Oxidation of Recombinant Human Parathyroid Hormone: Effect of Oxidized Position on the Biological Activity

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Purpose. To determine the oxidation products of recombinant human parathyroid hormone (rhPTH) treated with H_2O_2, the amino acid residue oxidized, and the biological activity of the oxidation products.
Methods. Oxidized residues were determined by CNBr cleavage, trypsin digestion and subsequent fast atom bombardment mass spectrometry. The biological activity of each oxidized rhPTH was examined in rat osteosarcoma cell adenylate cyclase assay.
Results. Three oxidized products were isolated, namely, Met at position 8 (Met8) sulfoxide, Met at position 18 (Met18) sulfoxide and both positions Met sulfoxide. It appears that the Met8 and Met18 oxidized forms are intermediates in the generation of the Met doubly oxidized form. All oxidized forms possessed reduced biological activity, more so for oxidation at Met8 than at Met18.
Conclusions. The region around Met8 is important for the activity of the parathyroid hormone.

KEY WORDS: recombinant human parathyroid hormone; methionine oxidation; adenylate cyclase activity; FAB-MS.

INTRODUCTION

Human parathyroid hormone (hPTH), is a peptide hormone of 84 amino acid residues (1). It regulates calcium homeostasis in serum by stimulation of calcium resorption in kidney and by enhancing resorption of calcified bone matrix (2). In addition, it also stimulates bone-forming processes (3). Thus, hPTH is expected to be effective against osteoporosis and as a consequence, several therapeutic studies have been under investigation (4).

From the pharmaceutical point of view, there is interest in the stability of this hormone under the conditions of isolation or storage. Degradation of this polypeptide can occur via the oxidation of Met residues. hPTH has two Met residues at the positions of 8 (Met8) and 18 (Met18) (1), however information concerning Met oxidation in hPTH has never been elucidated.

In the present study, we examined oxidations of hPTH by H_2O_2 using a recombinantly produced peptide (rhPTH). We determined the oxidized position, time course of oxidation and the change of biological activity.

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ABBREVIATIONS: FAB-MS-fast atom bombardment mass spectrometry; hPTH-human parathyroid hormone; rhPTH-recombinant human parathyroid hormone; TFA-trifluoroacetic acid.

MATERIALS AND METHODS

Production and Purification of rhPTH

rhPTH was produced by the method of Gardella et al. (5). In brief, rhPTH was obtained as a fusion protein with human growth hormone from *E. Coli* which contained the hPTH expression vector pGTP-1. A linker containing the recognition sequence of thrombin was used to join the two regions. rhPTH was released from this fusion protein by cleavage with thrombin. Subsequently, the cleaved product was purified by reversed-phase HPLC. Biological activity of rhPTH produced by this method was similar to that of synthetic hPTH and native intact hPTH (6,7).

Oxidation of rhPTH

rhPTH (40 μM) was incubated with 1mM H_2O_2 (Santoku Chemical Industries, Tokyo, Japan) in 100mM borate-NaOH-HCl buffer (pH 10.0). All oxidation reactions were carried out at room temperature. Oxidation was stopped by injection into HPLC.

HPLC Separation of Oxidized rhPTH

All HPLC separations were carried out using Waters HPLC system (Waters, Tokyo, Japan) which includes a pump (type 600), a automatic sample injector (WISP 712), a UV detector (type 490, fixed at 215 nm) and a data module (type 740). rhPTH and oxidized products were purified by a YMC-Pack ODS-A A312 column (150x 6.4 mm, YMC, Kyoto, Japan) using a water/acetonitrile/trifluoroacetic acid (TFA) solvent system. Solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in 60% acetonitrile/water. The column was equilibrated with 40% B, eluted with 40% to 50% B for 10 min and then with 50% to 60% B over 40 min by linear gradient at a flow rate of 1 ml/min. The separated fractions were collected and lyophilized.

CNBr Cleavage

rhPTH and oxidized products (20 μg) were dissolved in 20 μl of N_2-saturated 0.1N HCl, and CNBr (Nakarai Tesque, Kyoto, Japan) was added to give a final concentration of 5 μg/μl. The reaction mixture was kept for 18h at room temperature.

Trypsin Digestion

rhPTH and oxidized products were dissolved in 1% ammonium bicarbonate (pH7.8) at a concentration of 1 mg/ml. TPCK-treated trypsin (COOPER Biomedical, Malvern, PA, USA) was added and incubation was carried out at 37° C for 16h at a substrate:enzyme ratio of 50:1 (w:w).

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Fast Atom Bombardment Mass Spectrometry (FAB-MS)

FAB-MS spectra were obtained with a 70-250SEQ double-focusing mass spectrometer (VG Analytical, Manchester, UK), equipped with a xenon FAB ion source. The FAB gun was operated at 8kV potential. A resolving power of 2000 at 10% of peak height was employed at an acceleration potential of 8kV. The matrix used was thioglycerol containing a small amount of TFA. About 1 μl of a given sample was added to the matrix.

Biological Activity

Adenylate cyclase activity in ROS 17/2.8-5 rat osteosarcoma cells was measured by the method of Sato et al. (8). Concentration of produced cAMP was determined by radioimmunoassay (cAMP Kit, Yamasa, Chiba, Japan). Amounts of intact and oxidized rhPTH were measured by means of amino acid analysis (Pico Tag Work station, Waters, Tokyo, Japan).

RESULTS

Time Course of Oxidation

In the process of oxidation by H_2O_2, four peaks were detected on a reversed-phase HPLC chromatogram (Fig. 1). These peaks were named A, B, C, and D (D was intact rhPTH) in their order of elution. The time courses of production of the four peaks are shown in Fig. 2. Peaks B and C increased until 180min, then decreased gradually. The amount of peak C was always larger than that of peak B. Peak A appeared slowly and continued to increase with time to become the major component.

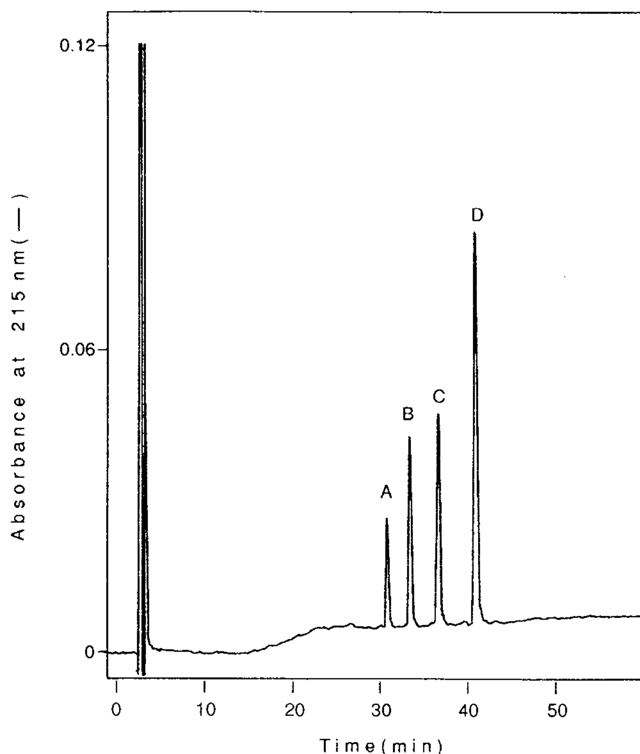

Fig. 1. Separation of the oxidized forms of rhPTH on HPLC. rhPTH (40μM) was treated with H_2O_2 (1mM) in 100mM borate-NaOH-HCl buffer (pH10.0) for 80min at room temperature. Peaks were named A, B, C and D in their order of the elution (D was intact rhPTH).

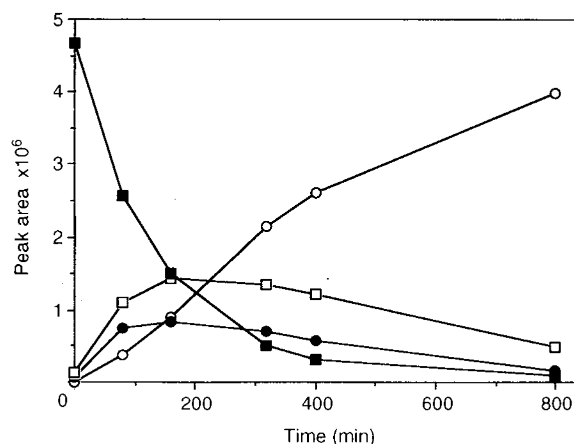

Fig. 2. Time course of the oxidation of rhPTH. rhPTH (40μM) was treated with H_2O_2 (1mM) in 100mM borate-NaOH-HCl buffer (pH10.0) at room temperature. Amounts of the products were represented as the peak area of the HPLC analysis. Open circle, peak A; closed circle, peak B; open square, peak C; closed square, peak D.

CNBr Cleavage of Oxidized Products

To determine the oxidized positions, the fractionated four peaks (A-D) were cleaved by CNBr and the products analyzed by FAB-MS without any purification steps. In mass spectra, CNBr cleaved peptide fragments were predominantly detected as a homoserine lactone form but in some instances, a small quantity of homoserine form was also observed. The summarized results are shown in Table I (mass values of the homoserine lactone form were employed). In peak A, no signal was detected in this analysis. This indicated that cleavage had not occurred at either Met position, thus peak A was assigned as rhPTH oxidized at both Met8 and Met18 which prevented cleavage. In peak B, only one peptide (m/z 2006.0) was observed which was designated as CN3 plus a 16 mass unit. Therefore, oxidation took place at position Met8 so as to prevent cleavage at this position whereas cleavage at Met18 was detected. In peak C, a signal m/z 858.6, which corresponds to the mass of peptide 1-8, was observed whereas cleavage at peptide position at Met18 was not detected. Thus, in peak C, oxidation had occurred at Met18 so as to prevent cleavage at this site.

Trypsin Digestion of Oxidized Products

The fractionated four peaks (A-D) were digested by trypsin and subsequently analyzed by FAB-MS, respectively. Table II shows the FAB-MS analysis of these peaks. Sixteen mass unit increments compared with the theoretical values of rhPTH were observed for peptides T1 and T2 in peak A. A similar mass unit increment was observed for peptide T1 with peak B and peptide T2 with peak C, respectively. In the other peptide fragments, no molecular weight change was observed.

Peptide T1 contains Met, at position 8 whereas T2 also contains Met, at position 18 (Fig. 3). Moreover, the observed

Table I. Mass values of CNBr cleaved products of oxidized rhPTH

Peptide	Position	Theoretical mass	Observed mass value (MH^+)			
			Peak A	Peak B	Peak C	Peak D
CN1	1–8	858.5	n.d.[a]	n.d.[a]	858.6	858.4
CN2	9–18	1102.6	n.d.[a]	n.d.[a]	n.d.	1102.5
CN3	1–18	1990.0[b]	n.d.[a]	2006.0[b]	n.d.	1989.2

[a] "n.d." represents not detected.
[b] Underlined value has 16 mass unit increment against theoretical mass value.

Table II. Mass values of tryptic digests of oxidized rhPTH

Peptide	Position	Theoretical mass	Observed mass value (MH^+)			
			Peak A	Peak B	Peak C	Peak D
T1	1–13	1455.8	1471.7[a]	1471.8[a]	1455.5	1455.9
T2	14–20	886.4	902.0[a]	885.9	902.2[a]	886.1
T3	21–25	702.4	702.1	702.0	702.5	702.1
T4	27–44	1946.1	1945.9	1946.1	1945.8	1946.1
T'4	28–44	1818.0	1818.4	1818.6	1818.3	1818.4
T5	53–65	1554.8	1554.8	1554.9	1554.7	1555.2
T6	66–80	1559.8	1559.9	1560.0	1559.7	1560.1

[a] Underlined values have 16 mass unit increment against theoretical mass values.

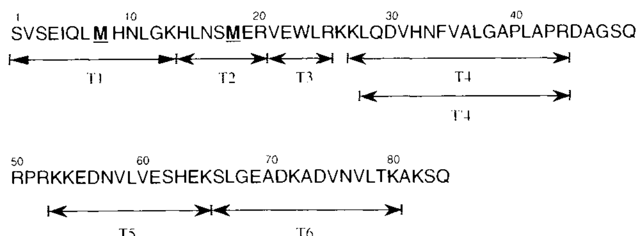

Fig. 3. Amino acid sequence of rhPTH. Tn show tryptic peptides observed in FAB-MS. Positions of Met are underlined.

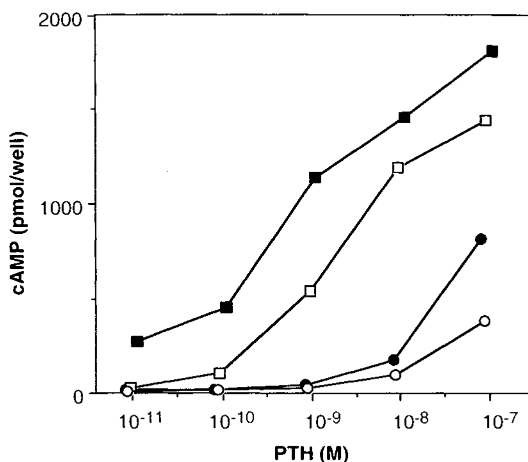

Fig. 4. Biological activities of oxidized products of rhPTH in ROS 17/2.8-5 rat osteosarcoma cell adenylate cyclase assay. The number of plated cell was 1×10^5 cells/well. cAMP concentration was determined in duplicate. Open circle, peak A; closed circle, peak B; open square, peak C; closed square, peak D.

molecular weight change by oxidation was 16. Therefore, all oxidized products, peaks A, B and C, were identified as a Met sulfoxide where oxidation occurred at: Met8 and Met18 in peak A, Met8 in peak B, and Met18 in peak C, respectively. In these peaks, other amino acid residues apart from Met were not oxidized by H_2O_2 under our experimental conditions (Table II).

Biological Activity of Oxidized rhPTH

The biological activities of the fractionated four peaks (A-D) were examined by measurement of rat osteosarcoma cell adenylate cyclase activity as shown in Fig. 4. Oxidation of Met by H_2O_2 reduced activity. Oxidation at Met8 (Peak B) reduced activity more potently than that at Met18 (Peak C). Potency of oxidation induced adenylate cyclase inhibition was in the order of: (Met8,Met18) > Met8 > Met18 > intact rhPTH.

DISCUSSION

Three forms of oxidation with H_2O_2 in rhPTH, at positions Met8, Met18 and both Met8 and Met18, were found, and all caused the formation Met sulfoxides. A pH 10 showed the highest oxidation rate of rhPTH in our preliminary study and was employed as a condition for oxidation (data not shown). To confirm the oxidized position, we used chemical and enzymatic degradation and subsequent FAB-MS. Since an oxidized Met was not subject to CNBr cleavage (9), the oxidized position was determined by the detection of the peptide fragments that were not produced after subjection to CNBr. In the analysis of tryptic digests, peptide fragments containing oxidized Met were detected by molecular weight increments, and most of the primary struc-

ture information of rhPTH was also obtained as has been previously reported (10). One advantage of this technique is that Met sulfoxide and Met sulfone, two possible forms of Met oxidation, are clearly distinguishable. Because, they show completely different molecular weight increments to that of intact Met, the sulfoxide having a 16, and sulfone a 32 mass unit increment, respectively.

The progression of Met oxidation was extrapolated from the results in Fig. 2. Either Met position was oxidized within the same time course whereas oxidation at both position was observed later. The final product in this study yielded a form of both Met oxidized, and singly oxidized forms as intermediates. Met18 was more readily susceptible to oxidation than Met8.

Biological activity, adenylate cyclase activity, was decreased after oxidation by H_2O_2. Oxidation at Met8 caused a remarkably larger suppression activity compared to that of Met18 (Fig. 4). This implies that Met8 acts as a key residue for activation of adenylate cyclase, that is, Met8 may play an important role in binding the hormone to receptors. The same order of decreased activity by oxidation has also been observed in bovine PTH (11) and in porcine PTH, which has only one Met, at position 8 (12). Analysis of the secondary structure of bovine PTH has shown that oxidation at Met8 produces substantial changes in folding (13). Thus for these reasons, it is indicated that the region around Met8 is important for the active structure of the hormone. The reduction of the activity by oxidation at Met18 may suggests that this region is another receptor recognition site, as has been mentioned in a previous study (14).

In conclusion, the oxidized products of rhPTH were Met8, Met18 and both Met8 and Met18 sulfoxide. The final product resulted in oxidation of both Met positions. Biological activity was decreased by oxidation at either Met position; oxidation at Met8 reduced the activity more markedly than that at Met18. This suggests that the region around Met8 is important for the active structure of the hormone.

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