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Photodegradation of Human Growth Hormone: A Novel Backbone Cleavage between Glu-88 and Pro-89

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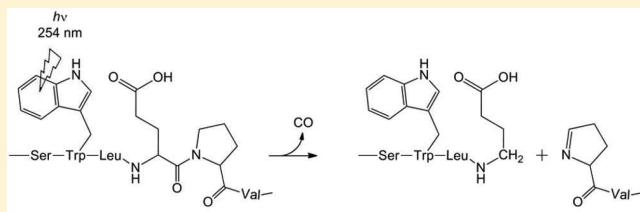
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S Supporting Information

ABSTRACT: The exposure of protein pharmaceuticals to light can cause loss of potency, oxidation, structural changes and aggregation. To elucidate the chemical pathways of photodegradation, we irradiated human growth hormone (hGH) at $\lambda = 254$ nm, $\lambda \approx 265\text{--}340$ nm, and $\lambda \approx 295\text{--}340$ nm (using the spectral cutoff of borosilicate glass) and analyzed the products by mass spectrometry. By means of LC-MS/MS analysis, we observed an unusual peptide backbone cleavage between Glu-88 and Pro-89. The crystal structure of hGH indicates that these residues are in proximity to Trp-86, which likely mediates this backbone cleavage. The two cleavage fragments observed by MS/MS analysis indicate the loss of CO from the amide bond and replacement of the Glu-C(=O)Pro bond with a Glu-H bond, accompanied by double bond formation on proline. The reaction is oxygen-independent and likely involves hydrogen transfer to the C_α of Glu-88. To probe the influence of the protein fold, we irradiated hGH in its unfolded state, in 1:1 (v/v) acetonitrile/water, and also the isolated tryptic peptide Ile-78–Arg-90, which contains the Glu-88–Pro-89 sequence. In both cases, the cleavage between Glu-88 and Pro-89 was largely suppressed, while other cleavage pathways became dominant, notably between Gln-84 and Ser-85, as well as Ser-85 and Trp-86.

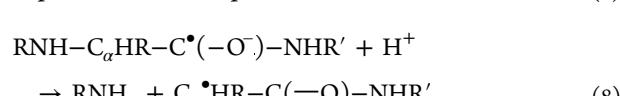
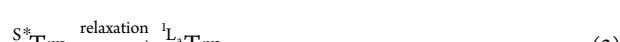
KEYWORDS: *human growth hormone, hGH, somatropin, photostability, peptide cleavage, protein degradation*



INTRODUCTION

Photodegradation represents a major degradation pathway of protein pharmaceuticals, which can occur at several points during fermentation, purification, formulation, and storage.¹ Potential consequences are increased heterogeneity, discoloration, loss of potency,^{2,3} conformational changes,⁴ and the formation of aggregates.^{3,5} Of all amino acids, tryptophan displays the highest absorbance of UV light⁶ and is, thus, a key player in photodegradation processes. Mechanistically, photon absorption of tryptophan populates its primary, prefluorescent excited singlet state, S^* (reaction 1), which undergoes either rapid ionization (reaction 2) or relaxation to the fluorescent state S_1 (reaction 3),^{7–9} also referred to as 1L_a state, by analogy to similar states of substituted naphthalenes. Besides undergoing fluorescence (reaction 4), the 1L_a state can convert to an excited triplet state via intersystem crossing (reaction 5)¹⁰ or undergo ionization (reaction 6).⁷ In proteins, the ionization of photolytically excited tryptophan residues results in the formation of a tryptophan radical cation with concomitant formation of a solvated electron,¹¹ which can reduce a nearby disulfide bond (reaction 7).^{12–15} Such electron transfers from tryptophan to disulfide bonds can be reversible and generate thiyl radicals, inferred from product analysis of photoirradiated lyophilized recombinant bovine somatotropin¹⁴ (bovine growth hormone), α -lactalbumin¹⁵ and *Fusarium solani pisi* cutinase.¹² In addition, electron transfer from tryptophan to peptide bonds has been experimentally observed^{16,17} and supported theoretically,⁹ leading to amide radical anions. These undergo

deamination (reaction 8), while a C-centered radical is formed, which can lead to various final products.^{16–18}



In this article, we report on the photodegradation of human growth hormone (somatropin, hGH), which belongs structurally

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to the class of four helix bundle proteins. The structurally closely related bovine growth hormone was found to photodegrade when irradiated between 300 and 410 nm in the solid state via initial electron transfer from tryptophan to the disulfide bond, followed by back electron transfer and generation of a pair of cysteine thiol radicals. These reacted with oxygen and eventually recombined to yield α -disulfoxide, thiosulfinate, or thiosulfonate.¹⁴ In contrast, as discovered in this study, the photo-irradiation of hGH specifically at 254 nm in aqueous solution leads to a photolytic peptide cleavage between Glu-88 and Pro-89, which represents a new degradation pathway likely involving intermediary amide radical anions.

We decided to expose hGH to three sets of photolytic conditions, photoirradiation at (i) $\lambda = 254$ nm, (ii) $\lambda = 265\text{--}340$ nm, and (iii) $\lambda = 295\text{--}340$ nm. The experiments at $\lambda = 254$ are generally representative of conditions proteins may experience during chromatographic purification coupled to UV-detection ($\lambda = 215\text{--}280$ nm)^{19,20} and also to conditions that have been recently proposed for viral decontamination of biotechnology products (irradiation at $\lambda = 254$ nm),^{5,21} while the experiments at $\lambda = 295\text{--}340$ nm are representative of the exposure of proteins to near-UV light, which is not filtered by glass.

EXPERIMENTAL SECTION

Materials. Human growth hormone (somatropin, hGH) was provided by Genentech, Inc. (South San Francisco, CA), as Nutropin AQ formulations. The formulations were dialyzed overnight against water with Slide-A-Lyzer dialysis cassettes of 3.5K molecular weight cut off (MWCO). Aliquots of ca. 0.1 mL were stored at -20°C . The concentrations of hGH stock solutions were measured by means of UV-spectroscopy at 277 nm, where $A_{277} = 0.82 \text{ cm}^{-1}$.²² Additionally, a solution of pure hGH was supplied by Genentech, Inc. Small levels of aggregates therein were removed by centrifugation (15 000g, 10 min) and clear solutions were stored at 4°C . TPCK-treated sequence-grade trypsin was obtained from Promega (Madison, WI). D₂O was supplied by Cambridge Isotope Laboratories, Inc., (Andover, MA). All other chemicals were of the highest commercial grade available and obtained from Sigma (Saint Louis, MO) or Fisher (Pittsburgh, PA). All solutions were prepared with MilliporeQ-water, and the chemicals were used as received.

Preparation of Samples. In some cases, the reduction and alkylation of hGH was performed prior to irradiation, which was achieved by treating hGH stock solutions (10 μL , 0.4 mM) for 15 min with 20 μL of solutions containing 10 mM dithiothreitol (DTT), 6 M guanidine, and 0.1 M NH₄HCO₃, buffered at pH 8. After reduction, the protein was precipitated with 0.5 M HClO₄ and washed with water. Protein pellets obtained were dissolved into 6 M guanidine hydrochloride and treated with a 10-fold excess of N-ethylmaleimide at pH 7.8 for 15 min. Then, solutions were diluted with water to 400 μL and dialyzed three times with Slide-A-Lyzer MINI 10K MWCO dialysis units into 15 mL of water for half an hour. Solutions prepared in D₂O were further enriched in D₂O before irradiation by dialysis with Slide-A-Lyzer MINI 10K MWCO dialysis units into 1.5 mL of D₂O for half an hour three times. All vials and glassware were washed with D₂O before usage.

Photoirradiation. The photoirradiation was carried out by means of a Rayonet photoreactor RPR-200 equipped with one to four 35 W low pressure mercury lamps (RPR-2537 Å) having an emission >80% at $\lambda = 254$ nm. Alternatively, four phosphor-coated

low pressure mercury lamps (RPR-3000 Å), which emit 95% of light between $\lambda = 265\text{--}340$ nm ($\lambda_{\text{max}} = 305$ nm) were used.²³ All equipment was from the Southern New England Ultra Violet Company (Branford, CT). Samples of 400 μL contained 10 μM (0.22 mg/mL) hGH and were buffered with 0.1 M phosphate at pH 7.4 or NH₄HCO₃ at pH 7.8. Samples prepared in D₂O were not buffered. Samples were irradiated in 13 \times 100 mm quartz glass tubes or in 13 \times 100 mm borosilicate glass (akin to Pyrex) tubes to cut off light below 295 nm. Ar saturation was carried out in the glass tubes immediately before the irradiation. Tubes were fitted with rubber septa and a stream of Ar was passed over the surface of the sample through a pair of hypodermic needles (inlet and outlet). The Ar was of technical grade, and residual oxygen was trapped by means of an OxiClear purifier (LabClear, Oakland, Ca). Glass vials were placed in a rotating Rayonet RMA-500 Merry-Go-Around unit with a distance of 3.8 cm (1.5 in.) from the lamps. According to the manufacturer, the light intensity reading at that distance orthogonal to the lamps is 210 W/m² when 16 lamps are employed.²³ Therefore, a light intensity of 52.5 W/m² is calculated for four lamps and our 20 min photoirradiations correspond to ca. 17.5 W·h/m². The ICH guideline for photostability is 200 W·h/m².²⁴ However, the ICH guideline makes use of a broad irradiation spectrum of 320–400 nm, whereas we used light at a specific wavelength.²⁴ Actinometry based on iodide–iodate^{25,26} was performed using the same experimental setup with two oppositely arranged RPR-2537 Å lamps and irradiation times of 12 s. We derived a flux of $(7.2 \pm 0.8) \times 10^{-9}$ mol photons/s (einstein/s) to the sample under such condition. Hence, we calculate that samples were subjected to a total of $(1.72 \pm 0.18) \times 10^{-5}$ einstein (mol photons) when irradiated for 20 min with four RPR-2537 Å lamps.

Reduction, Alkylation, and Tryptic Digestion. After irradiation, hGH was precipitated with 0.5 M HClO₄ and

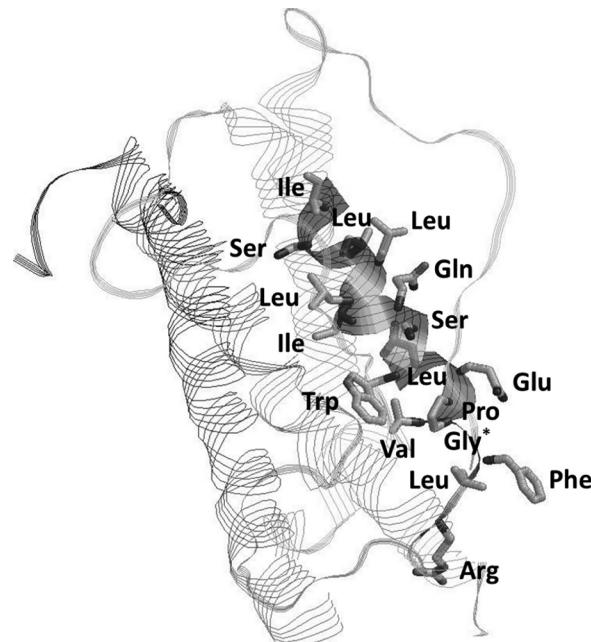


Figure 1. Tryptic fragment T9 in the crystal structure of hGH³³ (PDB 1HGU) displayed with RasMol.⁵⁷ There is an inconsistency in the crystal structure of residue Gly-91 (indicated with asterisk) with the sequence of hGH published in UniProt Knowledgebase (UniProtKB) _ENREF_56,⁵⁸ which shows a glutamate residue at position 91.

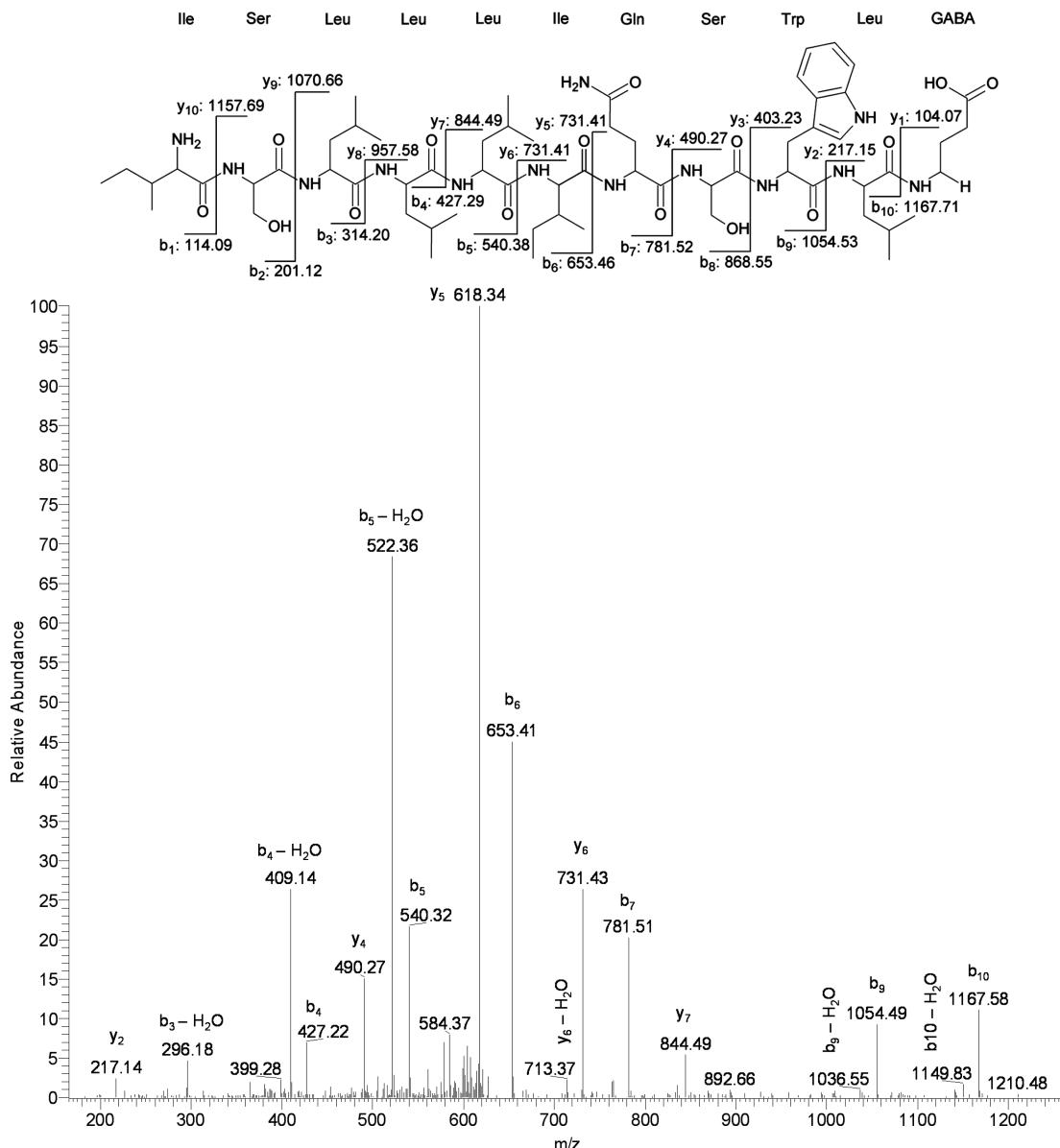


Figure 2. MS/MS spectrum of the doubly charged parent ion with m/z 635.89 of T9a. The C-terminal Glu-derivative with a m/z value of 104.7 is assigned to 4-aminobutanoic acid (GABA). The sample contained 0.4 mL of 10 μM hGH in 0.1 M phosphate at pH 7.4 and was irradiated at $\lambda = 254$ nm for 20 min with four RPR-2537 Å lamps corresponding to $(1.72 \pm 0.18) \times 10^{-5}$ einstein (mol photons). After precipitation, two washing steps, and tryptic digestion the resulting peptides were analyzed by an LTQ-FTICR mass spectrometer.

washed twice with water. If protein disulfides were to be reduced, protein pellets were treated for 15 min with 20 μL of solutions containing 10 mM DTT and 6 M guanidine hydrochloride buffered at pH 8 with 0.1 M NH_4HCO_3 . After reduction, the protein was precipitated with 0.5 M HClO_4 and washed with water. For alkylation, protein pellets were dissolved into 6 M guanidine hydrochloride and treated with a 10-fold excess of *N*-ethylmaleimide at pH 7.8 for 15 min. Tryptic digestions were carried out in acetonitrile (ACN)/ H_2O (10/90, v/v), buffered at pH 7.8 with 0.1 M NH_4HCO_3 . The initial trypsin/protein ratio was 1:50 (w/w), and samples were incubated at 37 °C for 8–10 h. Digests were stored at –20 °C prior to analysis. All experiments and analysis were done with at least one replication.

Analysis on a SYNAPT-G2 Mass Spectrometer. Samples were analyzed by HPLC-MS/MS on an Acquity UPLC system (Waters Corp., Milford, MA), equipped with a Vydac 218MS

capillary column (25 cm × 500 μm C18, 5 μm) from Grace (Deerfield, IL), connected to a Synapt G2 (Waters Corp., Milford, MA). Tryptic digests (10 μL , 10 μM) were eluted with a linear gradient of 10–60% (v/v) ACN in aqueous formic acid (0.06%, v/v), adjusted within 30 min, followed by a second linear gradient of ACN in aqueous formic acid (0.06%, v/v) to 80% (v/v) within 10 min. The flow rate was 20 $\mu\text{L}/\text{min}$. The SYNAPT-G2 hybrid mass spectrometer was operated for maximum resolution with all lenses optimized on the $[\text{M} + 2\text{H}]^{2+}$ ion from the $[\text{Glu}]^1$ -fibrinopeptide B. The cone voltage was 45 V, and Ar was admitted to the collision cell. The spectra were acquired using a mass range of 50–2000 amu (amu = atomic mass unit). The data were accumulated for 0.7 s per cycle. CID MS/MS spectra were acquired by setting the MS¹ quadrupole to transmit a precursor mass window of ± 0.1 m/z . Experimental raw data files were analyzed by the MassLynx Software (Waters Corp., Milford, MA).

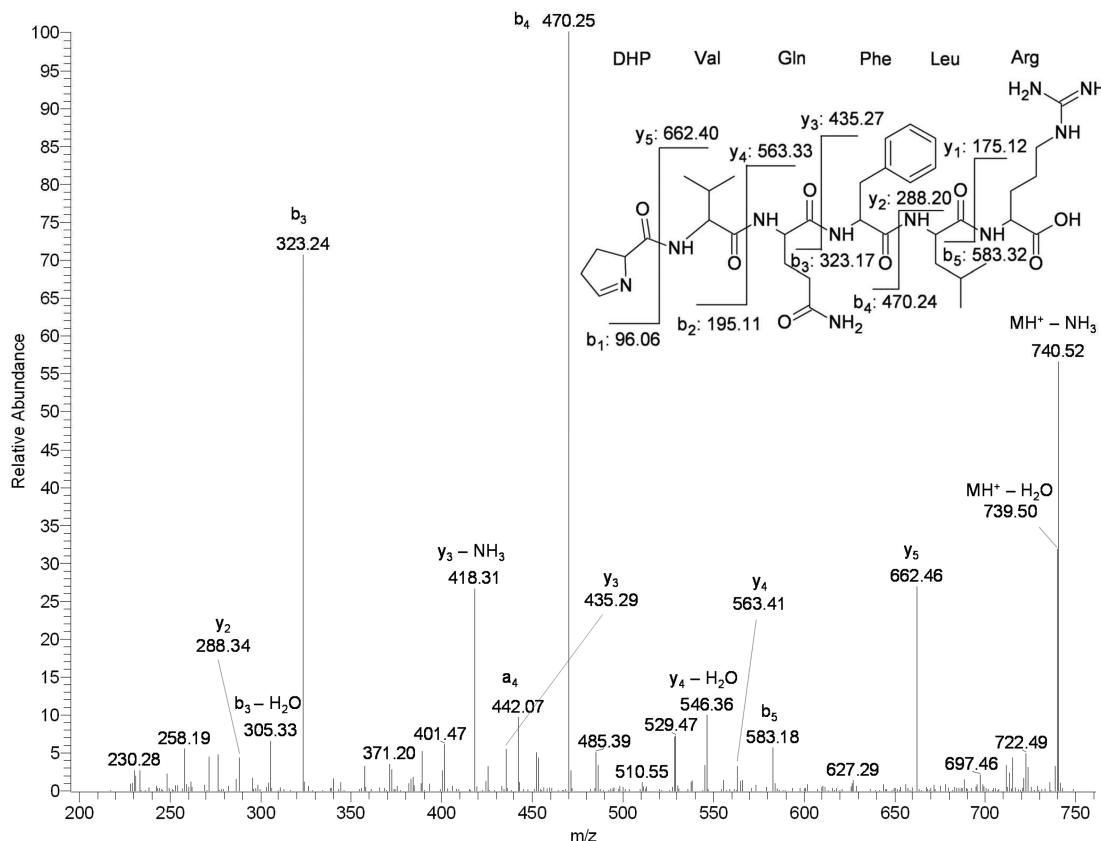


Figure 3. MS/MS spectrum of the singly charged parent ion with m/z 757.46 of T9b. The N-terminal Pro-derivative with a m/z value of 96.06 is assigned to a dehydroproline (DHP), here represented by 1,5-dehydroproline; 1,2-dehydroproline is considered as an equally feasible product. Conditions as in Figure 2.

Analysis on an LTQ-FT-ICR Mass Spectrometer. The hybrid linear quadrupole ion trap Fourier transform-ion cyclotron resonance (LTQ-FT-ICR) mass spectrometer (ThermoFinnigan, Bremen, Germany) was operated as described.²⁷ The chromatography was performed with an LC Packings Ultimate Chromatograph (Dionex, Sunnyvale, CA) system equipped with a Vydac column (25 cm \times 1 mm C18, 3.5 μm). Data analysis was performed with the Xcalibur software package (ThermoFinnigan, Bremen, Germany).

Calculation of Oxidation Yields. We calculated yields of methionine oxidation through the peak areas in the extracted ion chromatograms (peak area of oxidized peptide/peak area of oxidized + native peptide) of the most abundant ions of the respective tryptic peptides. For this quantitation, a first approximation, we assumed that electrospray-ionized peptides of the oxidative modifications have similar mass spectrometric properties as the respective ions of the native peptides due to the small mass differences and similar structures. The loss of tryptic fragments, including disulfide linkages, from hGH was calculated based on peak areas and referenced to the more photolysis-resistant fragment T12 (LEDGSPR) as internal standard. The yield of the cleavage between Glu-88 and Pro-89 was derived by HPLC analysis.

HPLC. The chromatographic analysis was performed with a Prominance HPLC system coupled to an SPD M-20A photo-diode array detector to monitor wavelengths between 210 and 400 nm, both from Shimazu (Shimadzu Scientific Instruments Inc., Columbia, MD). Tryptic digests (100 μL , 10 μM) were eluted with a linear gradient of 12.6–50.4% (v/v) ACN in

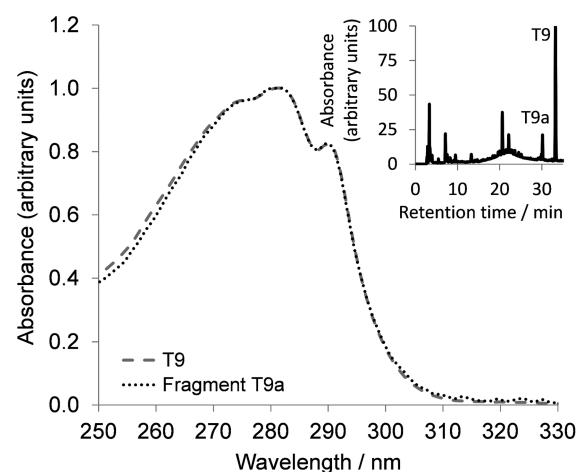


Figure 4. Absorbance spectrum of T9 and cleavage fragment T9a (I-77-GABA-88), recorded during an HPLC analysis. Spectra recorded a few seconds after the elution of the peptide were subtracted to account for the background absorbance of the mobile phase, and the spectra were normalized at 280 nm. Conditions as in Figure 2.

aqueous formic acid (0.1%, v/v) within 40 min. The flow rate was 1 mL/min.

Fluorescence Spectroscopy. A Shimazu RF-5000U fluorescence spectrometer was applied. The bandwidths for excitation and emission were set to 5 nm. Samples of 400 μL containing 10 μM hGH were analyzed in a 500 μL , 1 mm \times 10 mm, quartz cuvette (Hellma USA, Plainview, NY).

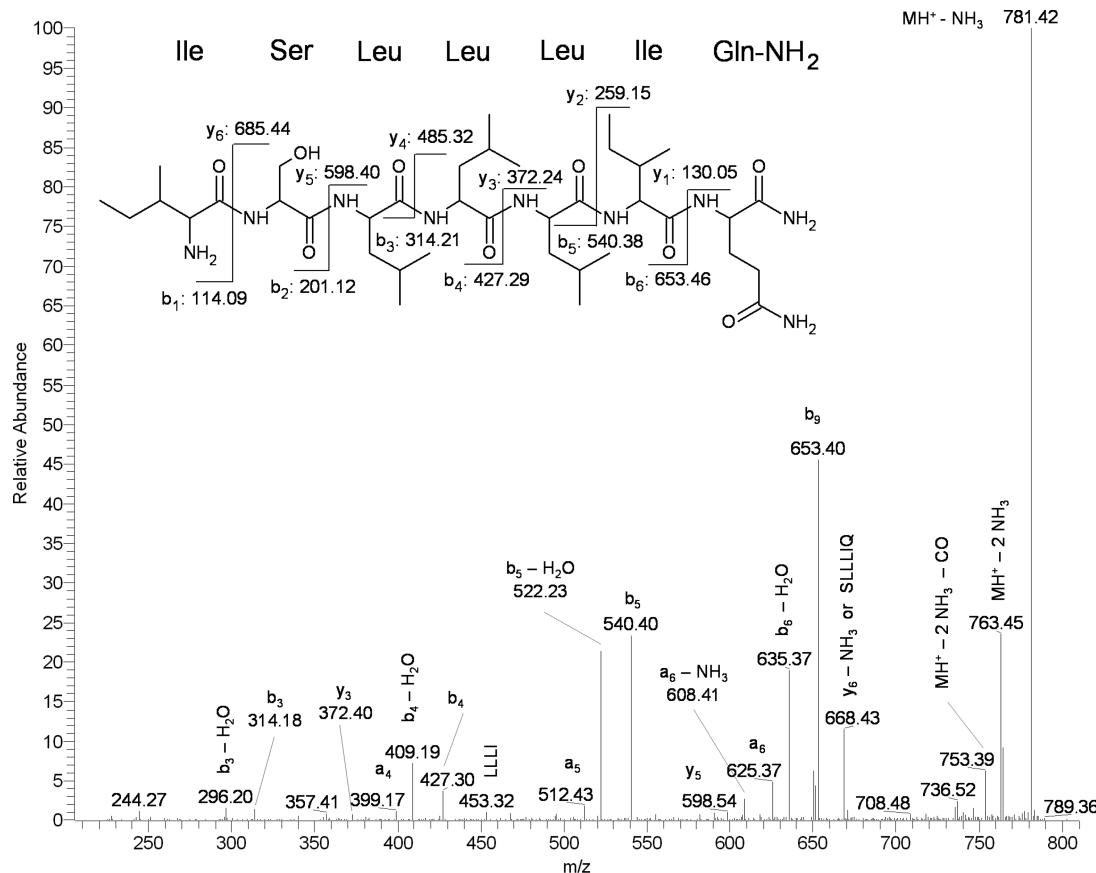


Figure 5. MS/MS spectrum of the singly charged parent ion with m/z 798.55, indicating peptide cleavage between Glu-84 and Ser-85. Conditions as in Figure 2.

RESULTS

Identification and Quantitation of Tryptophan Photodegradation Products and the Cleavage of T9 between Glu-88 and Pro-89. We photoirradiated aqueous solutions of native hGH at $\lambda = 254$ nm at pH 7.4 (0.1 M phosphate) and analyzed the tryptic digest for photodegradation products. LC-MS analysis revealed that the tryptic peptides originally containing the disulfide linkages, the methionine residues, or the single tryptophan residue were degraded to the highest extent. We will first focus on the photodegradation of the tryptophan-containing tryptic fragment T9 (ISLLIQSWLEPVQFLR), shown in Figure 1, to introduce a novel cleavage pathway between Glu-88 and Pro-89 (other photodegradation products will be summarized below). We did not detect significant oxidation of tryptophan to kynurenine ($\text{Trp} + 4$ Da), hydroxytryptophan, or oxindolealanine ($\text{Trp} + 16$ Da), as well as dihydroxytryptophan, tryptophan hydroperoxide, or N-formylkynurenine ($\text{Trp} + 32$ Da), suggesting that ultimate tryptophan degradation is not the primary pathway by which the fragment T9 degrades. In fact, primarily two cleavage fragments of T9, with molecular masses of 1270.8 and 785.7 Da, were detected as degradation products. The MS/MS spectrum of the N-terminal cleavage fragment of T9, denoted as T9a, is shown in Figure 2, and the C-terminal cleavage product of T9, denoted as T9b, is shown in Figure 3, together with tentative structural assignments based on the sequence interpretation. The peptide T9 is cleaved between Glu-88 and Pro-89 with loss of CO from the amide bond and replacement of the $\text{Glu}-\text{C}(=\text{O})\text{Pro}$ bond with a $\text{Glu}-\text{H}$ bond.

No experiments were carried out to detect CO, which requires specialized methodology.²⁸ Pro-89 is converted to a product with a molecular mass equivalent to Pro - 2 Da, suggesting the formation of dehydroproline. The yield of this cleavage process in the tryptic peptide T9 was derived by means of HPLC-UV spectrometric analysis at 289 nm in order to exclusively measure the absorbance of tryptophan. The absorbance spectrum of the tryptophan-containing cleavage product T9a and that of the native peptide T9 are very similar (Figure 4), suggesting that the two peptides have identical absorption coefficients at 289 nm. We derive a relative abundance of 14% of T9a after photoirradiation with four RPR-2537 Å lamps for 25 min, corresponding to a total of $(2.16 \pm 0.23) \times 10^{-5}$ einstein (mol photons). The quantum yield cannot be derived from these results since the number of photons absorbed by the sample is unknown. In order to assess the oxygen-dependence of the cleavage reaction, we photoirradiated hGH in air-, O_2^- , or Ar-saturated solutions. No oxygen dependence for the yield of the Glu-Pro cleavage was observed. To test whether the hydrogen atom, which replaces the C-terminal peptide bond in T9a at Glu-88, originates from the solvent, we irradiated hGH in D_2O . After photoirradiation, the protein was precipitated with HClO_4 , washed twice with H_2O , and digested in aqueous H_2O solution. We did not detect a significant covalent incorporation of deuterium into T9a (4%) compared with that into undegraded T9 (6%) by MS analysis. To probe whether the Glu-Pro cleavage is caused by a monophotonic or biphotonic process, the photoirradiation was carried out with either one or four lamps, changing the dose rate while the photoirradiation dose was kept constant.

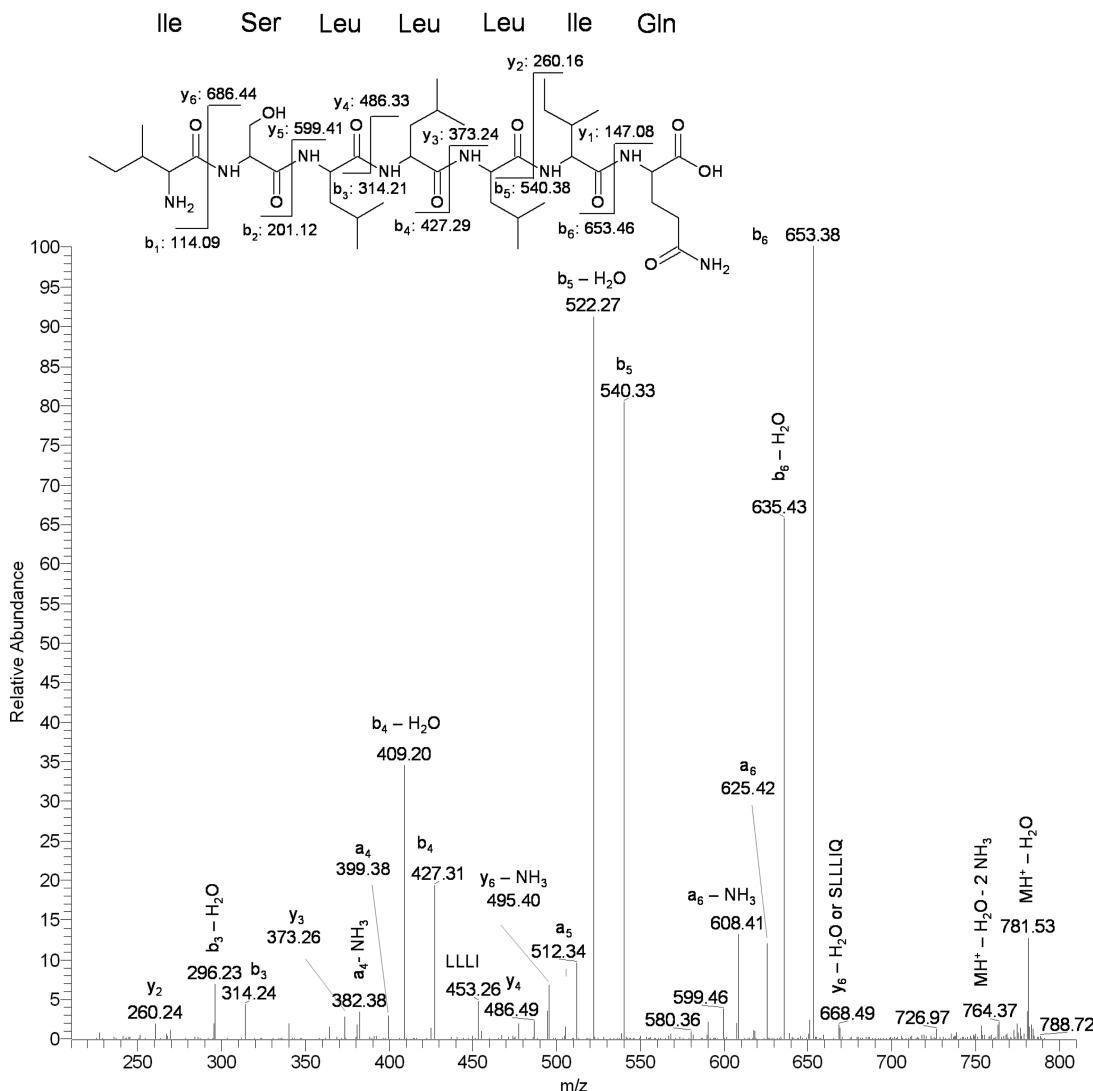


Figure 6. MS/MS spectrum of the singly charged parent ion with m/z 799.53, indicating peptide cleavage between Glu-84 and Ser-85. Conditions as in Figure 2.

No significant difference in the yield of the cleavage was observed, which would have suggested a biphotonic process. However, the linear light intensity dependency of the yields observed is not strict evidence for a monophotonic process.²⁹ The wavelength dependence was studied between $\lambda = 240$ and 295 nm. First, we tested whether wavelengths with $\lambda < 240$ nm from the RPR-2537 Å lamps contributed to the cleavage. For this, the photoirradiation was carried out in the presence of a 240 nm long pass filter: the quartz tube containing hGH was placed into a quartz beaker filled with 0.5% acetic acid acidified with 0.5 mM HCl. This solution absorbs light with a cutoff at ca. 240 nm according to a UV spectroscopic measurement. No difference in the yield of T9 cleavage was observed compared with control experiments with the same setup except the absence of the 240 nm long pass filter, indicating that it is not light of $\lambda < 240$ nm from the RPR-2537 Å lamps that induces the cleavage. Further, we photoirradiated hGH by means of four RFR3000 lamps, which emit ca. 95% of light between $\lambda = 265$ and 340 nm ($\lambda_{\text{max}} = 305$ nm) and ca. 80% between 280 and 320 nm.²³ When irradiated in either quartz or borosilicate glass (to cut off light at 295 nm), the Glu88–Pro89 cleavage was not observed, even at longer irradiation times of 4 h, during which

the protein suffered significant degradation according to LC-MS analysis far beyond that observed after 20 min of irradiation with four RFR2537 lamps.

Effect of Protein Conformation. We probed the role of the protein fold on the cleavage between Glu-88 and Pro-89. For this, we selected to unfold hGH with ACN because ACN is chemically rather inert and does not absorb the incident light. By means of fluorescence spectroscopy, monitoring the tryptophan residue (exc 280 nm, em 350 nm), we observed that the fluorescence intensity of tryptophan increased with increasing ACN content, until a plateau was reached at 40% (v/v) ACN in unbuffered solution (Figure S1 in Supporting Information). The fluorescence intensity under such conditions is similar to that of hGH in the presence of 40% (v/v) 1-propanol in water, where complete hGH unfolding was observed.³⁰ Therefore, we conclude that ACN at $\geq 40\%$ (v/v) in water results in hGH unfolding. When hGH was unfolded in 50% (v/v) ACN and subsequently photoirradiated, a significant reduction (7-fold) of the Glu–Pro cleavage within T9 was observed while the overall loss of native T9, due to other degradation routes, was similar. Instead, we detected the formation of other fragments of T9 with m/z 798.5, 799.5, 885.6, and 886.6

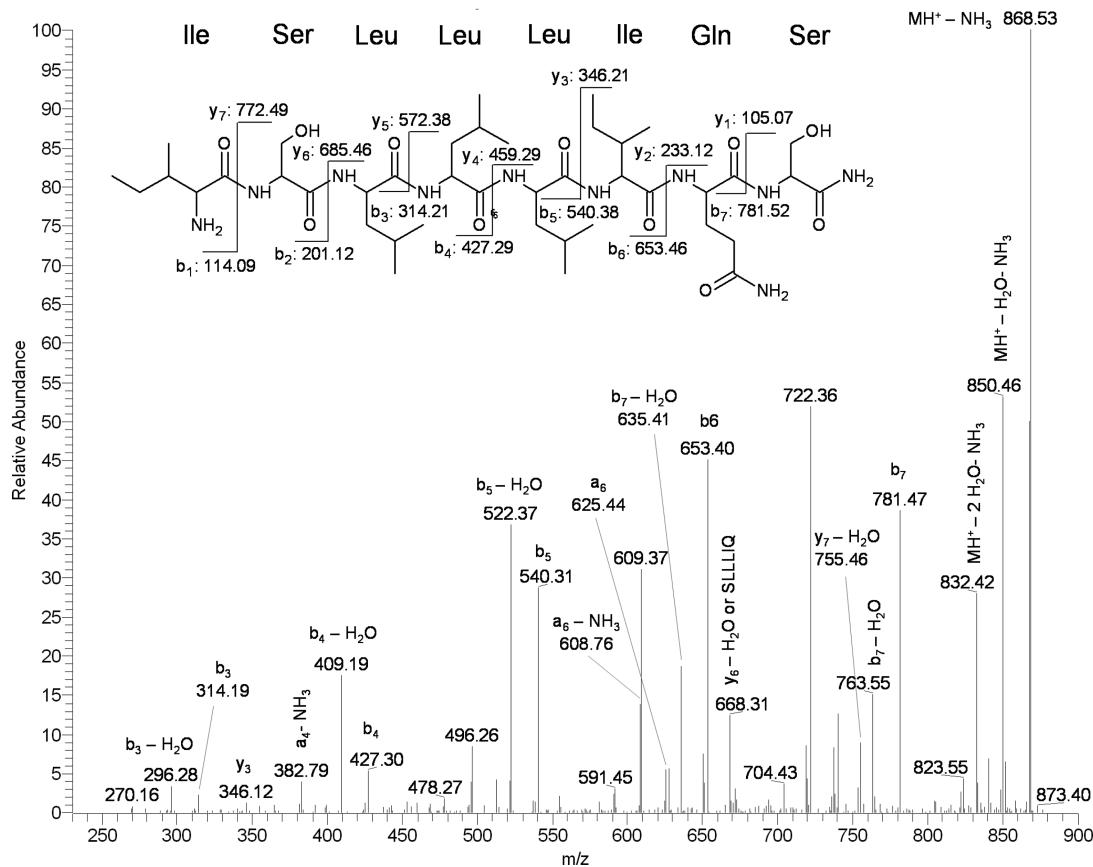
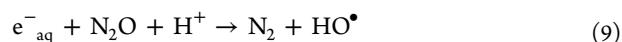


Figure 7. MS/MS spectrum of the singly charged parent ion with m/z 885.58 indicating peptide cleavage between Ser-85 and Trp-86. Conditions as in Figure 2.

(i.e., peptides with an intact N-terminal Ile-78). These products are derived from peptide cleavages between Gln-84, Ser-85, and Trp-86, and the formation of C-terminal free amides and carboxylic acids, respectively, on the N-terminal cleavage fragments. The generation of the C-terminal free amides was inhibited when samples were saturated with Ar, suggesting involvement of carbon- and oxygen-centered radicals. The MS/MS spectra and proposed structures of products are shown in Figures 5–8. We were unable to identify the C-terminal counter fragments (i.e., peptides with an intact C-terminal Arg-94). We then photoirradiated the isolated peptide T9 in aqueous solution, which led to the same cleavage products as the photoirradiation of hGH in 50% (v/v) ACN, that is, no significant cleavage between Glu-88 and Pro-89 but cleavages between Gln-84, Ser-85, and Trp-86. These results suggest that the Glu-Pro cleavage observed for the photoirradiation of native hGH requires an intact protein fold.

Mechanistic Studies. To test whether tryptophan photoionization, and specifically solvated electrons, are involved in the Glu-Pro cleavage, the photoirradiation was carried out in N_2O -saturated solution at pH 7.4 in 0.1 M phosphate buffer for 10 min with 4 RPR-2537 Å lamps. Here, N_2O scavenges hydrated electrons to yield hydroxyl radicals (HO^\bullet) (reaction 9) and hydroxyl radicals can convert tryptophan to hydroxytryptophan (Trp-OH; reaction 10). Under such conditions, we observed a ca. 80% loss of the tryptic fragment T9 compared with nonirradiated samples, together with a rather selective oxidation, yielding two main peaks detected by LC-MS, both with m/z 1036.10 corresponding to doubly charged ions. Tandem MS analysis identified tryptophan oxidation on T9, featuring Trp + 16 Da derivatives. Minor

products formed are Trp + 32 Da and Trp + 4 Da derivatives, likely *N*-formylkynurenine (NFK) and kynurenine (KYN), respectively. The fluorescence spectra after such irradiation are in agreement with the formation KYN and NFK (data not shown). Excitation at 340 nm, to avoid fluorescence contribution from tryptophan and hydroxytryptophan, gives an emission spectrum with $\lambda_{\text{max}} = 435$ nm in agreement with the formation of NFK; excitation at 400 nm, to avoid fluorescence contribution from NFK, gives an emission spectrum with $\lambda_{\text{max}} = 464$ nm, roughly in agreement with the formation of KYN.³¹



The presence of N_2O did not prohibit but reduced the yield of the Glu-Pro cleavage by 69%, that is, from a 14% to a 4% yield relative to the loss of T9, compared with photoirradiation in air. The addition of 5% (v/v) methanol to the N_2O -saturated aqueous solutions of native hGH (in order to scavenge potentially formed HO^\bullet) had no significant additional effect on the cleavage of T9, indicating that any potential reaction of HO^\bullet with T9 did not induce the cleavage into T9a and T9b. Methanol would scavenge free HO^\bullet generating hydroxymethyl radicals (HOCH_2^\bullet), which have reducing properties and would not lead to tryptophan oxidation products in the absence of oxygen.³² The formation of the Trp + 16 Da derivatives was not influenced by the addition of MeOH, suggesting that only HO^\bullet formed in the vicinity of tryptophan are not accessible to MeOH. Irradiation with four RPR-3000 Å lamps, that is, light between $\lambda = 265$ and 340 nm, in quartz tubes or between

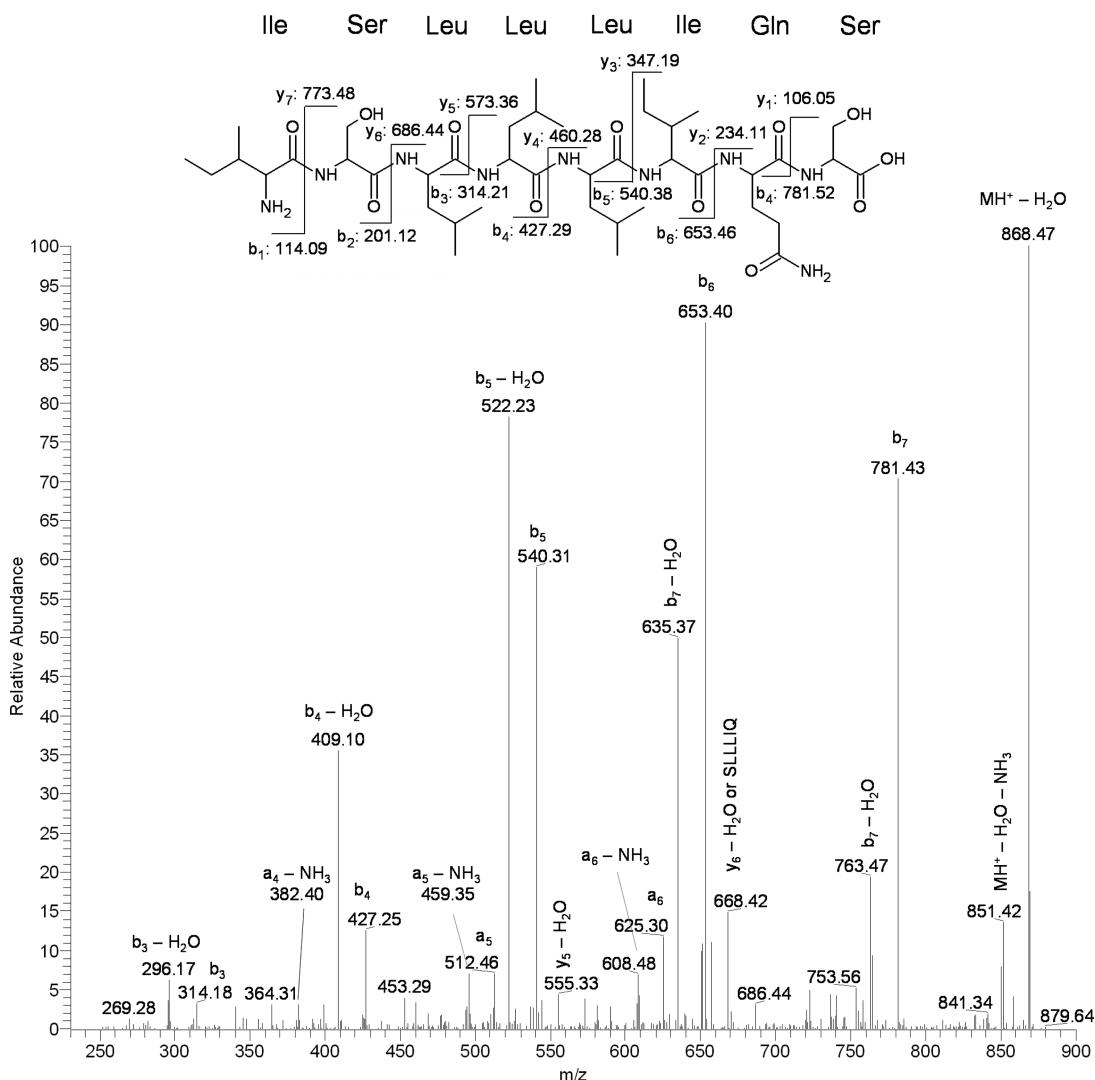


Figure 8. MS/MS spectrum of the singly charged parent ion with m/z 886.56 indicating peptide cleavage between Ser-85 and Trp-86. Conditions as in Figure 2.

$\lambda = 295$ and 340 nm in borosilicate tubes, in the presence of N_2O , led to the degradation of T9 due to tryptophan oxidation to mainly +16 Da derivatives. To exclude any photochemical involvement of the disulfide groups in the Glu-Pro cleavage, the disulfides were reduced with DTT and alkylated with NEM. Photoirradiation at $\lambda = 254$ nm of these reduced and alkylated hGH samples at pH 7.4 in 0.1 M phosphate buffer did not result in any significant change in the photodegradation of T9 compared with unreduced samples, indicating no role of disulfide photolysis in the formation of T9a and T9b.

Additional Degradation Pathways at $\lambda = 254$ nm. The two originally disulfide-linked tryptic peptides, T6-SS-T16 and T20-SS-T21, degraded by ca. 80% (major products are peptides containing reduced cysteine) during 5 min of irradiation of hGH by four RPR-2537 Å lamps compared with nonirradiated samples and are, thus, the two most photolabile moieties. The characterization of products and degradation pathways of these fragments will be reported elsewhere (see also Discussion). The methionine-containing tryptic peptides underwent also significant photo-oxidation, where Met-14, Met-125, and Met-170 were oxidized to methionine sulfoxide derivatives to an extent of 45%, 14%, and 22%, respectively, during 20 min of irradiation with four RPR-2537 Å lamps. In addition, we detected three

degradation products originating from the tryptic peptide T1 (FPTIPLSR), which degraded by ca. 70% during 20 min of irradiation with four RPR-2537 Å lamps at pH 7.4. The MS/MS spectra and proposed structures can be found in Figures 9–11. The major photolysis product displays a m/z value of 839.47. The MS/MS analysis (Figure 9) suggests a loss of 91.08 Da from the Phe-1 residue, that is, transformation of phenylalanine to ethanedial (glyoxal). The two minor products show ca. 1 order of magnitude lower signal intensities compared with the major product with m/z 839.47. One modification with m/z 783.48 can be rationalized by hydrolytic cleavage of Phe-1 from T1. However, the second minor modification, with m/z 781.48, would correspond to the loss of Phe-1 and an additional loss of 2 Da from Pro-2, suggesting the formation of dehydroproline.

■ DISCUSSION

Mechanism of Product Formation. Among all amino acids, tryptophan displays the strongest UV absorbance and is, therefore, a prime site for photo-oxidation.^{2–4,6} However, in our experiments, during which hGH was irradiated with $\lambda = 254$ nm light, the more common photo-oxidation products of tryptophan such as hydroxytryptophan, oxindole alanine, KYN, and NFK were not formed in significant yields unless N_2O was present.

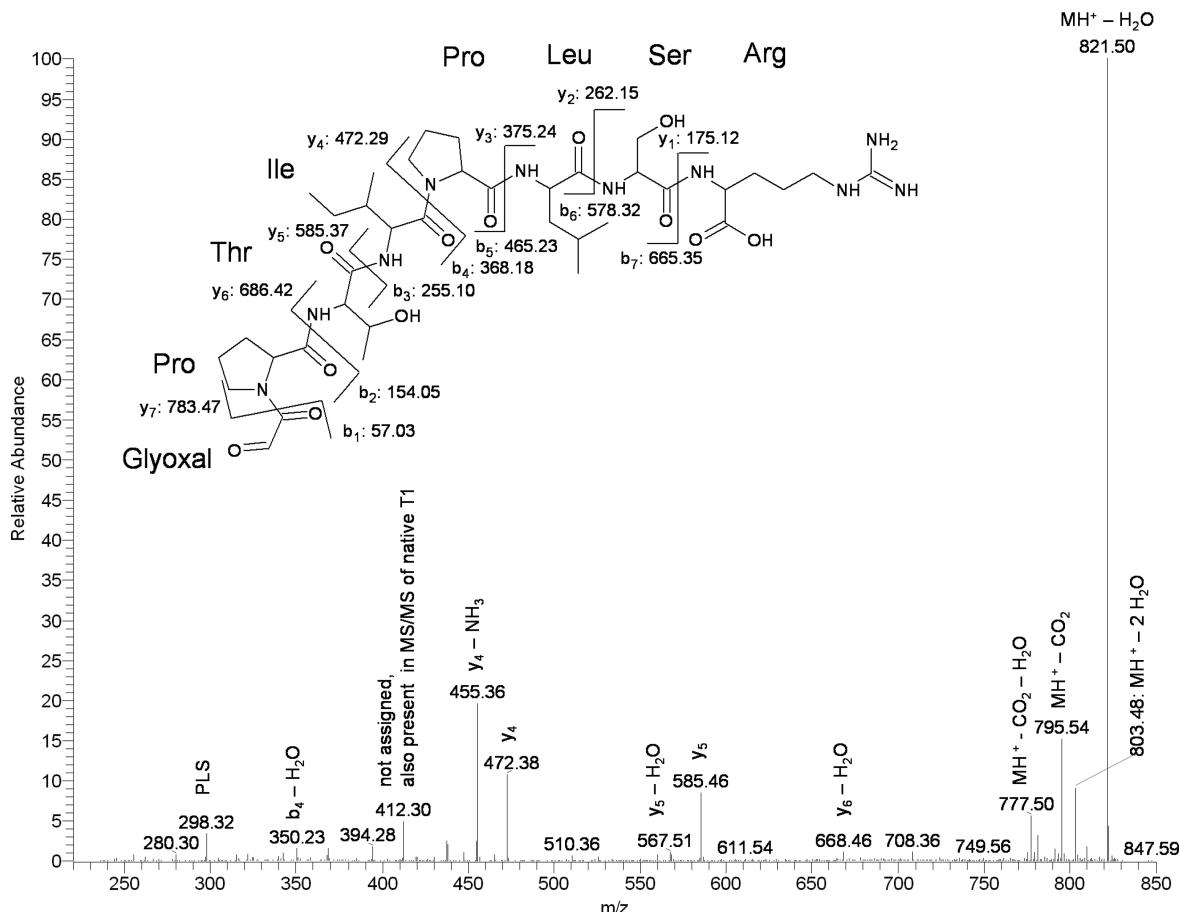


Figure 9. MS/MS spectrum of the singly charged tryptic parent ion with m/z 839.47. The N-terminal derivative of Phe-1 is assigned to ethanedial (glyoxal). Conditions as in Figure 2.

Instead, we observed a novel cleavage of the protein backbone between Glu-88 and Pro-89, two amino acids that are located spatially close to the tryptophan residue (see crystal structure in Figure 12).³³ We suspect that tryptophan acts as a photosensitizer since the absorption of glutamate and proline is negligible at $\lambda = 254$ nm. The cleavage does not depend on oxygen. It is not carried out through hydrophilic oxygen-centered radicals, since we did not detect the Glu-Pro cleavage upon exposure of hGH to oxygen-centered radicals (RO^\bullet , ROO^\bullet), generated by thermolysis of a diazo compound, AAPH.³⁴ However, under such conditions tryptophan oxidation was observed,³⁴ which may proceed directly by oxidation with AAPH-derived oxygen-centered radicals or secondary radicals derived thereof. Thus we propose the mechanism shown in Scheme 1. The photoionization of tryptophan leads to one-electron reduction of the Glu-Pro peptide bond (reaction 17), generating an intermediate amide radical anion (species II). The amide radical anion ultimately releases CO, followed by hydrogen transfer from the original proline residue to the carbon-centered radical on the original glutamate residue. The release of CO can be achieved by a homolytic bond cleavage of the $C_\alpha-C(=O)$ bond accompanied by a heterolytic cleavage of the amide bond $N-C(=O)$ (reaction 18), yielding a C_α -centered radical on Glu-88 (species III) and a free N-terminal Pro-89 (species IV). Such peptide backbone fragmentations are known to also take place as minor pathways during electron capture dissociation in mass spectrometry.³⁵ Since oxygen does not inhibit the Glu-Pro cleavage, the intermediate C_α -centered

radical at glutamate (species III) must rapidly abstract a H-atom (reaction 19) from the spatially close proline residue (species IV) before a reaction with dioxygen can take place. The concentration of oxygen in air-saturated water is about $240-280 \mu M$ in the temperature range of $22-30^\circ C$ measured in the rayonet reactor.³⁶ The rate constant for the reaction of oxygen with C-centered radicals approaches the diffusion-controlled limit, $k \approx 10^9 M^{-1} s^{-1}$.³⁷ Hence, the pseudo-first-order rate constant for the reaction of oxygen with species III would approximately be $k' = 1 \times 10^9 M^{-1} s^{-1} \times (2-3) \times 10^{-4} M = (2-3) \times 10^5 s^{-1}$ provided the effective oxygen concentration within the hydrophobic core of hGH is similar to that in solution. The intramolecular hydrogen abstraction proposed (reaction 19) has, accordingly, a rate constant greater than $3 \times 10^5 s^{-1}$. For intermolecular hydrogen atom transfer between amino acid side chains, rate constants from 10^5 to $10^6 M^{-1} s^{-1}$ were derived.³⁸ Such a hydrogen abstraction reaction is in agreement with our result that the transferred hydrogen does not originate from the solvent, that is, we detected no specific deuterium incorporation into peptide fragment T9a when the reaction was performed in D_2O . This latter experiment also excludes an alternative mechanism of product formation, namely, hydrolysis of the Glu-Pro bond followed by decarboxylation. In D_2O , such a mechanism would have led to the incorporation of one D into the product fragment T9a. In our proposed mechanism, the proline residue (species VI) is ultimately converted to dehydroproline (species VII) through a subsequent electron transfer to the tryptophan radical cation to

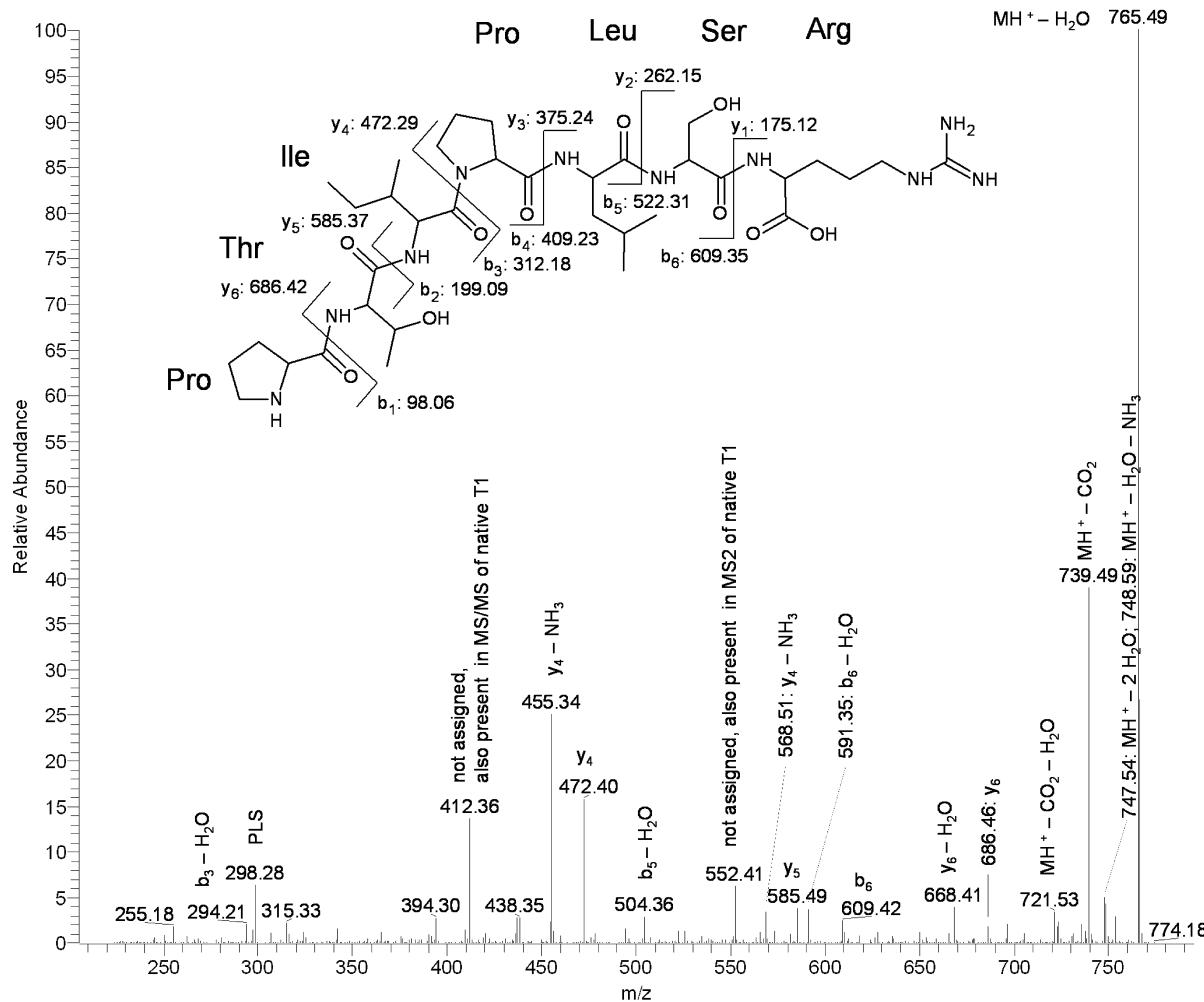


Figure 10. MS/MS spectrum of the singly charged tryptic parent ion with m/z 783.48, indicating peptide bond cleavage between Phe-1 and Pro-2. Conditions as in Figure 2.

regenerate the tryptophan (reaction 16). The latter reaction has to proceed faster than the potential reaction of the proline C-centered radical (species IV) with dioxygen. Alternatively, reactions 19 and 16 may take place in concerted manner.

The saturation of samples with N_2O to scavenge solvated electrons resulted in a significant oxidation of tryptophan to two products, characterized by a mass increase of +16 Da (either hydroxytryptophan or oxyindolealanine) at $\lambda = 254$ nm, $\lambda > 265$ nm, and $\lambda > 295$ nm. We suspect that at all wavelengths, solvated electrons are generated, which generate HO^\bullet by reaction with N_2O (reaction 9), with HO^\bullet capable of initiating tryptophan oxidation (reaction 10). Instead, the yield of the cleavage fragment T9a was significantly reduced in presence of N_2O . This can be rationalized in two ways: first, since the photoionization of tryptophan, followed by reactions 9 and 10, competes with the cleavage reaction, the yield of the latter will be reduced. Second, T9a contains a tryptophan residue, which can react with HO^\bullet to undergo further degradation. However, we exclude that solvated electrons solely induce the Glu-Pro cleavage since it was not completely suppressed by N_2O . A total inhibition of T9a formation would be expected based on the high rate constant of reaction 9, $k_9 = 9.1 \times 10^9 M^{-1} s^{-1}$,³⁹ and the high solubility of N_2O in water, $1.94 \times 10^{-6} mol cm^{-3} atm^{-1}$.⁴⁰ We calculate a half-life for solvated electrons of $t_{1/2} = \ln(2)/(9.1 \times 10^9 M^{-1} s^{-1} \times 1.94 \times 10^{-6} M) = 3.93 \times 10^{-8} s$.

The rate constant of solvated electrons with the amide function in peptide bonds is nearly equally fast, typically $k = (1-4) \times 10^9 M^{-1} s^{-1}$.⁴¹ Therefore N_2O may not scavenge all electrons within the protein. Further, solvated electrons are also generated during irradiation with $\lambda > 254$ nm,⁴² which under our experimental conditions, did not lead to the Glu-Pro cleavage either in the absence or presence of N_2O . Photoionization of tryptophan can occur from either the primary prefluorescent excited state or the fluorescent 1L_a state.⁷⁻⁹ The latter will be populated at $\lambda = 265$ nm and, to a smaller extent, at $\lambda > 295$ nm and is, therefore, not the precursor state leading to the Glu-Pro cleavage. Instead, electron or hydrogen atom transfer from an energetically higher prefluorescent excited state such as $N-H \pi-\sigma^*$ Rydberg state could be involved in the Glu-Pro cleavage.⁴³ Such electron transfer must be direct and not greatly affected by N_2O . Hydrogen atom transfer is less likely to be affected by the presence of N_2O due to the much lower reactivity of hydrogen atoms with N_2O , $k = 2.1 \times 10^6 M s^{-1}$, compared with that of the solvated electron with N_2O ,³⁹ hence, hydrogen transfer might provide a rationale why the Glu-Pro cleavage was not completely suppressed in presence of N_2O . The addition of 5% (v/v) methanol to the N_2O -saturated solution to scavenge HO^\bullet in solution did not influence the photodegradation of T9, which indicates that free HO^\bullet in solution were not involved in the Glu-Pro cleavage nor

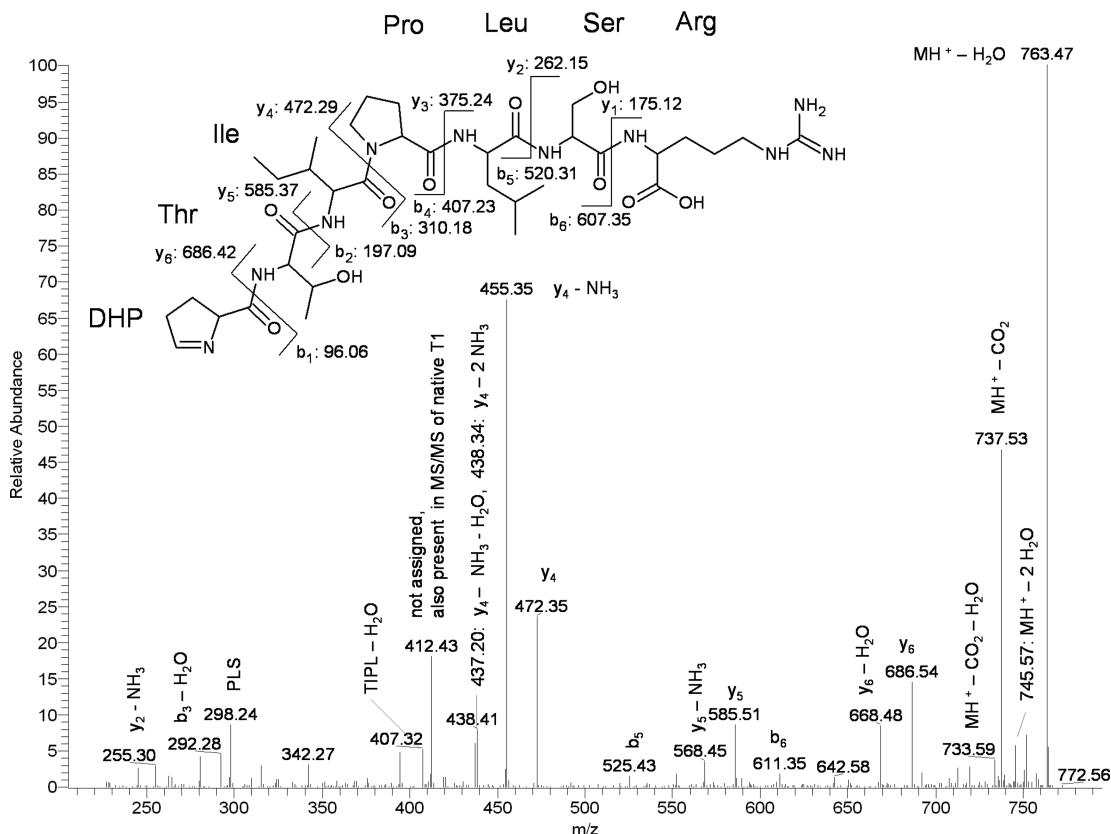


Figure 11. MS/MS spectrum of the singly charged tryptic parent ion with m/z 781.48, indicating peptide bond cleavage between Phe-1 and Pro-2 and double bond formation on Pro-2. The N-terminal derivative of Pro-2 is assigned to a dehydroproline (DHP), here represented by 1,5-dehydroproline; 1,2-dehydroproline is considered as an equally feasible product. Conditions as in Figure 2.

in the oxidation of tryptophan in presence of N_2O . However, we cannot completely exclude that methanol radicals reacted with traces of oxygen to form peroxy radicals, which in turn are capable of oxidizing tryptophan. Methanol has a dipole moment of 1.68 D, while that of N_2O is 0.166 D. Hence N_2O is more likely to diffuse into the hydrophobic core of hGH to react with electrons generated during photolysis. The HO^\bullet may thereby form within the protein and react with tryptophan ($k = 1.3 \times 10^{10} M^{-1} s^{-1}$)³⁹ before they are scavenged by methanol. Noteworthy, a mechanistically similar backbone cleavage N-terminal to proline in singly protonated peptides containing an N-terminal arginine has been observed after photoirradiation with vacuum ultraviolet light at $\lambda = 157$ nm.⁴⁴ Here, this particular cleavage pathway depends strongly on the peptide conformation. At $\lambda = 157$ nm, the $C_\alpha-C(=O)$ bond can be homolytically cleaved in a direct fashion. We did not consider such a process plausible for the Glu-Pro cleavage in hGH due to the low absorbance of the peptide backbone at 254 nm; however, absorbance spectra are subject to the local environment/stereoelectronics, and such a pathway as shown in Scheme 2 cannot be fully excluded, especially if the respective quantum yield is high.

An alternative hypothetical pathway leading to the homolytic cleavage of the $C_\alpha-C(=O)$ bond would be energy transfer from the electronically excited tryptophan to the Glu-Pro peptide backbone. Direct electronic energy transfer from tryptophan to the backbone seems unlikely due to unavailability of energetically feasible electronic transitions onto the Glu-Pro backbone.⁴⁵ However, vibrational energy transfer could take

place via two different pathways. The first pathway is via internal conversion of an electronically excited tryptophan state to the ground state and subsequent intermolecular vibrational redistribution of the internal energy. Since vibrational redistribution is fast compared with the breaking of an activated bond such a pathway is questionable.⁴⁶ Second, vibrational coupling between the electronically excited tryptophan state and ground state could lead directly to localized high-energy vibrations on the peptide backbone. Such coupling of electronic states with local high energy vibrations is believed to play a major role in excitation dynamics in photosynthetic light-harvesting systems,^{47,48} but whether such localized high energy vibrations could lead to $C_\alpha-C(=O)$ bond rupture remains hypothetical.

Significance. We found that the observed Glu-Pro cleavage is highly dependent on the protein fold of hGH, which indicates that a particular spatial arrangement of the amino acids involved is required for the reaction. It thus remains to be shown whether this photolytic degradation pathway is a peculiarity of hGH or of ubiquitous nature. We note that an analogous cleavage pathway may take place between Phe-1 and Pro-2 (Figure 11). We suspect that proline potentially represents a preferred amino acid for such radical anionic cleavage reactions, specifically as Hill et al. demonstrated that Phe-Pro and Pro-Phe dipeptides were by far more photolabile than the Phe-Gly and Gly-Phe dipeptides.¹⁸ Further, it is reported that proline-containing peptides react faster with solvated electrons than expected based on their pK_a values and aliphatic structure.⁴⁹ These observations suggest that

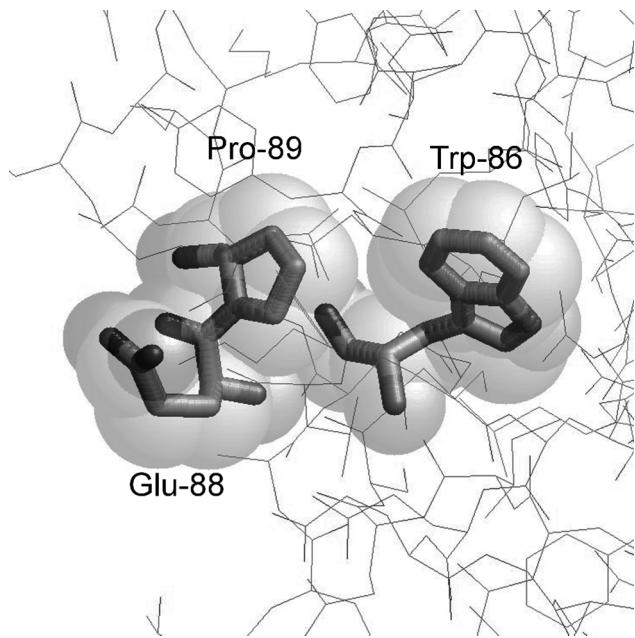


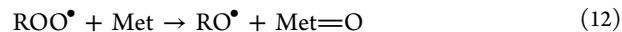
Figure 12. Excerpt from the crystal structure of hGH³³ (PDB 1HGU) displayed with RasMol⁵⁷; Trp-86, Glu-88, and Pro-89 are displayed in the stick and partly transparent spacefill style.

proline is a preferred amino acid for the formation of peptide amide radical anions, and subsequent degradation reactions at proline are more probable than at other amino acids.

Protein backbone cleavage, as observed in this study, represents one of the most critical degradation pathways that can jeopardize the entire integrity of the protein. Such cleavage reactions in proteins may result in the loss of potency, but also in protein aggregation if hydrophobic protein areas of the resulting fragments become solvent-exposed. Photoirradiation around $\lambda = 254$ nm has been considered for the decontamination

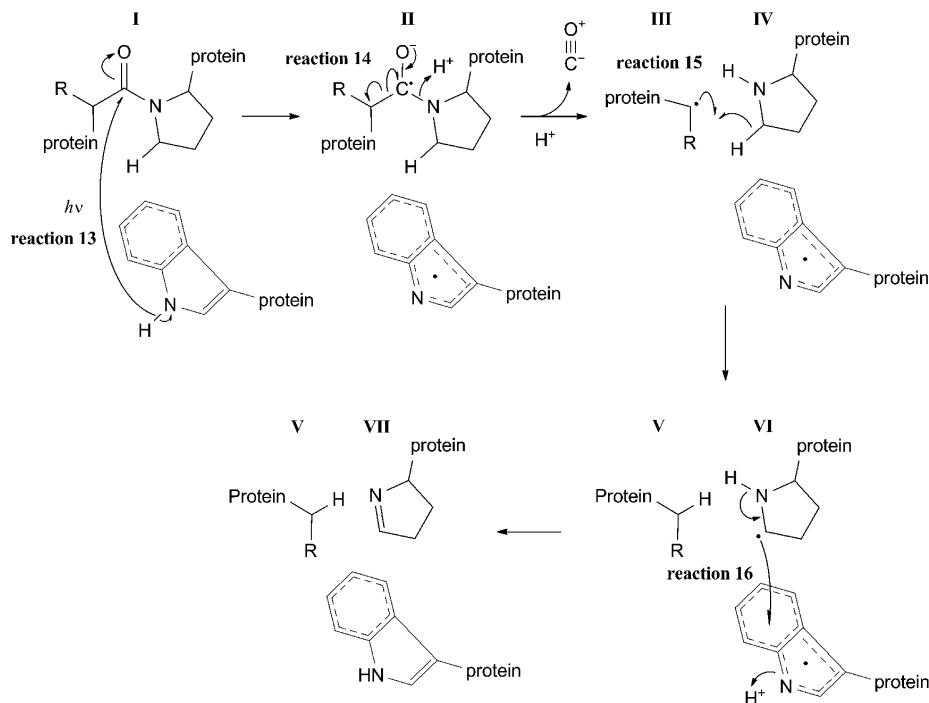
of protein preparations from viruses and, therefore, requires a careful selection of light doses to minimize such photolytic degradation.⁵ In hGH, the cleavage products were only formed during irradiation with $\lambda = 254$ nm light; however, we cannot exclude that similar cleavage reactions take place in other proteins with a different wavelength dependence. Another significant finding is that monochromatic light at $\lambda = 254$ nm with an irradiance of only 8% compared with that suggested in the ICH guideline leads to photodegradation of the protein.

Additional Degradation Products. Besides cleaving the Glu-Pro peptide bond in T9, the photolysis of hGH at $\lambda = 254$ nm degraded the disulfides and methionine residues. At wavelengths $\lambda < 300$ nm, disulfide bonds are homolytically cleaved,^{50,51} which can result in the formation of a multitude of different degradation products, documented with small model peptides.⁵² In proteins, the irradiation at $\lambda = 254$ nm led to the formation of thiols, thiohemiacetals, and thioether linkages.⁵³ Sulfur-centered radicals, formed after irradiation, are likely to abstract hydrogens from C–H bonds, and the resulting C-centered radicals will react with oxygen to yield peroxy radicals (reaction 11). These, in turn, can oxidize methionine (reaction 12),⁵⁴ a prime degradation product observed in our experiments:

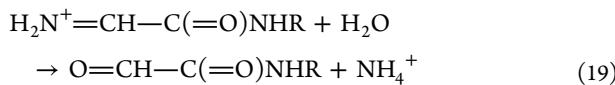
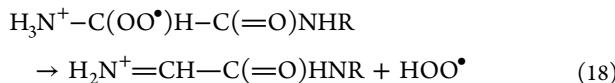
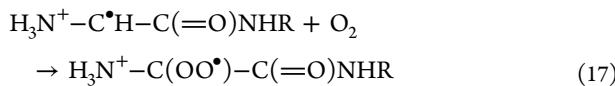
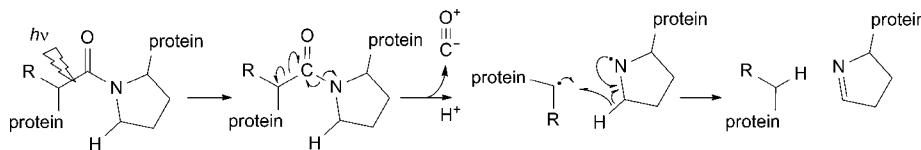


In addition, we noted a cleavage between Phe-1 and Pro-2. We propose the following degradation pathway for the main product with m/z 839.47 (Figure 9): First, the photoirradiation leads to elimination of a benzyl radical (PhCH_2^\bullet) from phenylalanine.⁵⁵ Second, the resulting C_α glycyl radical will react with oxygen to form a C_α peroxy radical (reaction 17), from which HOO^\bullet and NH_4^+ will be eliminated in aqueous solution (reactions 18 and 19).⁵⁶

Scheme 1. Primary Mechanism for Glu–Pro Cleavage



Scheme 2. Alternative Mechanism for Glu–Pro Cleavage



The product with m/z 783.48 may be formed as suggested for the elimination of glycine from the Phe–Gly dipeptide during photolysis.¹⁸ Most interestingly, the photolysis product with m/z 781.46 could be formed in analogy to the Glu–Pro cleavage: photolytically induced electron transfer from Phe-1 to the Phe–Pro peptide bond, followed by release of CO and double bond formation at proline as displayed in Scheme 1.

Irradiation of the unfolded protein at $\lambda = 254$ nm leads to different cleavages within T9, that is, between Gln-84, Ser-85, and Trp-86. The formation of the amide products with m/z 798.53 (Figure 5) and m/z 885.58 (Figure 7) points to an oxidative cleavage mechanism,⁵⁶ which is in agreement with the observation that yields were oxygen dependent. However, since we were unable to identify the C-terminal fragments, we cannot derive a reaction mechanism at present.

CONCLUSION

In conclusion, by means of mass spectrometry, we detected an unexpected peptide backbone cleavage between Glu-88 and Pro-89 in hGH after irradiation at $\lambda = 254$ nm. We hypothesize that electron transfer from Trp-86 to the Glu–Pro peptide bond initiates the fragmentation reaction.

ASSOCIATED CONTENT

Supporting Information

Figure S1 showing the fluorescence of hGH as a function of organic solvent. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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