH exposed at both pH 2.0 and pH 4.0 (*51*).

The fluorescence emission spectra of hGH taken at pH 2.0 and 4.0 (Figure 2C) are essentially identical, but in acid solution the intensity is reduced by ∼30% with respect to that observed at neutral pH. Of note, the maximum of the emission spectra is not changed on shifting the pH from neutral to acid solution, implying that the polarity of the microenvironment near Trp86 remains essentially unchanged (*53*, *54*). On the other hand, the denaturation of the protein in 6 M Gdn·HCl (Figure 2C) leads to a red shift of the maximum of emission from about 343 to 353 nm, thus indicating full exposure of the Trp86 residue.

Limited Proteolysis of hGH. The proteolysis of hGH by pepsin at pH 2.0 was performed in 0.01 M HCl/0.15 M NaCl at 25 °C using an E/S ratio of 1/300 (by weight). The kinetics of proteolysis was followed by SDS−PAGE gel electrophoresis (Figure 3A) and RP-HPLC (Figure 3B). The SDS−PAGE analysis of the proteolysis reaction at pH 2.0 (Figure 3A), under reducing conditions, reveals the formation of a protein fragment of ∼17 kDa, which is quite resistant to further digestion by pepsin. The RP-HPLC chromatogram of the reaction mixture after 15 min reaction (Figure 3B) shows two main peaks, preceded by many small peaks of earlier eluted peptide material. The analysis of the two main

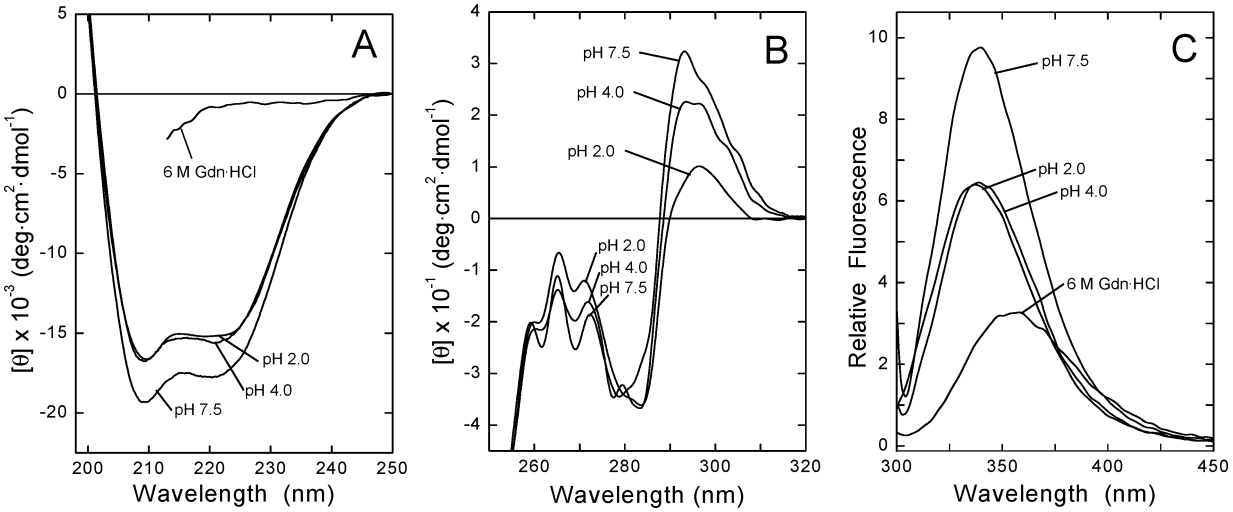

FIGURE 2: Spectroscopic analysis of hGH at pH 2.0, 4.0, and 7.5. Far-UV CD (A), near-UV CD (B), and fluorescence emission spectra (C) of hGH were measured at 25 °C, pH 7.5 (10 mM Tris·HCl/0.15 M NaCl), pH 4.0 (10 mM sodium citrate/0.15 M NaCl), pH 2.0 (10 mM HCl/0.15 M NaCl), or in 6 M Gdn·HCl in Tris buffer, pH 7.5. Fluorescence spectra were taken by exciting samples at 295 nm.

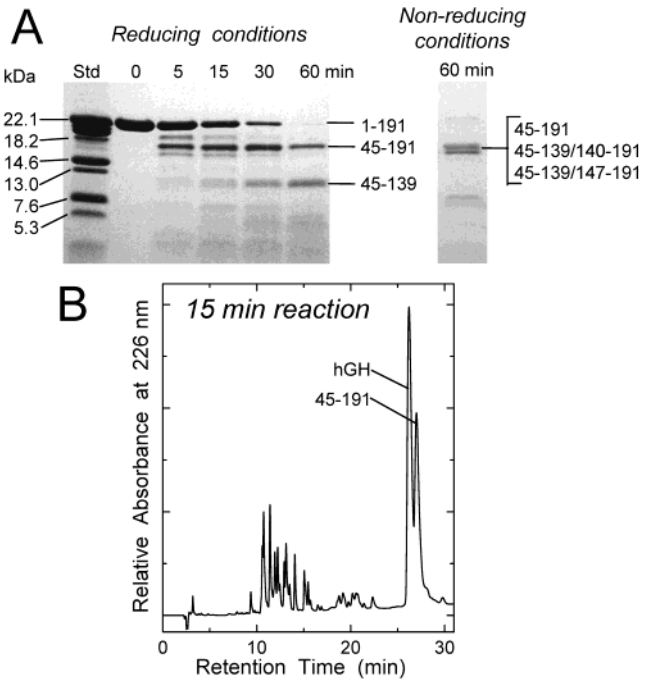

FIGURE 3: Limited proteolysis of hGH at pH 2.0. Proteolysis was conducted at 25 °C (E/S 1/300, by weight) in 10 mM HCl/0.15 M NaCl, pH 2.0. (A) Time course analysis by SDS−PAGE of the peptic digestion of hGH. The BrCN fragments of hGH (Std; see Materials and Methods) served as molecular markers, and their masses in kilodaltons are shown on the left side of the gel. The identity of the hGH fragments are indicated by the numbers shown in the right side of the gels. (B) RP-HPLC analysis of the proteolytic mixture of hGH with pepsin after 15 min reaction at 25 °C. The chromatographic peaks containing intact hGH and the proteolytic fragment 45−191 are indicated in the chromatogram. A Vydac C_4 column (4.6 × 150 mm) was employed.

peaks by N-terminal sequencing and ESI-MS provided evidence that the first one contains the intact hormone, whereas the second contains fragment 45−191 (Table 1). Therefore, the main fragment of ∼17 kDa seen in the SDS−PAGE gel is fragment 45−191, resulting from the peptic fission of the peptide bond Phe44−Leu45.

After 60 min reaction, in the SDS−PAGE gel (Figure 3A), besides the band of fragment 45−191, a major band of about 11 kDa is seen. Analysis of a protein digest obtained after 60 min reaction (not shown) reveals that, upon prolonged reaction of hGH with pepsin at pH 2.0, the protein is cleaved also at peptide bonds Phe139−Lys140 and Phe146−Asp147, besides at Phe44−Leu45. These peptic hydrolyses lead to nicked/gapped species of fragment 45−191, namely, the two-chain disulfide-cross-linked species 45−139/140−191 and 45−139/147−191. Under the reducing conditions of the SDS−PAGE gel, these nicked/gapped forms of fragment 45−191 give a band of ∼11 kDa corresponding to fragment 45−139, while the C-terminal portions of these species (residues 140−191 and 147−191) are poorly stained in the SDS−PAGE gel, likely due to their lower molecular mass. The same protein sample, when analyzed by SDS−PAGE under nonreducing conditions (i.e., without treating the sample with β-mercaptoethanol), gives a broad band of about 17 kDa, which contains fragment 45−191 and its nicked/gapped forms (Figure 3A, right). The band corresponding to fragment 45−139, seen in the gel under reducing conditions (Figure 3A, left), does not appear in the gel obtained under nonreducing conditions (Figure 3A, right),

Table 1: Molecular Masses and N-Terminal Sequences of Peptic Fragments of hGH[a]

protein or fragment species	N-terminal residues			molecular mass (Da)	
	first	second	third	found	calculated
hGH (1−191)	F	P	T	22125.5	22125.1
45−191	L	Q	N	16781.9	16780.1
1−44	F	P	T	5362.9	5363.0
1−139/140−191	F/K	P/Q	T/T[b]	22144.7	22143.1
1−139/147−191	F/D	P/T	T/N[b]	21261.0	21260.1
45−139/140−191	L/K	Q/Q	N/T[b]	16800.1	16798.0
45−139/147−191	L/D	Q/T	N/N[b]	15915.5	15915.0

[a] The fragments of hGH were obtained by limited proteolysis of the hormone with pepsin at pH 2.0 or 4.0 as described in the text. The N-terminal sequence analysis was performed on the protein fragments isolated after RP-HPLC of the proteolytic mixture. The amino acids (single-letter code) identified in the first three cycles of Edman degradation are indicated in the table, even if for some protein fragment species much longer amino acid sequences have been determined. Molecular masses were calculated from the amino acid sequence of hGH, assuming cysteine residues to be in their disulfide form. Experimental molecular masses were determined by ESI-MS. [b] Multiple N-terminal sequences were observed.

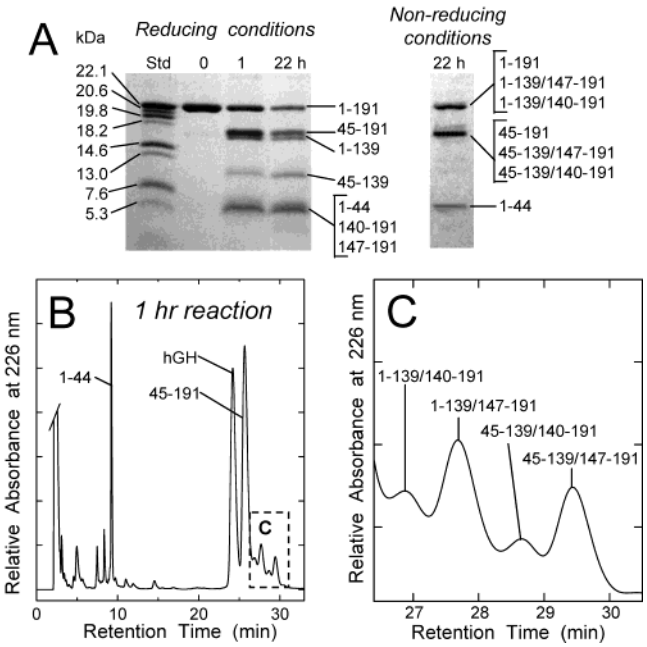

FIGURE 4: Limited proteolysis of hGH at pH 4.0. Proteolysis was conducted at 4 °C (E/S 1/10, by weight) in 10 mM sodium citrate/0.15 M NaCl, pH 4.0. (A) Time course analysis by SDS−PAGE of the peptic digestion of hGH. The BrCN fragments of hGH (Std) were used as molecular markers, and their molecular masses in kilodaltons are indicated at the left side of the gel. The identity of the hGH fragments is indicated by the numbers given at the right side of the gels. A sample of the proteolytic mixture after 22 h reaction at pH 4.0 was also analyzed under nonreducing conditions (A, right). (B) RP-HPLC analysis of the proteolytic mixture of hGH with pepsin after 1 h reaction at pH 4.0, 4 °C. The chromatographic peaks containing hGH and fragments 1−44 and 45−191 are indicated. The chromatogram in panel C is a blow-up of a section (c) of the chromatogram shown in panel B. The numbers near the chromatographic peaks refer to the identity of the nicked/truncated hGH species. A Vydac C_{18} column (4.6 × 150 mm) was employed.

since fragment 45−139 remains linked via disulfide bridges to fragments 140−191 and/or 147−191 (see Figure 1).

Limited proteolysis of hGH with pepsin was also conducted in 10 mM sodium citrate/0.15 M NaCl, pH 4.0, at 4 °C and with an E/S of 1/10 (by weight). Proteolysis experiments, conducted at 25 °C and with a lower E/S, showed the same pattern of the reaction conducted at 4 °C (data not shown). However, to produce and isolate hGH fragments (see below), the lower temperature of reaction was considered more suitable to keep them folded and so more resistant to further proteolytic degradation (*39*). Because the reaction is considerably slower at 4 °C and the activity of pepsin is lower at pH 4.0 with respect to that displayed at pH 2.0 (*55*), we also increased the E/S in order to promote a faster proteolysis of hGH.

A time−course analysis of the pepsin digest of hGH at pH 4.0 was carried out using both SDS−PAGE (Figure 4A) and RP-HPLC (Figure 4B). The SDS−PAGE analysis, under reducing conditions, reveals that the proteolysis of hGH at pH 4.0 is much slower than that observed at pH 2.0 and that the protein is not completely degraded even after 22 h. Proteolytic digestion of hGH at pH 4.0 is slow, but a burst phase is observed in the first hour of reaction, and then the reaction is slowed. Even after 22 h there is still some intact hGH in the proteolysis mixture (see Figure 4A). This observation can be correlated to the aggregation tendency of hGH at a pH lower than neutrality. The time-dependent aggregation phenomena of the hGH substrate at pH 4.0 could hamper the hydrolysis of the hormone by pepsin and hence make nonquantitative the proteolysis reaction. Indeed, we have observed that during proteolysis at pH 4.0 a protein precipitate is slowly formed and this can be redissolved upon increasing the pH to neutrality. It is worth mentioning that hGH is stored in an aggregated form in mature secretory granules in the pituitary gland, where the pH is about 5.0. The physiological aggregates of hGH are stable in the granules at an acidic pH, but they can be dissolved at neutral pH (*32*−*34*).

After 1 h reaction at pH 4.0, the 22 kDa band of the intact hormone is seen in the SDS−PAGE gel, together with two nearby bands of about 17 kDa and a band of about 5 kDa, these last not observed with the proteolysis performed at pH 2.0 (see Figure 3A). The RP-HPLC chromatogram of the mixture obtained after 1 h digestion (Figure 4B) shows the presence of three main peaks. The N-terminal sequence analysis and ESI/MS of the peptide material of these peaks (Table 1) provided evidence that the peptide eluted from the column at 9.2 min is fragment 1−44, and the peak with the retention time of hGH corresponds indeed to the hormone, whereas the peptide eluted at 26 min is fragment 45−191. Fragment 1−44 can be identified also in the SDS−PAGE gel as the band at about 5 kDa. Of note, the N-terminal fragment 1−44 was not identified in the proteolysis mixture at pH 2.0 (see Figure 3), implying that under more acid conditions this fragment is easily degraded by pepsin.

The RP-HPLC chromatogram (Figure 4B) of the protein mixture after 1 h reaction shows four minor peaks of peptide material eluted from the column later than fragment 45−191. This region of the chromatogram has been magnified in Figure 4C, and the identity of the various hGH fragment species is indicated by the numbers given near the four peaks. It can be noted that these species correspond to nicked or gapped forms of hGH and fragment 45−191, resulting from proteolysis at the level of Phe139−Lys140 and Phe146−Asp147 peptide bonds. These nicked/gapped species can be evaluated also in the SDS−PAGE analysis of an hGH sample digested for 22 h at pH 4.0 (see Figure 4A). Under reducing conditions, the band of fragment 1−139 is poorly separated from that of fragment 45−191, and fragment 45−139 gives a band at about 11 kDa, whereas the C-terminal fragments 140−191 and 147−191 migrate in the SDS−PAGE gel together with fragment 1−44 and lead to a broad band of about 5 kDa. When the same protein sample is analyzed by SDS−PAGE under nonreducing conditions (Figure 4A), only three bands are seen in the stained gel. The band at higher molecular mass corresponds to hGH and its nicked/gapped forms and the band of about 17 kDa to fragment 45−191 and its nicked/gapped forms, whereas the band at about 5 kDa is fragment 1−44.

Conformational Analysis and Complementation of hGH Fragments 1−*44 and 45*−*191.* Gel filtration on a Superdex-75 column of an equimolar mixture of fragments 1−44 and 45−191, after incubation for 30 min at room temperature in Tris buffer, pH 7.5, eluted as a symmetrical peak and with a retention time identical to that of intact native hGH (Figure 5). Therefore, gel filtration experiments clearly indicate that the two fragments associate into a stable 1/1 complex under physiological conditions, leading to the nicked species of

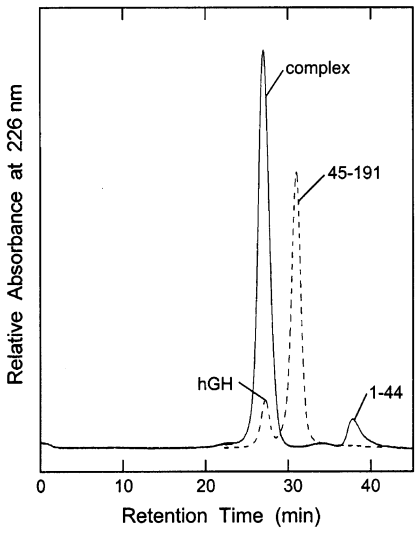

FIGURE 5: Gel filtration analysis of the 1−44/45−191 complex. The complex (solid line) was prepared by mixing fragment 1−44 and fragment 45−191 using a 7% molar excess of fragment 1−44 over fragment 45−191. The chromatographic profile of a mixture (about 1/9 ratio, by weight) of hGH and fragment 45−191 (dashed line) is also shown. The analyses were performed utilizing a Superdex-75 column equilibrated and eluted with 10 mM Tris·HCl/0.15 M NaCl, pH 7.5, at a flow rate of 0.4 mL/min. The effluent from the column was monitored by absorbance measurements a 280 nm.

hGH 1−44/45−191. Of note, when an equimolar mixture of the fragments was gel filtered at pH 2.0, the two fragments eluted from the column well separated (not shown).

Far-UV CD spectra of isolated fragments 1−44 and 45−191, reported in Figure 6A, indicate that they retain secondary structure even when isolated from the rest of the 191-residue chain of the hormone. The N-terminal fragment 1−44 shows a negative dichroic signal at 203 nm but also a distinct shoulder at 220 nm, this last being related to the α-helical secondary structure (*48*). Fragment 45−191 displays a far-UV CD spectrum characterized by the two minima at 209 and 220 nm, both being characteristic signatures of a helical secondary structure (*48*). In the near-UV region (Figure 6B) fragment 45−191 shows a CD spectrum characterized by a well-defined positive CD signal centered at about 290 nm, attributed to Trp86 (*51*). Moreover, the near-UV CD spectrum of this fragment shows the bands of Phe residues in the region between 255 and 270 nm, but it lacks the negative band at 277 nm, which has been assigned to the Tyr residues in intact hGH (*51*, *52*). Conversely, fragment 1−44 does not show any dichroic activity in the near-UV region (not shown), implying that this fragment in isolation does not acquire significant tertiary structure.

The complex 1−44/45−191 at neutral pH shows a far-UV CD spectrum similar to that of intact hGH, but with a lower α-helical content than hGH (Figure 6A). On the basis and limitations of the methods employed to evaluate the content of secondary structure in proteins from their far-UV CD spectra (*48*), an ∼5% decrease in helical content can be calculated for nicked hGH with respect to that of the intact protein. In the near-UV CD (Figure 6B), nicked hGH shows the same minima and maxima of native hGH, but the intensities of the dichroic signals are somewhat lower than those of the intact protein. Moreover, the complex 1−44/45−191 shows a lower signal intensity of the CD band at

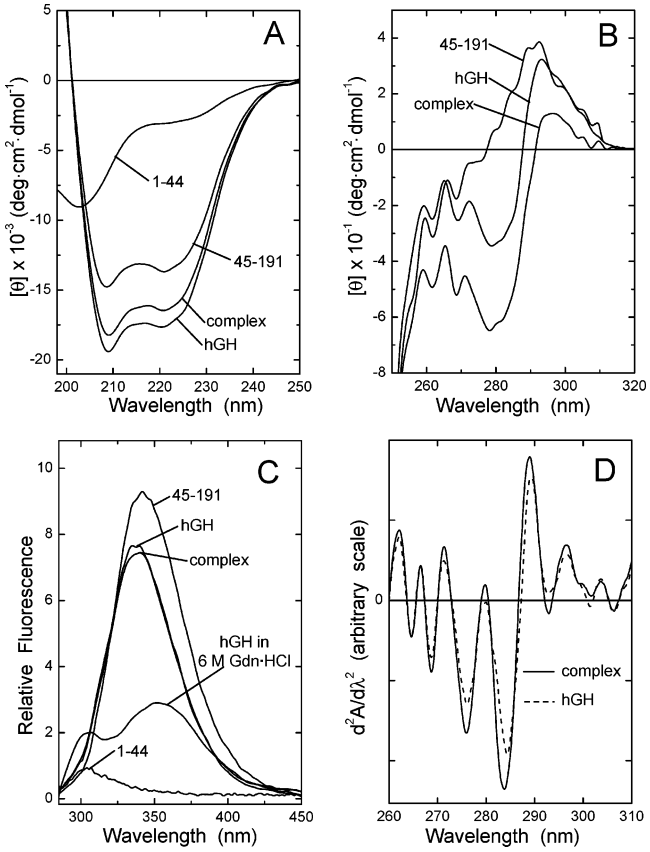

FIGURE 6: Spectroscopic analysis of the hGH complex 1−44/45−191. Far-UV (A) and near-UV (B) CD spectra of hGH, fragment 1−44, fragment 45−191, and the complex 1−44/45−191. Fluorescence emission spectra (C) of hGH, fragment 1−44, fragment 45−191, and complex 1−44/45−191. Spectra were obtained by exciting samples at 280 nm. Second-derivative ultraviolet absorption spectra (D) of hGH and of the complex 1−44/45−191. The peak-to-trough distances between the maximum at 287 nm and minimum at 283 nm and that between the maximum at 295 nm and the minimum at 290.5 nm were used to calculate the Tyr exposure according to Ragone et al. (*47*). Spectra were taken at 25 °C in 10 mM Tris·HCl/0.15 M NaCl, pH 7.5, and in 6 M Gdn·HCl in Tris buffer for determining the full exposure of Tyr residues.

about 290 nm, which can be ascribed to a slight change of the microenvironment near the single Trp86 residue. Nevertheless, considering that the near-UV CD spectrum can be taken as a very sensitive fingerprint of the 3D structure of a protein (*51*), we may conclude that the CD data indicate that nicked hGH retains most of the conformational features of the intact protein.

The fluorescence emission spectra of hGH, fragment 1−44, fragment 45−191, and complex 1−44/45−191 in 10 mM Tris·HCl/0.15 M NaCl, pH 7.5, are shown in Figure 6C. Fragment 1−44 does not contain Trp, but three Tyr residues in positions 28, 35, and 42 of the chain. In fact, it shows a fluorescence emission with a maximum near 303 nm, typical of Tyr (*54*). Fragment 45−191 displays a strong fluorescence emission spectrum with a maximum of emission at 346 nm, due to Trp86. The emission spectrum of complex 1−44/45−191 is similar to that of hGH in terms of position of the maximum (λ_{max} 343 nm) and intensity, thus indicating that the polarity of the microenvironment of Trp86 in the complex is similar to that of native hGH (*53*, *54*). Of interest, the spectra of fragment 45−191, hGH, and complex 1−44/45−191 do not show Tyr fluorescence emission near 303

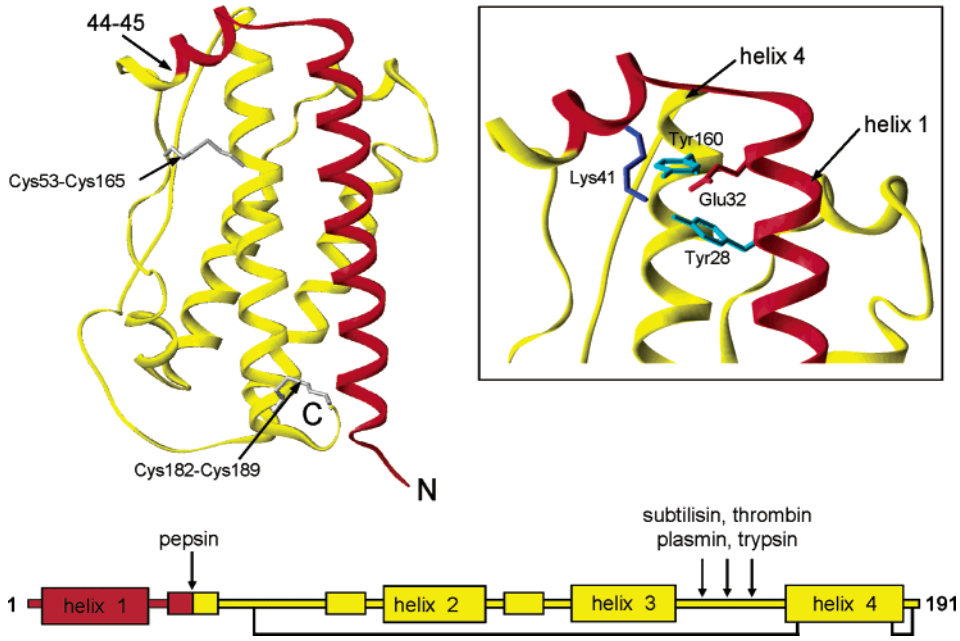

FIGURE 7: Schematic 3D structure and secondary structure of hGH. (Top left) The four major helices and the three minor helices are shown as ribbons, the remaining residues are represented as a string, and the two disulfide bridges (Cys53−Cys165 and Cys182−Cys189) are indicated by gray sticks. Numbers near the polypeptide backbone show the location of the amino acid residues relevant to the discussion of the results of this study. Segments of the 3D structure of hGH corresponding to fragment 1−44 and fragment 45−191 are shown in red and yellow, respectively. The model was constructed from the X-ray structure of hGH (PDB file 3HHR) using the program WebLab ViewerPro 4.0 (Molecular Simulations Inc., San Diego, CA). (Top right) Relative topology of Tyr160 and Tyr28 in the 3D structure of hGH. The orientations of the phenolic groups of the two Tyr residues relative to the side chains of Lys41 and Glu32 are also indicated. (Bottom) Scheme of the secondary structure of hGH. The main boxes indicate the helical segments of the four-helix bundle in hGH, and smaller boxes indicate the three short helical segments, whereas disulfide bonds are represented by a solid line. Fragment 1−44 (red) and fragment 45−191 (yellow) are colored as in the 3D model of hGH (top). The main sites of cleavage of hGH by pepsin at low pH (this study) and by subtilisin, thrombin, plasmin, and trypsin at neutral pH (5) are indicated by arrows.

nm, implying an efficient energy transfer Tyr−Trp and thus a vicinity of these aromatic residues in these protein species (*54*). On the other hand, Tyr fluorescence appears in the emission spectrum of hGH dissolved in 6 M Gdn·HCl, i.e., if the hormone is completely denatured. Moreover, the maximum of fluorescence emission of Trp86 in the denatured hormone occurs at 353 nm (Figure 6C), thus implying a full exposure of this amino acid residue (*53*).

Second-derivative UV spectroscopy can be used to estimate the solvent exposure of Tyr residues in proteins, taking advantage of the fact that the peak-to-trough distances in the 280−295 nm region are related to the polarity of the medium in which Tyr residues are embedded (*47*). Second-derivative spectra of hGH and of the complex 1−44/45−191 in 10 mM Tris·HCl/0.15 M NaCl, pH 7.5, share common features, but some differences are seen in the 280−295 nm region (Figure 6D). We have calculated the average exposure to solvent (α) of the eight Tyr residues in hGH and in the complex 1−44/45−191. First of all, the method has been validated with the native hormone, for which an average exposure of 80% was calculated for Tyr residues, which accounts for about six of the eight Tyr residues exposed. This estimate is in agreement with the crystallographic structure of the hormone, which shows that six out of eight Tyr residues are accessible to the solvent (*23*). Also nitration data indicated that only six Tyr residues in native hGH are nitrated at neutral pH, whereas all eight Tyr are nitrated under denaturating conditions (*56*). When the method was applied to the hGH complex 1−44/45−191, a 100% exposure of Tyr residues was calculated, accounting for the full exposure of the eight Tyr residues.

DISCUSSION

Acid-Induced Conformational State of hGH. The initial site of proteolysis of hGH by pepsin at pH 2.0 is at peptide bond Phe44−Leu45, which is located in the first minihelix of hGH (residues 38−47) (Figure 7). The resulting fragments 1−44 and 45−191 display a significant difference in their susceptibility to proteolysis, since only fragment 45−191 is rather resistant to proteolysis at pH 2.0. This is in agreement with far-UV CD measurements (not shown) which provided evidence that at pH 2.0 fragment 45−191 is highly helical (not shown), while fragment 1−44 is largely unfolded at pH 2.0. Thus, if one accepts the notion that an unfolded polypeptide is that required for rapid proteolysis, we can explain why fragment 1−44 is easily degraded, while fragment 45−191 is instead rather resistant to hydrolysis.

At pH 2.0 there is a strong destabilization of helix 1 (residues 9−34) of the hormone, due to the weakening of many interactions of this helix with the remaining part of the protein. In particular, helix 1 forms salt bridges with helix 4 (His18···Glu174), helix 3 (Arg16···Asp116), and the first minihelix (Glu32···Lys41) (*23*). Upon lowering the pH, there is a protonation of the acidic residues, and these stabilizing interactions between helix 1 and the other helices are lost, as observed by NMR measurements (*28*). In particular, at low pH the disruption of the salt bridge Glu32···Lys41 can lead to a less structured or flexible state of the chain region encompassing the peptide bond Phe44−Leu45, thus explaining the limited proteolysis phenomenon. Therefore, after hydrolysis of the peptide bond Phe44−Leu45 at pH 2.0, the fragment 1−44 comprising helix 1 is cleaved off from the

protein core and, being largely unfolded, is easily digested by pepsin. Complementation studies of fragments 1−44 and 45−191 (see also below) conducted at pH 2.0 have shown that they are unable to form a complex at pH 2.0, as given by the fact that the two fragments eluted separately from a gel filtration column equilibrated at pH 2.0 (not shown).

At pH 4.0 the initial site of proteolysis is also at the level of the peptide bond Phe44−Leu45, but at variance from proteolysis experiments conducted at pH 2.0, fragment 1−44 can be isolated. It is likely that the nicked form of hGH, produced by fission of a single peptide bond, is stable at pH 4.0 and that the N-terminal portion of the nicked species is kept in place, thus retaining interactions of helix 1 with the rest of the molecule. Indeed, gel filtration experiments of the proteolysis mixture conducted at pH 4.0 have shown that fragments 1−44 and 45−191 elute with the same retention time as intact hGH (not shown), thus providing evidence that they are able to associate into a 1/1 complex. At pH 4.0, besides the main cleavage at the Phe44−Leu45 peptide bond, pepsin cleaves the hormone also at the loop region 134−149, but these cleavages are minor with respect to the main site of hydrolysis leading to fragments 1−44 and 45−191. The secondary proteolytic sites at Phe139−Lys140 and Phe146−Asp147 can be taken as an indication that, at pH 4.0, the partly folded state of hGH at low pH acid is in equilibrium with the fully native form of the hormone at neutral pH. This hypothesis appears to be substantiated by the fact that these additional sites of cleavage are embedded in the chain region, which is the site of attack of the hormone at neutral pH by several proteases (*5*).

Crystallographic studies of hGH complexed to its receptor prompted the hypothesis that the first minihelix 38−47 could be induced by interaction of the hormone with the receptor (*23*). However, a subsequent analysis of a mutant of hGH crystallized in the monomeric form allowed the conclusion that this helix is also present in hGH in isolation (*57*). In our opinion, the existence of the first minihelix in hGH appears to be confirmed by the fact that the chain region encompassing the minihelix is relatively stable to proteolysis at neutral pH, since under physiological conditions the main sites of limited proteolysis of hGH occur at the loop region 134−149 (see Figure 7) (*5*). This proposal is in line with the fact that the sites of limited proteolysis in globular proteins occur at the level of flexible loops and never at chain regions embedded in a regular secondary structure such as the α-helix (*38*, *39*). Therefore, it is reasonable to propose that, at acidic pH, the minihelix 38−47 undergoes an unfolding process, whereas other regions of the protein remain folded. Recent NMR data confirmed that hGH at pH 2.0 retains its structure at the level of the four helices and that the main regions of unfolding are essentially located at the level of loops (*31*). Of interest, with the hormone exposed to pH 2.0, NMR data provide evidence that the loop region which connects helix 3 to helix 4 displays a novel tertiary interaction, bringing into proximity residues 143 and 144 with chain region 56−60 of the hormone (*31*). Therefore, this acid-mediated novel interaction could explain why pepsin hydrolyzes hGH preferentially at the level of the first minihelix much faster than at the loop region 134−149.

To summarize, our proteolysis data indicate that at pH 2.0, and in particular at pH 4.0, the acid-induced conformational state of hGH is that of a well-packed globular structure, but with the chain region 38−47 encompassing the first minihelix highly flexible or unfolded. Therefore, proteolysis experiments, together with those obtained by NMR spectroscopy (*31*), indicate that the partly folded state of hGH at low pH is a "highly ordered" molten globule (*58*, *59*). The conformational state of hGH under acidic conditions can be compared to the molten globule of other four-helix bundle proteins, such as interleukin-4 and apocytochrome b_{562}, which retain a native-like helical structure at low pH or upon removal of the heme group at neutral pH, respectively (*60*, *61*).

Complementation of Fragments 1−44 and 45−191. Analysis by CD spectroscopy of the conformational features of fragments 1−44 and 45−191 revealed that the N-terminal fragment 1−44 is largely unfolded, while fragment 45−191 in isolation is well packed and folded into a helical structure under physiological conditions (Figure 6A). Gel filtration experiments demonstrated that the fragments 1−44 and 45−191 form a rather stable complex (Figure 5). Analysis of the structural features of the complex 1−44/45−191 by CD, fluorescence emission, and second-derivative UV absorption spectroscopy demonstrated that the complex adopts a native-like structure, but with some structural details that differ from those of the intact protein. The first minihelix 38−47 very unlikely is retained in nicked hGH since the peptide bond fission is located exactly at the middle of the helical segment and short helices are strongly destabilized by end effects. In fact, the complex has a slightly lower helical content with respect to that of the intact hormone (see Figure 6). Moreover, the nicked protein shows a full exposure of Tyr residues. The two Tyr residues (Tyr28 and Tyr160) that in hGH are shielded from the solvent (*23*) are located near the first minihelix in the 3D structure of the hormone and are hydrogen bonded between themselves, as well as with Lys41 and Glu32 (Figure 7, right). In the complex 1−44/45−191, the hydrolysis of the peptide bond Phe44−Leu45 prompts an enhanced flexibility at the level of the first minihelix 38−47 and thus likely a disruption of these interactions and a concomitant exposure of the two Tyr residues (see Figure 7).

The conformational features of nicked hGH 1−44/45−191 can be compared to those of the 20 kDa form of the hormone. This physiological species of hGH is generated by an alternative splicing of the hGH mRNA precursor, and it lacks residues 32−46, which correspond to the region of the first minihelix (*3*, *23*). The far-UV CD analysis of the 20 kDa hGH shows a lower content of secondary structure with respect to that of the 22 kDa hormone, and this difference can be attributed to the absence of residues 32−46, which in 22 kDa hGH are in an α-helical conformation (*4*). Also, the complex 1−44/45−191 shows a slightly lower content of helical structure relative to that of the intact protein (Figure 6A), and again this reduction can be related to the absence of the first minihelix 38−47. Moreover, experiments of nitration of the Tyr residues of the 20 kDa hGH indicated that the deletion of residues 32−46 leads to the exposure of one to two Tyr residues (*4*). These observations are in agreement with the results of second-derivative UV spectroscopy of the complex (see Figure 6D), indicating that two Tyr residues, which are hidden to the solvent in the intact native hormone, become exposed in the nicked form of hGH.

We can conclude that fragment 1−44 and fragment 45−191 can form a native-like complex, although there is evidence that the chain region encompassing the first minihelix of the native hormone remains rather flexible or unfolded. The fact that the 20 kDa hGH is folded and exhibits a growth-promoting activity as the 22 kDa hGH suggests that the chain region 32−46 of the protein is not required to adopt the native structure of the four-helix bundle of hGH. Therefore, the molecular features of the 20 kDa variant of hGH may help us to understand why nicked hGH can adopt the native fold of the intact protein.

In developing strategies for preparing complementing fragments of a protein, Taniuchi et al. (*62*) elaborated the concept of permissible sites of cleavage. In a number of cases of complementing fragments, it has been observed that the sites of cleavage are located at chain regions not embedded in hydrogen-bonded, regular secondary structure but at flexible loops exposed to the surface of the protein. Therefore, limited proteolysis is the method of choice for producing complementing fragments, since by using this technique it is possible to perform preferential peptide bond fissions at flexible loops only (*38*, *39*). Present data indicate that the chain fission at Phe44−Leu45 is a permissible site for a complementing fragment system of hGH, and thus the corresponding chain region does not impair the formation of the four-helix bundle of the hormone. Actually, this region even can be deleted in the 20 kDa variant of hGH, without impairing overall folding and function of the hormone. We may mention herewith that previously it has been demonstrated that the reduced and S-carboxymethylated fragments 1−134 and 135−191 form a complex displaying conformational features similar to those of native hGH and retaining about 30% of the biological potency of the intact hormone (*21*). Again, the site of cleavage 134−135 lies at the level of the long loop connecting helix 3 and helix 4 (see Figures 1 and 7).

Biological Implications. There is currently the hypothesis that hGH may behave as a prohormone, which requires proteolytic hydrolysis to induce the diversity of effects observed (*5*−*8*). On this basis, it seems that hGH exerts its growth-promoting activity as intact hormone, whereas many of its actions on carbohydrate and lipid metabolism are induced by fragments produced by proteolysis of the hormone (*8*). Of more specific interest for the studies herewith reported is that hGH in vivo has both insulin-like and diabetogenic effects. Two fragments of hGH exert in vivo these two opposite activities, i.e., fragment 1−43 which shows insulin-potentiating effects (*14*, *15*) and fragment 44−191 which has diabetogenic activity (*16*−*18*). Both fragments have been detected in serum and at the level of the pituitary gland (*14*−*18*). Fragments 1−43 and 44−191 are two complementary parts of hGH, and it is believed that they are formed in vivo by proteolytic cleavage of the hormone, even if at present the specific protease responsible of their production has not been identified (*18*). The hGH fragments 1−44 and 45−191 herewith described differ by only one amino acid residue from the two physiologically relevant fragments 1−43 and 44−191 exhibiting activities on carbohydrate metabolism. It should be noted, however, that the proteolytic cleavage of the 191-residue chain of hGH that in vivo produces the 5 and 17 kDa N- and C-terminal fragments, respectively, is not totally specific, even if it appears that the most prominent proteolytic cleavage lies between residues 43 and 44 (*8*, *14*−*18*). Therefore, likely in vivo the polypeptide chains of the fragments possess ragged ends. In several studies, fragments 1−43 and 44−191 were (arbitrarily) chosen as protein targets for their production by synthetic or recombinant methods, and their biological properties were studied in detail. Therefore, it is likely that also the hGH fragments 1−44 and 45−191 occur in vivo and share the biological properties of the previously investigated fragments 1−43 and 44−191.

Our view is that the in vitro proteolysis phenomenon of hGH at low pH by pepsin likely mimics that occurring in vivo. We advance the hypothesis that the highly structured molten globule state of hGH at acidic pH could be implicated in the generation of the two biologically active fragments. It could be well that under acidic conditions there is an unfolding of the region of the first minihelix 38−47, which becomes flexible and consequently preferentially cleaved by a protease. In our study we used pepsin, since it is active at low pH and it has a broad substrate specificity, but certainly it is not a candidate protease for the physiological processing of the hormone in vivo. Nevertheless, the proteolytic processing of hGH at acid pH here described can be of physiological significance. Indeed, it has been demonstrated that hGH complexed to its receptor is directed to lysosomes (*37*). The lower pH inside the lysosomes triggers the dissociation of hGH from the receptor, and subsequently both proteins are digested by proteases. The proteolysis of hGH in lysosomes has not been studied in detail yet, but it was demonstrated that proteolysis is inhibited by leupeptin and pepstatin, inhibitors of serine and aspartic proteases, respectively (*37*). Of note, also pepsin is an aspartic protease and is inhibited by pepstatin. Moreover, hGH is stored in the pituitary gland in mature secretory granules in an aggregated form (*32*, *33*), and in these granules the pH is acidic (*35*). Since fragments 1−43 and 44−191 have been isolated from the pituitary gland, these granules are likely the place where limited proteolysis of the hormone occurs.

CONCLUDING REMARKS

Partly folded states or MGs of proteins have attracted a strong interest from numerous investigators in the past decade (*63*−*65*), since it has been suggested that MGs may have a physiological significance and play a role in ligand binding and translocation across membranes (*65*), as well as in protein aggregation phenomena (66−*68*). Numerous proteins contain largely disordered chain regions (*69*−*72*), and some proteins appear to be even intrinsically or "natively" unfolded under their normal conditions in the cell (*72*). Therefore, it has become increasingly clear that proteins are dynamic systems that can adopt partly folded states and, in particular, that the function of proteins cannot be interpreted solely on the basis of their static 3D structures. These observations appear to challenge the long-lasting protein structure−function paradigm (*69*, *71*). In the present case of hGH, disordered chain region(s) may be functional for producing, by limited proteolysis of the protein, several hGH fragments that are able to display some of the many activities of this prohormone. These flexible regions may include chain segment 130−140 at neutral pH (*5*), as well as segment 38−47 (this study) when the hormone is exposed to an acidic environment in the cell, as actually it occurs in vivo.

The novel system of complementing fragments of hGH herewith described appears to be very useful for investigating features of structure, function, and folding of this very important hormone (*73*, *74*). In particular, it was suggested that the formation of a noncovalent complex upon fragment complementation can mimic the protein folding process of the entire protein chain, with the difference that the folding of the complementing fragments is an intermolecular process (*74*). Since fragments 1−44 and 45−191 in isolation do acquire a partly folded state and only upon their complementation do their overall 3D structures become native-like, it seems that these fragments can be used as a useful experimental system to analyze the initial, intermediate, and final stages of the overall folding pathway of hGH, a prototype four-helix bundle protein.

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