Reaction of Bovine Growth Hormone with Hydrogen Peroxide†

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ABSTRACT: Methionine residues in bovine pituitary growth hormone were selectively modified with hydrogen peroxide in Tris buffer at pH 8.5 at 25°. The results indicate that three of the four methionine residues were oxidized. The nonoxidized residue was found to be located at position 179 along the polypeptide chain. The oxidized protein was compared to the native hormone as to the relative rates of trypsin digestion, behavior in exclusion chromatography, circular dichroism, ultraviolet difference and fluorescence spectroscopy, and biological activities. The data show that the oxidation is specific, and that both structural and functional parameters are preserved. It was also shown that the pattern of methionine oxidation in sheep pituitary growth hormone on peroxide treatment is identical with that of the bovine hormone.

A previous study from this laboratory reported on the specific modification of tyrosyl residues in bovine and sheep pituitary growth hormones with tetranitromethane (Glaser *et al.*, 1973). It was shown that of the six tyrosyl residues present in each molecule, two were completely modified, two were partially modified, and two were unreacted at identical sites along the linear chain. Various physical, chemical, and biological data showed the close structural similarity of the two modified hormones, both in relation to their parent compounds and to each other.

A study of the accessibility of methionine residues to hydrogen peroxide under various solvent conditions was undertaken in the present work. Several related investigations on protein hormones have been previously reported. In bovine parathyroid hormone (Tashjian *et al.*, 1964), pig adrenocorticotropin (Dedman *et al.*, 1957, 1961; Dixon and Stack-Dunne, 1955), and human calcitonin (Neher *et al.*, 1968), oxidation of methionine residues to the corresponding sulfoxide derivatives was accompanied by significant losses in biological function. In the first two examples cited, restoration of biological activity was achieved *via* reduction with thiol reagents. In addition, the report on bovine parathyroid hormone presented immunological evidence that the loss in biological activity was not accompanied by major conformational alterations. The subject of this report is the selective oxidative of methionine residues in BGH[1] and the effects on functional and structural parameters.

Materials and Methods

The pituitary growth hormones from beef (Li, 1954) and sheep (Papkoff and Li, 1958) pituitaries were isolated according to methods previously described. Each was further purified on Sephadex G-100 (Pharmacia) in 0.05 M Tris buffer (pH 8.5). Glycine and 2-amino-2-(hydroxymethyl)-1,3-propanediol were obtained from Eastman Organic Chemicals, Rochester, N. Y., and used without further purification. Catalase was purchased from Sigma Chemicals, St. Louis, Mo. Iodoacetic-*1*-^{14}C acid was obtained from International Chemical and Nuclear Co., Irvine, Calif. Urea was purchased from Mallinckrodt Chemical Co., St. Louis, Mo., and was recrystallized from 95% ethanol before use. Dioxane was obtained from Matheson Coleman and Bell, East Rutherford, N. J. Toluene-^{14}C was received from Packard Instrument Co., Downers Grove, Ill. All other chemicals were reagent grade. Deionized water was used throughout.

Kinetics of the Oxidation Reaction. The protein was dissolved (~5 mg/ml) in the appropriate solvent containing 0.0005 M EDTA, and hydrogen peroxide (30%) was added to a final concentration of 0.3 M. The solution was stirred at 25°. At various times, 0.2-ml aliquots were removed and added to a solution containing 333 units of catalase in 1 ml of phosphate buffer at pH 7.0; one unit of the enzyme will hydrolyze 1 μmol of H_2O_2/min at pH 7.0 and 25°. After ~10 min the solution was dialyzed against 0.001 N $HClO_4$ and lyophilized. Specific alkylation of the nonoxidized methionine residues was achieved by the addition of 2.75 ml of 8 M urea (pH 3.5) containing 15.1 mg of iodoacetic-*1*-^{14}C acid (sp act. = 4.45×10^{-2} Ci/mol) according to the procedure of Wallis (1972). After 48 hr at 37°, the reaction was terminated by extensive dialysis against 0.1 N glycine-HCl buffer of pH 3.6 until the radioactivity in the outside solution was reduced to that of buffer alone. After measuring the protein concentration of each solution, its radioactivity was determined on a Packard Model 3320 Tri-Carb liquid scintillation spectrometer as follows: 0.5 ml of the protein solution in the glycine buffer was added to 10 ml of scintillant mix (1 l. of dioxane, 143 g of naphthalene, 4.5 g of 2,5-diphenyloxazole, and 0.58 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene). The counting efficiency in this system was determined using toluene-^{14}C as a standard and found to be 78%.

Preparation of Peroxide-Oxidized BGH. The protein (120 mg, 4.74 μmol) was dissolved in 25 ml of Tris buffer (0.05 M, pH 8.5) containing 0.0005 M EDTA at 25°. Hydrogen peroxide was added to a final concentration of 0.3 M. The reaction mixture was stirred for 5 hr and then submitted to gel filtration on a Sephadex G-25 column. After dialysis and lyophilization of the unretarded material, the dimeric product was separated from higher molecular weight aggregates by exclusion chromatography on Sephadex G-100 (3 × 68 cm) in the same buffer. After dialysis the product was obtained on lyophilization.

Amino acid analyses, rate of tryptic digestion, circular dichroism, and ultraviolet fluorescence spectra were performed by procedures previously described (Glaser *et al.*, 1973). The protein concentration of the unmodified hormone was obtained using a value $E^{1\%}_{1\text{ cm},277\text{ nm}} = 7.30$ as determined

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[1] Abbreviations used are: BGH, bovine pituitary growth hormone; SGH, ovine pituitary growth hormone; CD, circular dichroism.

previously (Bewley and Li, 1972). The same value was employed for peroxide-oxidized derivatives of BGH and SGH.

Autoradiographic Procedure for Identification of Non-oxidized Methionine. Peroxide-oxidized BGH (3.0 mg) was allowed to react with 3.0 mg of iodoacetic-*1*-^{14}C acid (sp act. = 3.4×10^{-1} Ci/mol) as described above. The product was dissolved in 0.1 M NH_4OAc of pH 8.5 and digested with trypsin (enzyme/substrate ratio, 1/25) for 24 hr; the lyophilized material was submitted to two-dimensional chromatography–electrophoresis. Chromatogtaphy was performed on Whatman No. 3MM paper with a solvent system consisting of *n*-butyl alcohol–acetic acid–water (4:1:5, v/v), and high voltage electrophoresis in formic acid–acetic acid–water buffer of pH 2.1 for 1 hr at 2000 V (Gilson high voltage electrophoretor, Model D). The chromatogram was spotted in the corners with a solution of iodoacetic-*1*-^{14}C acid, put into direct contact with Du Pont Cronex X-ray film for 60 hr and developed with a Kodak RP-x-omatic processor. Only one radioactive spot was visible on the film. Treatment of the peptide map with ninhydrin gave a positive response corresponding exactly to the radioactive spot. After elution with 0.1 N NH_4OH the solution was lyophilized and hydrolyzed for amino acid analysis.

Bioassay. The growth promoting potency of the modified protein was estimated by the rat tibia test (Greenspan *et al.*, 1949).

Results

Oxidation Reaction. The rate of oxidation of methionine residues in BGH under various conditions is illustrated in Figure 1. The rapid oxidation of all four methionine residues was achieved at pH 2.85, while in solutions of pH 8.5 and 11.5 only three out of four residues were affected. In basic media, complete oxidation was achieved only in the presence of 8 M urea.

Exclusion chromatography of the product formed in the Tris buffer (without denaturant) on Sephadex G-100 gave a dimeric fraction with a V_e/V_0 ratio of 1.79 in 60% overall yield. This compares with a V_e/V_0 ratio of 1.60–1.65 for the dimer fraction of BGH (Bewley and Li, 1972). The observed retardation in the elution pattern may be due either to absorption of the protein onto the gel or to a slight shift in equilibrium toward the monomeric form. All experiments described below were carried out on this dimeric fraction.

Amino Acid Analysis. The amino acid analyses (for experimental conditions for hydrolysis, see Glaser *et al.*, 1973) of the oxidized derivatives prepared at pH 2.85 and at 8.5 are given in Table I; the values are in good agreement with native material. The methionine and methonine sulfoxide contents were determined as follows: the H_2O_2-treated protein was alkylated with iodoacetic acid and then performic acid oxidation was employed to convert methionine sulfoxide residues to the corresponding sulfone. The content of methionine sulfone as determined by amino acid analysis gave the number of methionine residues which were oxidized by H_2O_2. The results from this procedure confirmed those from liquid scintillation counting. Three methionine sulfone and 0.7–0.8 methionine residue (methionine plus homoserine plus homoserine lactone) were obtained for each oxidized protein derivative.

Identification of the Unreacted Methionine Residue. A single peptide is clearly visible on the autoradiograph.[2] On elution and amino acid analysis, the composition[3] was found to be $Lys_{1.0}Val_{1.0}Met_{0.7}$. From the reported sequence[4] of BGH (Fernandez *et al.*, 1972) and SGH (Li *et al.*, 1972) it is clear the isolated peptide is Val-Met-Lys. Methionine-179 is thus resistant to the oxidation in the Tris buffer. Since it has been established that BGH at pH 8.5 exists as a dimer (Bewley and Li, 1972), it was interesting to compare this result to the oxidation at pH 11.5, where the protein is completely in a monomeric form. Autoradiography was carried out exactly as described for the Tris product, and the results were identical. Therefore, in basic media, Met-179 was not available for attack by hydrogen peroxide. On the other hand, in 8 M urea at pH 8.5 or in a solution of pH 2.85, all methionine residues were oxidized (see Figure 1).

Rate of Tryptic Digestion. The relative rates of proteolysis of BGH, as measured by alkali uptake, H_2O_2-treated BGH (prepared in the Tris buffer), and performic acid oxidized BGH were compared. It was found that the H_2O_2-treated

TABLE I: Amino Acid Analyses of Native and H_2O_2-Oxidized Bovine Pituitary Growth Hormone.

Amino Acid	Native BGH	H_2O_2-Treated BGH	
		pH 8.5[b]	pH 2.85[c]
Tryptophan[a]	1	1	1
Lysine	10.4	9.8	10.1
Histidine	3.1	3.1	2.9
Arginine	12.2	10.8	11.4
Aspartic acid	16.6	16.6	16.6
Threonine	13.5	12.0	12.8
Serine	11.4	11.6	11.6
Glutamic acid	22.9	23.6	23.6
Proline	5.8	6.5	5.9
Glycine	11.2	11.2	10.9
Alanine	12.8	13.8	14.0
Half-cystine	3.6	3.8	4.4
Valine	6.4	6.5	5.8
Methionine	3.9	0.8[d]	0.7[d]
Isoleucine	7.6	6.9	7.4
Leucine	26.2	25.8	25.8
Tyrosine	5.9	5.4	6.2
Phenylalanine	12.4	12.5	13.0
Methionine sulfone	0	3.0[d]	3.0[d]

[a] Determined from the fluorescence at 350 nm after tryptic digestion in comparison with the tryptic digest of native BGH. [b] Oxidation was performed at 25° in 0.05 M Tris (pH 8.5) for 5 hr. [c] Oxidation was performed at 25° in perchloric acid (pH 2.85) for 5 hr. [d] Determined from a separate experiment. The proteins were alkylated with iodoacetic acid and then performic acid oxidation was employed to convert the methionine sulfoxide residues to the corresponding sulfone. The methionine values represent the summation of methionine + homoserine + homoserine lactone.

[2] The autoradiograph was submitted to the scrutiny of the reviewers and the authors would be glad to forward it to any interested reader.

[3] The value for methionine is a summation of the values for methionine, homoserine, and homoserine lactone.

[4] The amino acid sequence in the region of methionine-177 in BGH (Fernandez *et al.*, 1972) is completely homologous to that of methionine-179 in SGH (Li *et al.*, 1972). The discrepancy in numbering is a result of small differences in the reported amino acid sequences and total number of amino acid residues for these proteins. Since the reported sequence of BGH was not complete, positions of amino acid residues are taken from the SGH structure.

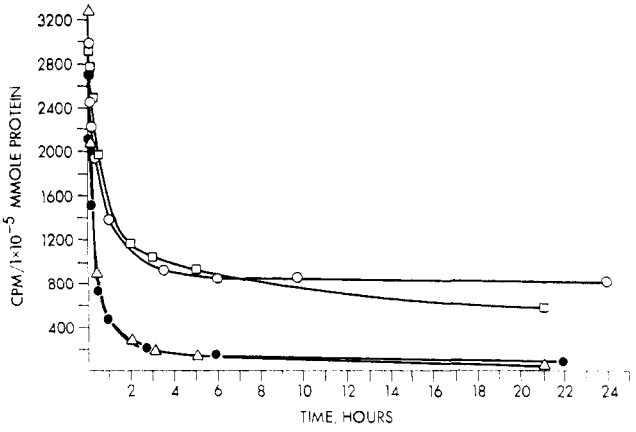

FIGURE 1: Rate of oxidation of methionine residues in BGH by 0.3 M H_2O_2 at 25°: (○) 0.05 M Tris (pH 8.5); (□) 0.1 M phosphate (pH 11.5); (●) perchloric acid (pH 2.85); (△) 0.05 M Tris containing 8 M urea (pH 8.5). The kinetics are followed by the subsequent reaction of nonoxidized methionine residues with iodoacetic-*1*-^{14}C acid in 8 M urea (pH 3.5).

protein was digested at about the same rate as native BGH, while performic acid oxidized material was digested at a considerably more rapid rate.

Circular Dichroism. The CD spectra of the oxidized BGH at pH 8.2 and the native hormone spectra were identical within experimental error. From the ellipticity values at 221 nm, α-helix contents have been calculated to be approximately 50%. In the region of side-chain absorption, the two spectra were again very similar, showing nearly identical bands.

Fluorescence. The relative fluorescence intensity of BGH and its methionine-oxidized derivative in the Tris solvent showed no change in the region of tryptophan fluorescence within experimental error. In order to examine the comparative behavior of these proteins in the presence of a reductant, dithiothreitol was added in large excess (1000-fold over disulfide bridges) and the increase in fluorescence intensity at 330 nm with time was noted (Figure 2). The native material increased fluorescence considerably more slowly than the modified material. The wavelength of maximum fluorescence does not change upon reduction.

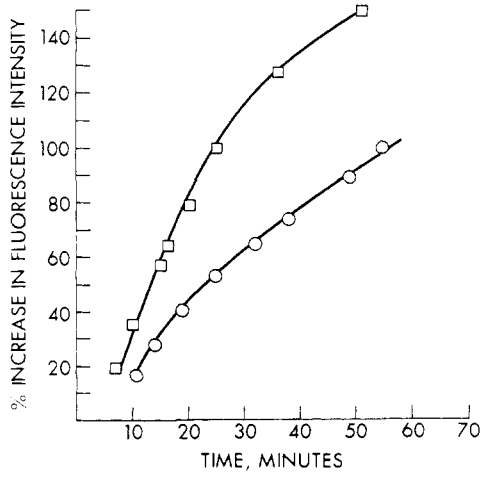

FIGURE 2: Rate of increase in the fluorescence intensity at 330 nm after addition of 2 mg of dithiothreitol to 2.5 ml of a protein solution (0.14 mg/ml) in 0.05 M Tris (pH 8.5) at 25°: (○) BGH; (□) BGH after oxidation with 0.3 M H_2O_2 in the same Tris solvent. An immediate quenching effect (5%) is observed on addition of the reducing agent.

TABLE II: Growth-Promoting Activity of BGH and Its Oxidized Products as Measured by the Rat Tibia Test.

Preparation	Total Dose (μg)	Tibia Width[d] (μ)
BGH	20	214 ± 10 (5)
	60	270 ± 17 (5)
BGH–urea[a]	60	261 ± 23 (4)
BGH–O[b]	20	227 ± 17 (3)
	60	284 ± 11 (5)
BGH–O[c]	60	211 ± 11 (4)
Saline	0	167 ± 12 (5)

[a] Treated with 8 M urea as in footnote *c* in the absence of H_2O_2. [b] The oxidation was carried out in 0.05 M Tris buffer (pH 8.5) at 25° for 5 hr. [c] The oxidation was carried out as in footnote *b* in the presence of 8 M urea. [d] Expressed as the mean ± standard error of the mean followed by the number of test animals in parentheses.

Biological Studies. Table II shows the tibia assay responses. Oxidized hormone prepared in the Tris buffer shows complete retention of its growth promoting activity when compared to native material. Good biological potency was also found when the hormone was incubated with urea; however, oxidation in 8 M urea solution caused a loss of biological potency.

Discussion

In Tris buffer of pH 8.5, three out of four methionine residues in BGH were selectively oxidized by H_2O_2 to the corresponding sulfoxide residues. Autoradiographic procedures show that the nonreacting methionine residue was located in position 179 along the peptide chain. Apparently, the region around this methionine was sufficiently hydrophobic to shield this residue from the oxidizing media. Half-cystine residue in position 181 may be an important factor in the lack of accessibility of the neighboring methionine to the solvent. The existence of BGH as a dimer in this buffer does not serve to explain the nonreactivity of methionine-179, as the same results were obtained in the phosphate buffer of pH 11.5 where BGH had previously been shown to exist in a monomeric form (Bewley and Li, 1972). However, solutions of pH 2.85 and 8 M urea did bring about a normalization of methionine residues to the extent that all four methionines were accessible to the oxidizing reagent.

Previous studies have demonstrated the many physical, chemical, and biological similarities of BGH and SGH (Bewley and Li, 1972; Glaser *et al.*, 1973; Li *et al.*, 1972; Fernandez *et al.*, 1972). In further support of this view, treatment of SGH with hydrogen peroxide in the Tris buffer (pH 8.5) gave identical results to those obtained with BGH, *i.e.* three out of four methionine groups were oxidized, and the unreacted methionine residue was shown by autoradiography to be residue 179.

The properties of the H_2O_2-treated BGH (Tris buffer, pH 8.5) were remarkably similar to those of the native hormone. For example, the initial rate of proteolytic digestion has been proposed as a measure of the protein flexibility (Markus, 1965). Protein oxidation, by either hydrogen peroxide or performic acid, does not affect lysine or arginine residues. Therefore, the relative rates of tryptic digestion of BGH and its oxidized derivatives should be a measure of the availability

of peptide bonds for digestion. The equal rate of digestion of BGH and its mildly oxidized derivative indicates that no major conformational change to a less compact form has occurred. This is in contrast to the performic acid oxidized material, which digests at a considerably more rapid rate.

The chemical modification of methionine groups does not directly affect chromophoric residues. Therefore, any secondary effects of the modification on the environment of these residues can be probed by a variety of comparative studies, including circular dichroism, ultraviolet spectroscopy, and fluorescence spectroscopy. The amide region of the CD spectra of BGH and its methionine oxidized derivative indicate that the secondary structures of both proteins were essentially identical with an α-helical content of $\sim 50\%$. Similarly, the close correspondence of bands in the region of side-chain absorption showed the asymmetric microenvironment about the various side-chain chromophores remains unchanged upon oxidation.

The absence of any difference spectrum in Tris buffer between BGH and its oxidized derivative was another good indication that the modification had not caused any substantial change in the environment of aromatic residues. Likewise, the identical fluorescence spectra of oxidized BGH in comparison to the native protein were evidence that the chemical modification had neither oxidized tryptophan residues nor resulted in a changed microenvironment around this residue. However, a difference was noted in the fluorescence at 330 nm (Figure 2) when these two proteins were treated with a large excess of dithiothreitol. The greater rate of increase in the derivative is indicative of a loss of rigidity about tryptophan. This may reflect a higher rate of unfolding and/or a higher rate of disulfide bond reduction of the oxidized derivative in comparison to the native hormone.

The oxidized hormone in which three out of four methionine residues were modified was fully active as measured by the rat tibia assay. When the fourth methionine residue in position 179 was oxidized in the presence of 8 M urea, the growth promoting potency was diminished (Table II). This suggests a possible role for Met-179 in the biological activity.

In a related investigation on the nature of methionine residues in BGH, Wallis (1972) has presented data showing all four methionine residues are alkylated with excess iodacetic acid in acidic media (0.2 M sodium formate (pH 3.6) with or without 8 M urea). By using iodoacetic-*2*-^{14}C acid in a twofold molar excess over protein (0.5 μmol of alkylating agent/μmol of methionine), it was also demonstrated that methionine II (*i.e.*, methionine residue 124) was considerably less reactive than any of the others, both in the presence and absence of denaturant. These results, in conjunction with our own, show that in going from acidic to basic media, conformational changes must occur which shield methionine-179 from the reaction media.

The experiments described in this report suggest a method currently under investigation for the preparation of protein fragments of BGH and SGH consisting of residues 1–179 and 180–191. Modification of methionine residues with hydrogen peroxide in the Tris buffer can be followed by a cyanogen bromide cleavage at methionine-179 (Gross, 1967). The free methionine residues in the 1–179 fragment can then be regenerated by reduction with thiol reagents. The purified fragments may then be studied by many of the methods described in this article.

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