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In Vitro Methionine Oxidation of Recombinant Human Leptin

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Purpose. To investigate the role and importance of the four methionines in recombinant human leptin, and the effect of methionine oxidation in leptin structural stability and biological activity.

Methods. Oxidized leptin derivatives were prepared in the presence of H_2O_2 and *t*-butylhydroperoxide, separated by RP-HPLC, and characterized by peptide mapping and LC/MS. Their biophysical and biological properties were studied.

Results. Six major species of oxidized leptins were detected: two mono-oxidized, one di-oxidized, two tri-oxidized, and one tetra-oxidized. Further oxidation at cystine disulfide was also detected. Kinetic analysis indicated that oxidation at Met^1 and Met^{69} proceeded first and independently. In 48 mM *t*-butylhydroperoxide, the pseudo first-order rate constants, k_1 and k_{69}, were 1.5×10^{-3} and 2.3×10^{-4} min^{-1}. No change in the secondary or tertiary structure was detected for Met^1 mono-oxidized and Met^1, Met^{69} di-oxidized leptins. The Met^1 mono-oxidized leptin retained full potency as compared to native leptin. A slight decrease of thermostability and a significant loss of the *in vitro* bioactivity were observed for Met^1, Met^{69} di-oxidized leptin. Both Met^{55} and Met^{137} were not oxidized in *t*-butylhydroperoxide but only in H_2O_2. They appeared to be much less accessible to oxidation and might interact with the hydrophobic core structure of the leptin molecule.

Conclusions. The oxidation of leptin occurred in the order of $Met^1 > Met^{69} >> Met^{55} \approx Met^{137}$, and the importance for maintaining leptin structural integrity was $Met^{55} \approx Met^{137} >> Met^{69} \approx Met^1$. Met^{69}, but not Met^1, plays a critical role in the protein stability and activity.

KEY WORDS: recombinant human leptin; leptin oxidation; oxidation kinetics; protein characterization; protein structure.

INTRODUCTION

The identification and cloning of the obese gene (*ob*) and the gene encoding *ob* receptor (*db*) has resulted in an increasing amount of basic and clinical research in human obesity (1,2). Mutations in the *ob* or *db* genes generate a variety of mouse phenotypes and result in obesity, infertility and diabetes (3,4). The *ob* protein, known as leptin, has been demonstrated to normalize body weight together with reduction of body fat, food intake, and serum glucose and insulin in *ob/ob* mice (5–8). This observation has led to pre-clinical and clinical studies to evaluate human leptin as a drug candidate for treatment of some types of obesity.

The major form of human leptin is not a glycoprotein (4) and can be expressed recombinantly in *E. coli* in biologically active form after proper refolding and oxidation (9,10). To evaluate the suitability of leptin for long term storage, the recombinant molecule has been subjected to rigorous tests of its molecular stability. One of the commonly seen degradation processes for proteins during storage is oxidation of methionine residues (11). Since recombinant human leptin contains four methionine residues, the possibility that oxidation of the molecule occurs has to be taken into consideration. As a result, the oxidized proteins could become biologically, chemically or physically unstable (12). In order to evaluate the impact of oxidation on the product stability and activity of recombinant human leptin, *in vitro* oxidation experiments were conducted and the reaction kinetics for individual methionines were carefully analyzed. The rate of oxidation was accelerated by the introduction of two chemical oxidants, hydrogen peroxide (H_2O_2) and *t*-butylhydroperoxide (TBHP). Both biophysical and biological parameters were characterized and compared among the oxidized forms and the native protein.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade, except when otherwise indicated. Endo-proteinase Asp-N was obtained from Boehringer Mannheim (Indianapolis, IN). Hydrogen peroxide and TBHP were from Sigma Chemical Co. (St. Louis, MO). Recombinant human leptin was produced using an *E. coli* direct expression system according to the procedures previously reported (13). The expressed protein was accumulated in inclusion bodies, allowed to fold and oxidize after solubilization, and purified by ion exchange chromatography (9). The recombinant protein is composed of a polypeptide chain of 147 amino acids with an intramolecular disulfide bridge formed between two cysteines at positions 97 and 147. The methionine at the N-terminus is present as a result of incomplete processing of the initiator methionine following translation.

H_2O_2 Oxidation

Oxidation of recombinant human leptin was carried out at room temperature in 100 mM sodium borate, pH 9.0. To 400 μl of protein (200 μg, 0.5 mg/ml) was added 5 μl of 3% (0.9 M) or 10% (3 M) H_2O_2 in water. The final concentration of the protein was 31 μM and H_2O_2 was 11 mM or 37 mM. Aliquots of the reaction mixture were periodically taken without filtering, and injected to pre-equilibrated reverse phase columns for HPLC separation of the oxidized species (see below). A control experiment without the addition of the oxidizing reagent was conducted at the same time.

TBHP Oxidation

The oxidation conditions were similar to the procedure described above. To 400 μl of leptin (200 μg, 0.5 mg/ml) was added 5 μl of TBHP (35%, 3.9 M). The final concentrations of the protein and TBHP were 31 μM and 48 mM. The progress of oxidation was monitored by reverse phase HPLC (see below).

Preparation of Met^1 Mono-Oxidized Leptin

To 600 μl of protein (1.5 mg, 2.5 mg/ml) in 100 mM sodium borate, pH 9.0, was added 5 μl of H_2O_2 (3%, 0.9 M).

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The final concentration of leptin was 155 μM and H_2O_2 was 7.4 mM. The reaction was incubated at room temperature for 1 hr and stopped by passing through a PD-10 desalting column (Pharmacia) pre-equilibrated with phosphate-buffered saline (PBS).

Preparation of Met^1, Met^{69} Di-Oxidized Leptin

The reaction was conducted similarly as the procedure described above. The protein solution (600 μl, 2.5 mg/ml) was mixed with 5 μl of H_2O_2 (10%, 3 M). The final concentration of the protein was 155 μM and H_2O_2 was 24.8 mM. The reaction was incubated at room temperature for 3 hr and stopped by the same method described above.

Reversed-Phase HPLC

Different oxidized leptin derivatives were separated by reverse phase HPLC. Chromatography was performed on an HP-1090 system equipped with a ChemStation and a diode array detector using a Vydac C-4 column (2.1 × 250 mm), which was pre-equilibrated with 47% mobile phase A (0.1% TFA) and 53% mobile phase B (0.1% TFA in 90% acetonitrile) at a flow rate of 0.2 ml/min at 40°C. UV detection was set at 215 and 280 nm. Separation was achieved by a linear gradient from 53% to 55% solvent B in 35 min. The composition of the oxidized leptin species was determined by area integration of the chromatogram.

Identification of Oxidized Proteins by Mass Spectrometry

Oxidized leptin derivatives separated by HPLC were collected and the protein molecular masses were determined using a Finnigan MAT TSQ-7000 (San Jose, CA), or a Sciex API-100 (Thornjill, Ontario, Canada) electrospray mass spectrometer. The analyses were performed by either using a direct infusion of the HPLC-collected protein solution at a flow rate of 2-5 μl/min into the ionspray interface, or through on-line LC-MS analysis which was performed by splitting the outlet line from the UV cell, allowing 25% eluant (50 μl/min) going directly to the electrospray mass spectrometer. The scan range on the mass spectrometer was set from m/z 900 to 2000, with an orifice voltage of 55 volts, step size of 0.25 Da, and a dwell of 0.5 ms. The chromatographic conditions for the LC/MS analysis were the same as described above.

Endo-Proteinase Asp-N Peptide Mapping of Soluble Oxidized Leptin

Oxidized leptin samples were digested with Asp-N endoproteinase in PBS with an enzyme to substrate ratio of 1:20 for 5 hr at room temperature. The digest was immediately injected onto a Vydac C-4 column (2.1 × 250 mm), pre-equilibrated in 5% solvent B (mobile phases were the same as above). The peptide mixture was separated by HPLC using a gradient starting from 5% to 10% B at 5 min, and to 50% B at 60 min, and finally to 90% B at 65 min. The flow rate was 0.2 ml/min and detector wavelengths were set at 215 and 280 nm. Native leptin was also digested to provide a reference map. The molecular masses of the digested peptides were determined using a Kompact MALDI III matrix-assisted laser desorption ionization mass spectrometer (Kratos Analytical, Ramsey, NJ). Sequential Edman degradation was performed on a HP G1000A automated protein sequencer (Hewlett Packard) or ABI model 473 and 477 sequencers (Applied Biosystems, Foster City, CA) using sequencing programs recommended by the manufacturer (Hewlett Packard Inc., Mountain View, CA).

Endo-Proteinase Asp-N Peptide Mapping of Precipitated Oxidized Leptin

The oxidized leptin precipitate was separated from the supernatant by centrifugation, and was washed with PBS. The precipitate was then dissolved in 6 M guanidine hydrochloride (GdnHCl) in PBS and diluted three-fold with PBS to obtain a final protein solution in 2 M GdnHCl in PBS prior to enzymatic digestion. With the same enzyme to substrate ratio of 1:20, the digestion was allowed to proceed for 7 hr before the subsequent analysis by LC-MS and sequential Edman degradation using the same procedure described above. The HPLC was performed using a Vydac C-4 column (4.6 × 250 mm), at a flow rate of 0.5 ml/min. The buffer gradient was the same as above for peptide map analysis. The LC-MS parameters used were the same as those used above for analyzing oxidized proteins, except for the scan range was set from m/z 450 to 2200, and step size was 0.2 Da. The split ratio of the eluant from the outlet line of UV cell was 9:1, with 10% eluant (50 μl/min) going directly to the electrospray mass spectrometer.

Peptide Nomenclature

The eleven native leptin peptides derived from Asp-N digestion were numbered P1 through P11 starting from the N-terminus sequence of the protein. The peptides containing methionine residues were: P1 containing Met^1, P4 containing Met^{55}, P5 containing Met^{69}, and P9 containing Met^{137}. The peptides containing cysteine residues were: P10 containing Cys^{97}, P11 containing Cys^{147}, Peptide P10-P11 was a disulfide-linked peptide at Cys^{97}-Cys^{147}.

Circular Dichroism (CD)

CD spectra were obtained using a Jasco J-720 spectropolarimeter and cuvettes with a 0.02 cm path length (for far UV CD) or a 1 cm path length (for near UV CD). Protein samples were clarified by sterile filtration using a 0.2 μm membrane, and concentrations were determined by UV absorption at 280 nm using an absorbance of 0.98 at 1 mg/ml concentration. All proteins were analyzed in PBS (10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.0). The α-helical content of protein was estimated according to Greenfield and Fasman (14).

In Vitro Bioassay

The *in vitro* biological activity of leptin was determined on interleukin-3-dependent murine hematopoeitic cells (15) which were transfected with the leptin/erythropoietin chimeric receptors (2,15,16). The cells were allowed to grow for two days in the presence of the various amounts of the assay samples. The cells were then incubated for approximately eighteen hours in the presence of ^3H-thymidine and their DNA was harvested.

Sample activity was determined directly from the equation of the linear portion of the standard dose response curve.

RESULTS

In Vitro Oxidation

Recombinant human leptin was oxidized in the presence of H_2O_2 or TBHP. Various concentrations of the oxidants were examined to determine the appropriate conditions for conducting kinetic studies. Leptin is stable at pH 9.0 at room temperature, and the detection of any Asn deamidation or Asp isomerization usually requires days of incubation at an elevated temperature (data not shown). The control experiment of leptin in the absence of oxidizing reagents showed no change on the HPLC profiles during the reaction time conducted for all the oxidation kinetics. The oxidation rate of leptin in TBHP was slower than in H_2O_2. The reaction was monitored in 11 and 37 mM H_2O_2 or in 48 mM TBHP at room temperature. When the reactions were allowed to proceed longer than 8 hr in 37 mM H_2O_2, the solution became cloudy and insoluble precipitation appeared, which indicated the occurrence of protein denaturation due to extended oxidation. The control experiment showed no change for the recovery and HPLC chromatogram profile for native leptin when incubated for the same time interval. The characterization of the precipitate by peptide mapping as described below showed that all methionine residues were oxidized.

Separation of Oxidized Leptin Products by Reverse Phase HPLC

The oxidized leptin products were separated by reverse phase HPLC (Figures 1) and the extent of oxidation was determined by the difference in molecular weight compared to native leptin (Table I). Figure 1 illustrates the reverse phase HPLC chromatograms of leptin oxidized in 48 mM TBHP and 37 mM H_2O_2 at various time intervals (top and bottom panels, respectively). In addition to the native leptin (peak A), several peaks were observed after oxidation. Native leptin eluted at 35.1 minutes and exhibited a molecular mass of 16158.0 Da which is in good agreement with the theoretical mass of 16155.7 Da (see Table I). Several early eluting peaks were detected after oxidation of leptin by H_2O_2 and TBHP. Peaks B, D1, D2, E, F, and G were observed after H_2O_2 oxidation, and peaks B, C1, C2, D1, and D2 were detected following TBHP oxidation. Following MS analyses of the modified proteins, peaks B, C1, and C2 were identified to be mono-oxidized leptins based on their determined molecular weights shown in Table I. These species exhibited masses of 16175.6, 16175.2, and 16175.7 Da which were close to the theoretical mass of 16171.7 Da for mono-oxidized leptin. Peaks D1 and D2 were seen following both treatments and were determined to have molecular masses of 16190.0 and 16190.5 Da. These peaks were di-oxidized leptins as their masses were approximately 32 Da greater than native protein. Only after H_2O_2 treatment were the further oxidized species, E, F, and G, observed. Peaks E and F had masses of 16207.4 and 16206.9 Da, and peak G displayed a mass of 16220.0 Da. The differences of 49 and 64 Da from the mass of native leptin indicated that peaks E and F were tri-oxidized derivatives, and peak G was a tetra-oxidized product. The final

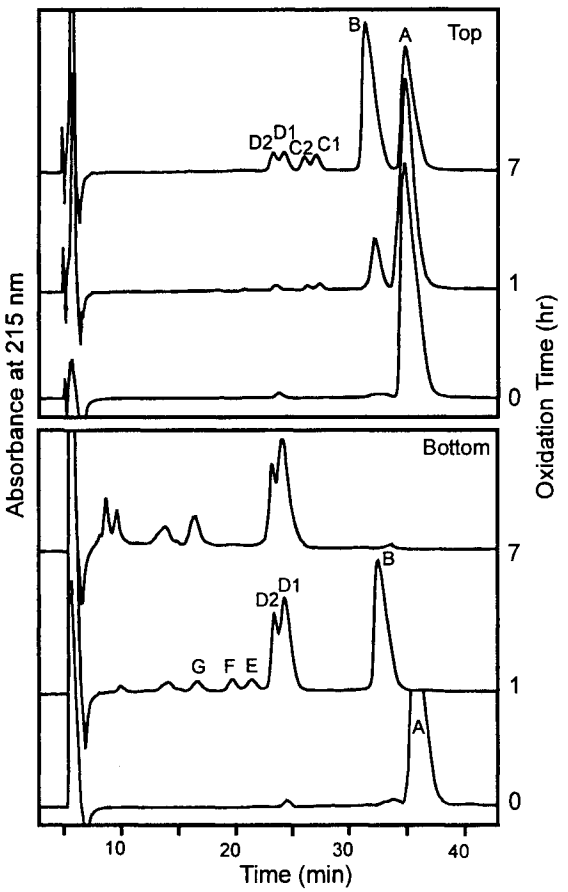


Fig. 1. Reverse phase HPLC chromatogram of oxidized leptins (20 μg each) at various time intervals (0, 1, and 7 hr). *Top panel*, reaction in 48 mM *t*-butylhydroperoxide (TBHP). *Bottom panel*, reaction in 37 mM hydrogen peroxide (H_2O_2). Peak A, native leptin; peak B, Met^1 mono-oxidized; Peaks C1, and C2, Met^{69} mono-oxidized; peaks D1 and D2, Met^1, Met^{69} di-oxidized; peak E, Met^1, Met^{55}, Met^{69} tri-oxidized; peak F, Met^1, Met^{69}, Met^{137} tri-oxidized; peak G, Met^1, Met^{55}, Met^{69}, Met^{137} tetra-oxidized.

precipitate was dissolved in 6 M GdnHCl and analyzed by on-line LC-MS. The derived protein masses indicated that there was a mixture of extensively oxidized species, which included penta-, hexa-, hepta-, octa-, and higher oxidized leptins. These oxidized leptins were detected in the solution in much lower amount. The characterization of peaks A to E as well as the highly oxidized species was achieved by peptide mapping as shown below.

Characterization of Oxidized Leptin Derivatives by Asp-N Peptide Mapping

Peaks A, B, C1, C2, D1, D2, and E were digested with endo-proteinase Asp-N; and the obtained peptide maps were compared to a leptin control peptide map to locate the oxidation site(s). Figure 2 illustrates the peptide maps obtained from leptin digests derived from peaks A, B, C1, D1, and E. Identification of methionine oxidation was confirmed by (1) the detection of early eluting peaks with concomitant disappearance of methionine-containing peptides and (2) direct sequence analysis and mass spectrometric determination of the modified peptides.

Table I. Molecular Masses of Native and Oxidized Human Leptin

Peak[a]	Oxidized leptin[b]	Observed mass[d] (theoretical[e])	Mass. diff.[f]
A	native	16158.0 (16155.7)	+0
B	mono-oxidized(Met^1)[c]	16175.6 (16171.7)	+17.6
C1	mono-oxidized (Met^{69})[c]	16175.2 (16171.7)	+17.2
C2	mono-oxidized (Met^{69})[c]	16175.7 (16171.7)	+17.7
D1	di-oxidized (Met^1,Met^{69})[c]	16190.0 (16187.7)	+32.0
D2	di-oxidized (Met^1,Met^{69})[c]	16190.5 (16187.7)	+32.5
E	tri-oxidized (Met^1,Met^{69},Met^{55})[c]	16207.4 (16203.7)	+49.4
F	tri-oxidized (Met^1,Met^{69},Met^{137})[c]	16206.9 (16203.7)	+48.9
G	tetra-oxidized (Met^1,Met^{69},Met^{55},Met^{137})[c]	16220.0 (16219.7)	+64.0

[a] Peak assignment for oxidized leptins eluted on reverse phase HPLC.
[b] Oxidized leptins were assigned based on molecular masses.
[c] The oxidized methionies in parenthesis were assigned based on Asp-N peptide mapping.
[d] Observed mass (M_r) on Finnigan MAT TSQ-7000 electrospray mass spectrometer.
[e] Theoretica mass for M_r.
[f] Mass difference between oxidized leptin and native leptin.

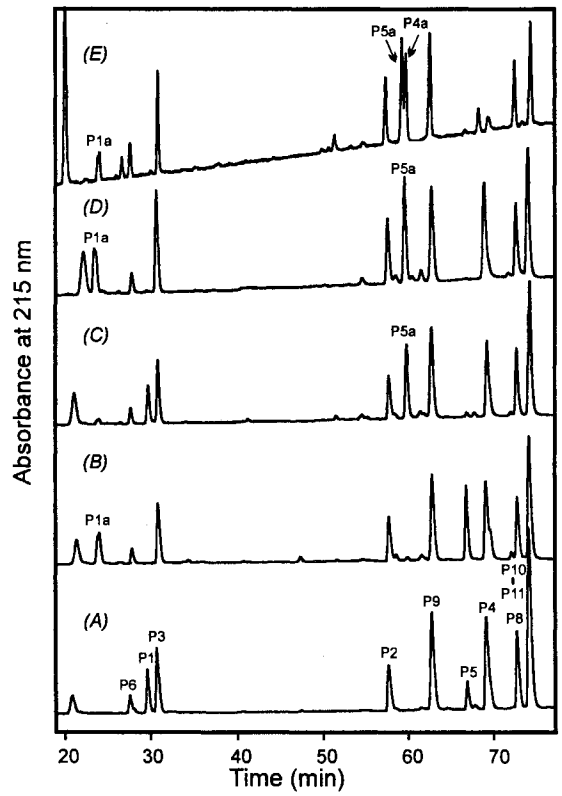


Fig. 2. Reverse phase chromatogram of endo-proteinase Asp-N peptide maps of oxidized leptins (10 μg each). (A) Native leptin. (B) peak B, Met^1 mono-oxidized leptin, which had peptide P1a instead of P1. (C) peak C1, Met^{69} mono-oxidized leptin, which had peptide P5a instead of P5. (D) peak D1, Met^1, Met^{69} di-oxidized leptin, which had peptides P1a, P5a instead of P1, P5. (E) peak E, Met^1, Met^{55}, Met^{69} tri-oxidized leptin, which had peptides P1a, P4a, P5a instead of P1, P4, P5. Peaks A to E were from HPLC analysis of oxidized leptin shown in Figure 1.

Peak A is the unmodified leptin and therefore its peptide map is identical to the reference map obtained from digestion of native protein.

The map obtained from peak B did not contain peptide P1 found in the map of peak A, instead a new peak, P1a, eluting at an earlier retention time was found. As indicated in Table II, peptide P1a has a molecular mass of 16.4 Da higher than peptide P1, indicating that P1a was the peptide containing the Met^1 sulfoxide. This data confirmed that peak B is the mono-oxidized leptin with the Met-sulfoxide located at the N-terminal Met residue. By similar analysis of the peptide map and the structural characterization summarized in Table II, the other modified peptides were identified. Peaks C1 and C2 had identical maps, in which the appearance of peptide P5a instead of peptide P5 indicated that they were mono-oxidized forms and both contained Met^{69} sulfoxide. Peaks D1 and D2 also showed identical peptide maps which contained two oxidized peptides P1a and P5a, and were identified as di-oxidized species containing Met^1 sulfoxide and Met^{69} sulfoxide. The peptide map from peak E revealed that three peptides, P1, P4, and P5, found in peak A shifted to earlier retention times, designated as peaks, P1a, P4a, and P5a, respectively. The data, therefore, confirmed that three methionine residues, Met^1, Met^{55}, and Met^{69}, were oxidized in peak E. Oxidation in peak F occurred at Met^1, Met^{69}, and Met^{137}, and peak G contained leptin with all four methionines oxidized (peptide map chromatograms not shown).

Characterization of the oxidized leptin precipitate was achieved by conducting endo-proteinase Asp-N digestion of the GdnHCl-denatured protein (Figure 3). The precipitate contained a mixture of highly oxidized leptins with methionines converted to either methionine sulfoxides or sulfones. Peptides of native leptin, P1, P4, P5, and P9, completely disappeared, whereas new peptides, P1a, P1b, P4a, P4b, P5a, P5b, P9a, P9b, P10a, P10b and P11a, were detected. Peptides P1a, P4a, P5a, and P9a were identified to contain methionine sulfoxide derivatives at Met^1, Met^{55}, Met^{69}, and Met^{137}, respectively as described above (Table II). Upon confirmation by mass spectrometry and N-terminal sequencing analysis, peptides P1b, P4b, P5b, and P9b are the sulfone derivatives of peptides P1, P4, P5, and P9. The difference of the molecular weight between the methionine- and methionine sulfone-containing peptides was 32 Da (see

Table II. Endo-Proteinase Asp-N Peptides of Native and Oxidized Leptins

Peptide	Sequence[b]	Observed mass[e] (Theoretical)[f]	Mass diff.[j]
P1	MVPIQK	942.4 (942.5)	0
P1a	**M(O)**[c]VPIQK	958.8 (958.5)[g]	+16.4
P1b	**M(O_2)**[c]VPIQK	974.8 (974.5)[h]	+32.4
P4	DFIPGLHPILTLSKM	1682.0 (1681.9)	0
P4a	DFIPGLHPILTLSK**M(O)**[c]	1697.9 (1697.9)[g]	+15.9
P4b	DFIPGLHPILTLSK**M(O_2)**[c]	1714.4 (1713.9)[h]	+32.4
P5	DQTLAVYQQILTSMPSRNVIQISN	2719.5 (2719.4)	0
P5a	DQTLAVYQQILTSM(O)[c]PSRNVIQISN	2736.5 (2735.4)[g]	+17
P5b	DQTLAVYQQILTSM(O_2)[c]PSRNVIQISN	2752.7 (2751.4)[h]	+33.2
P9	DMLWQL	805.5 (805.4)	0
P9a	DM(O)[c]LWQL	821.5 (831.4)[g]	+16.0
P9b	DM(O_2)[c]LWQL	837.8 (837.4)[h]	+32.3
P10[a]	DLLHVLAFSKSCHLPWASGLETL	3126.2 (3126.5)	0
P11[a]	DLSPGC		
P10a	DLLHVLAFSKSC(O_3)[d]HLPWASGLETL	2586.2 (2585.3)[i]	+48.9[k]
P10b	C(O_3)[d]HLPWASGLETL	1375.5 (1374.8)[i]	+49.0[l]
P11a	DLSPGC(O_3)[d]	639.3 (640.2)[i]	+48.1[m]

[a] Peptide P10-P11 is a disulfide-linked peptide through Cys^{97} and Cys^{147}.
[b] Sequence observed from sequential Edman degradation.
[c] **M(O)** and **M(O_2)** represent methionine sulfoxide and methionine sulfone, respectively.
[d] **C(O_3)** represents cysteic acid.
[e] Observed mass (MH^+) on Sciex API-100 electrospray mass spectrophotometer.
[f] Theoretical mass for MH^+.
[g] Theoretical mass for the peptides with methionine sulfoxide modification.
[h] Theoretical mass for the peptides with methionine sulfone modification.
[i] Theoretical mass for the peptides with cysteic acid modification.
[j] Mass difference between oxidized and native peptides.
[k] Mass difference of peptide P10a and peptide P10 (theoretical mass, MH^+ = 2537.3 Da).
[l] Mass difference of peptide P10b and the native peptide (theoretical mass, MH^+ = 1326.5 Da).
[m] Mass difference of peptide P11a and peptide P11 (theoretical mass, MH^+ = 591.2 Da).

Table II), while PTH-methionine sulfone derivative eluted earlier than PTH-methionine by on-line RP-HPLC analysis during sequence analysis (data not shown). The ratio of sulfoxide to sulfone for Met^1, Met^{55}, Met^{69}, and Met^{137} were individually estimated to be P1a : P1b (5 : 1), P4a : P4b (10 : 1), P5a : P5b (6 : 1), and P9a : P9b (18 : 1). Approximately 60% of the disulfide-linked peptides, P10—P11, between Cys^{97} and Cys^{147} disappeared and new peptides, P10a, P10b, and P11a, were found. In these new peptides, the cystine that forms the disulfide bridge in native leptin was oxidized to form cysteic acids. The cysteic acid-containing peptides, P10a and P11a, had molecular masses of 2586.2 and 639.3 Da, respectively, which were

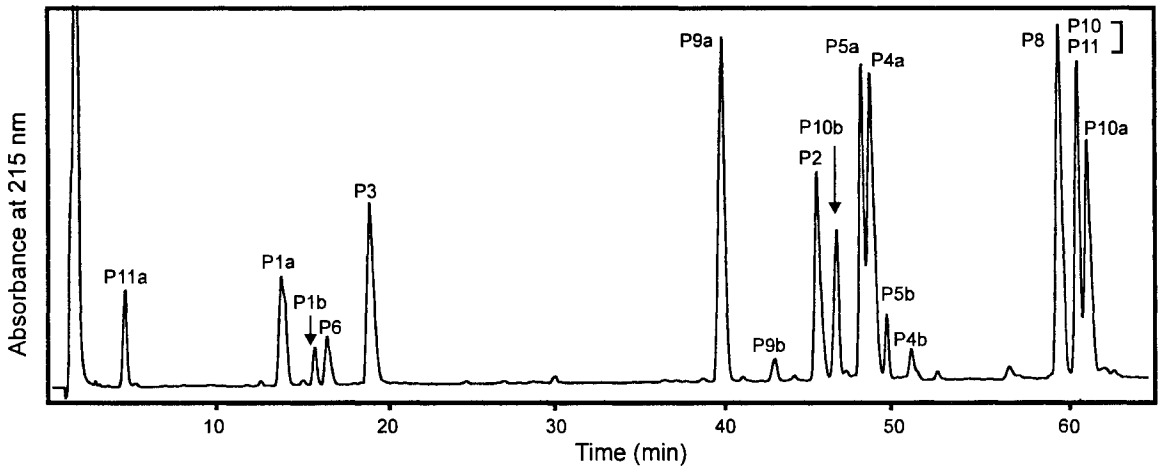


Fig. 3. Reverse phase chromatogram of a highly oxidized leptin endo-proteinase Asp-N peptide map (10 μg each). Modification in each peptide: P1a, Met^1 sulfoxide; P1b, Met^1 sulfone; P4a, Met^{55} sulfoxide; P4b, Met^{55} sulfone; P5a, Met^{69} sulfoxide; P5b, Met^{69} sulfone; P9a, Met^{137} sulfoxide; P9b, Met^{137} sulfone; P10a, $Cys(O_3)^{97}$cysteic acid; P10b, $Cys(O_3)^{97}$cysteic acid; P11a, $Cys(O_3)^{147}$ cysteic acid.

approximately 48 Da greater than the reduced native peptides, P10 and P11 (theoretical masses, 2537.3 and 591.2 Da, respectively). Peptide P10b was a truncated form of peptide P10a. It was generated from the proteolytic cleavage of the peptide bond at the amino side of the cysteic acid at leptin sequence position 97. Therefore, the oxidation of Cys^{97}-Cys^{147} resulted in the disruption of the disulfide bond and the formation of $Cys(O_3)^{97}$ and $Cys(O_3)^{147}$. The location of methionine sulfone and cysteic acids was also confirmed by electrospray MS/MS peptide fragmentation analysis (data not shown).

Effect of Detergent on Leptin Oxidation

In an attempt to obtain soluble tetra-oxidized species for further biophysical analysis, moderate amounts of detergents or alcohols were introduced to the reactions. Solutions containing either 0.1% Tween 20, 0.1% octylglucoside, 0.5 M sucrose, 10% glycerol, or 10% ethanol in oxidation buffer were prepared. Although the oxidation rate was slower in 10% glycerol, none of the conditions could prevent the tetra-oxidized leptin from forming insoluble precipitate. The oxidation of disulfide bond to cysteic acids, however, was significantly inhibited in the presence of detergents and alcohols.

Oxidation Kinetics

The kinetics of leptin oxidation was obtained by plotting the integrated peak area of the oxidized leptin in percentage as a function of oxidation time. Figure 4 shows a representative kinetic plot of leptin in the presence of 37 mM H_2O_2. Met^1 mono-oxidized leptin (peak B) was detected immediately upon oxidation. It grew quickly in the early time-course and reached approximately 45% in one hour, then gradually decreased with longer oxidation time. Met^1, Met^{69} di-oxidized leptin (peaks D1 and D2 combined) increased steadily and became the major product in the reaction solution at the end of 7 hr. The maximum amount of di-oxidized leptin in the reaction solution was detected after 5 hr at which it reached approximately 65% of the total protein. The percentage of total tri-oxidized leptins was the combined integration area of peaks E and F. They were only detected in minute quantities, maximized to approximately 7% in 1 hr, and then disappeared after longer oxidation. A small amount of tetra-oxidized leptin (peak G) was detected during HPLC analysis and slowly increased to a maximum of 10%. It was not very soluble in aqueous solution and was found mainly in the precipitate. When the reaction was allowed to continue further, more precipitate formed and the major soluble components in the reaction were di-oxidized leptin (peaks D1 and D2) and the tetra-oxidized leptin (peak G), which was present in a much lesser amount.

The oxidation of leptin in the presence of TBHP generated three oxidized species, Met^1 and Met^{69} mono-oxidized leptins and Met^1, Met^{69} di-oxidized leptin. Figure 5 shows the representative kinetics in 48 mM TBHP. Met^1 and Met^{69} mono-oxidized leptins were detected initially, with a concomitant decrease of the native leptin. Met^1 (peak B) was oxidized at a significantly faster rate than Met^{69} (peaks C1 and C2). The Met^1, Met^{69} di-oxidized species (peaks D1 and D2) grew steadily throughout the entire time course. No tri- or tetra-oxidized species were detected throughout the reaction time course.

We used the obtained kinetic data to establish a kinetic model for TBHP oxidation. In order to simplify the kinetic equations and reduce the number of kinetic parameters for calculation, we made the following assumptions. First, oxidation occurred only at two sites, Met^1 and Met^{69}, from methionine

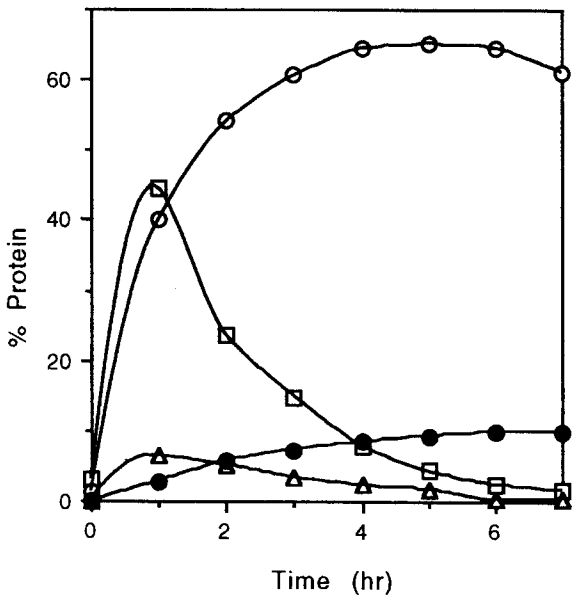


Fig. 4. Leptin oxidation kinetics in the presence of 37 mM hydrogen peroxide (H_2O_2). The percentage of oxidized leptins in total protein versus reaction time was plotted. Met^1 mono-oxidized (□); Met^1, Met^{69} di-oxidized (○); Met^1, Met^{69}, Met^{55} and Met^1, Met^{55}, Met^{137} tri-oxidized (Δ); Met^1, Met^{69}, Met^{55}, Met^{137} tetra-oxidized (●).

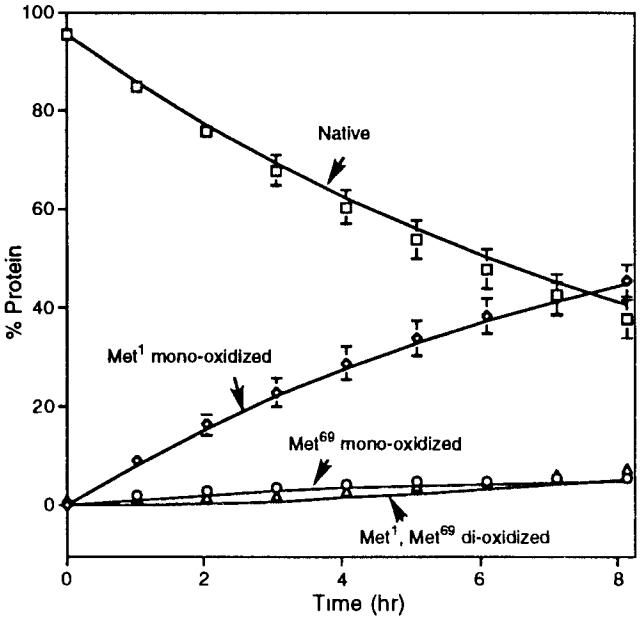
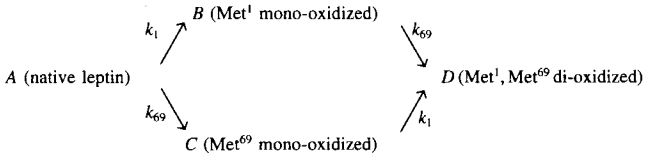


Fig. 5. Leptin oxidation kinetics in the presence of 48 mM TBHP. The percentage of oxidized leptins in total protein versus reaction time was plotted. Native leptin (□); Met^1 mono-oxidized (◇); Met^{69} mono-oxidized (○); Met^1, Met^{69} di-oxidized (Δ). The error bars represent standard deviations of three repetitive experiments. The experimental data was superimposed with the simulated kinetic model (represented in lines) as described in the text.

to methionine sulfoxide. Second, oxidation of Met^1 and Met^{69} were non-cooperative; therefore, the rate of oxidation of native leptin to Met^1 mono-sulfoxide and the rate of oxidation of Met^{69} mono-sulfoxide to Met^1, Met^{69} di-sulfoxide would be the same, which is assigned as k_1. The rate of oxidation of native leptin to Met^{69} mono-sulfoxide and the rate of oxidation of Met^1 mono-sulfoxide to Met^1, Met^{69} di-sulfoxide would also be the same (assigned as k_{69}). Based upon a similar analysis described previously (17), the kinetic model used to analyze the data can be depicted as follows:



The differential equations describing the system are

$$\frac{dA}{dt} = -(k_1[A] + k_{69}[A])$$

$$\frac{dB}{dt} = k_1[A] - k_{69}[B]$$

$$\frac{dC}{dt} = k_{69}[A] - k_1[C]$$

$$\frac{dD}{dt} = k_{69}[B] + k_1[C]$$

The analytical solutions for these equations can be expressed as the following.

$$A = A_0 e^{-(k_1+k_{69})t}$$
$$B = A_0(e^{-k_{69}t} - e^{-(k_1+k_{69})t})$$
$$C = A_0(e^{-k_1 t} - e^{-(k_1+k_{69})t})$$
$$D = A_0 - A_0(e^{-k_1 t} + e^{-k_{69}t} - e^{-(k_1+k_{69})t})$$

When the oxidant is present in large excess, k_1 and k_{69} are the pseudo-first-order rate constants and can be determined from linear regression analysis fitting of the data obtained from early time points ($t \sim 0$). Under TBHP oxidation, k_1 was 1.5×10^{-3} min^{-1} and k_{69} was 2.3×10^{-4} min^{-1}. The Met^{69} mono-oxidized species was not observed in the 37 mM H_2O_2 oxidation condition described above; however, this species was detected at lower H_2O_2 concentrations. The pseudo-first-order rate constants, k_1 and k_{69}, in 11 mM H_2O_2 were determined to be 2.6×10^{-2} min^{-1} and 1.0×10^{-3} min^{-1}, respectively. In order to examine the veracity of the assumptions made for the basis of our kinetic model, an overlay of the experimental data with the simulated kinetics is shown in Figure 6. The simulated kinetics was plotted using the pseudo-first-order rate constants k_1 (1.5×10^{-3} min^{-1}) and k_{69} (2.3×10^{-4} min^{-1}) from 48 mM TBHP reaction, with the kinetic equations described above.

Physicochemical Analysis

Met^1 mono-oxidized, and Met^1, Met^{69} di-oxidized leptins can be generated under the optimum oxidation conditions as described in the Methods and the obtained samples contain above 80% of the desired protein as analyzed by RP-HPLC. Since the oxidation rate of Met^{69} was much slower than Met^1,

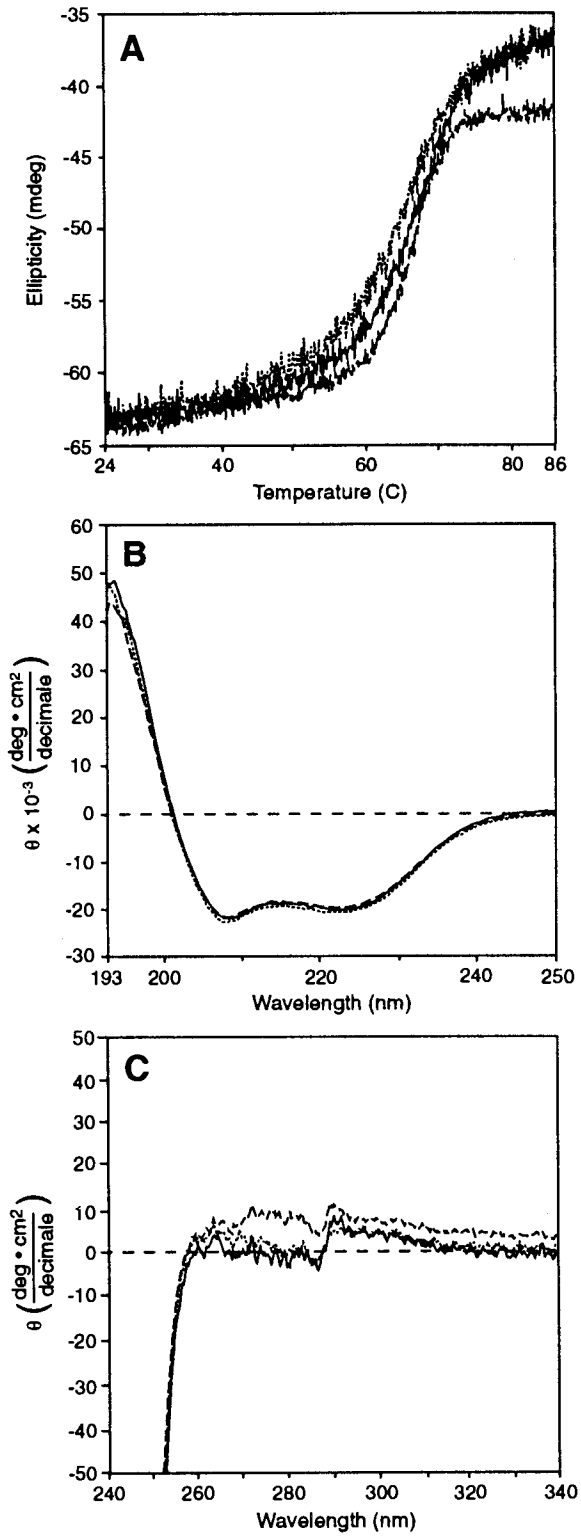


Fig. 6. CD spectra of native leptin (solid lines), Met^1 mono-oxidized (dashed lines), and Met^1, Met^{69} di-oxidized (dotted lines). *Panel A* represents the thermostability monitored at ellipticity at 222 nm in a CD analysis. *Panel B* represents the far UV spectra. *Panel C* represents the near UV spectra.

the Met^{69} mono-oxidized species could not be recovered as a major product under the reaction conditions examined. Attempts to obtain tri- and tetra-oxidized leptins under non-denaturing conditions were also unsuccessful, as precipitate was observed under all conditions tested. The physicochemical data was therefore only available for three species, native, Met^{1} mono-oxidized, and Met^{1}, Met^{69} di-oxidized leptins. The thermostability of the leptin derivatives was determined by monitoring changes in the ellipticity at 222 nm with increasing temperature. Both native and Met^{1} mono-oxidized leptin began to melt at 58°C, while the di-oxidized leptin melted at 54°C (Figure 6A). The far UV-CD spectra of these three species were identical, indicating a similar α-helical content among these three forms (Figure 6B). Following extended oxidation, a mixture of di-oxidized leptin and a small amount of tetra-oxidized species was obtained, which revealed less α-helix (data not shown), reflecting that the tetra-oxidized species present in the sample may have lost some secondary structure. The near UV-CD spectral analysis indicated that native, mono-oxidized, and di-oxidized leptins exhibited similar spectral profiles (Figure 6C). However, the di-oxidized leptin sample containing small amount of tetra-oxidized leptin again showed minor difference from the control (data not shown). These data suggested that the conformational stability of the mono-oxidized and di-oxidized leptins was grossly similar to that of the native leptin, and that the presence of oxidized Met^{1}, or Met^{69} did not disrupt the global structure of the leptin molecule. The procedures used to isolate the oxidized leptin species did not alter the stability and structure of unmodified leptin.

In Vitro Bioassay

Met^{1} mono-oxidized leptin showed equivalent bioactivity compared to the native leptin in an *in vitro* cell-proliferation assay. The Met^{1}, Met^{69} di-oxidized leptin, however, retained only approximately 20% potency as compared to native leptin.

DISCUSSION

When leptin was oxidized with H_2O_2, all four methionine residues and a disulfide bond were susceptible to oxidation. The methionine residues were converted to sulfoxides and sulfones, while the single disulfide was oxidized to form cysteic acids. In the presence of TBHP, only Met^{1} and Met^{69} were oxidized to methionine sulfoxides. The results indicate that TBHP oxidation is more gentle and specific to the methionine residues on the surface of a protein, which is consistent with results reported previously with other proteins (12).

A pair of Met^{69} mono-oxidized leptin peaks were observed during reverse phase HPLC analysis, despite the fact that both had identical peptide maps. This phenomenon also occurred with a pair of di-oxidized leptin peaks which were identified as Met^{1}, Met^{69} di-oxidized leptin. In both cases, Met^{69} was oxidized. One possible explanation is that these pairs were derived from the asymmetric oxidation of Met^{69}. The two electron lone pairs on the sulfur atom of the Met^{69} side chain are both likely to be the recipient of an oxygen atom at similar reaction rates during oxidation. The spatial orientation of these two forms could be slightly different and the free rotation of the methionine side chain is probably restricted in an orderly folded tertiary structure. This may result in the formation of two stereoisoforms that elute separately during reverse phase HPLC. However, the sulfoxide-containing peptides generated by endo-proteinase Asp-N digestion would not be differentiated by reverse phase chromatography due to the freedom of side chain bond rotation in these peptides.

The kinetic model for the oxidation of Met^{1} and Met^{69} to methionine sulfoxides in the presence of TBHP was based on the assumption that oxidation at the two sites was non-cooperative. A simulated kinetics was generated based on the pseudo-first-order rate constants k_1 and k_{69} obtained from TBHP oxidation, which superimposes nicely with the experimental data. As the hypothesized kinetic model closely resembles the actual mechanism of TBHP oxidation, the two methionine residues undergo oxidation independently of each other as expected. On the other hand, H_2O_2 oxidation of leptin generated many further oxidized species. The Met^{1}, Met^{69} di-oxidized leptin was not the end product, and could be further oxidized to form tri- and tetra-oxidized species and other derivatives. The oxidation kinetics in H_2O_2, therefore, was much more complicated than that for TBHP oxidation.

Our data appear to indicate that Met^{1} and Met^{69} are highly accessible to oxidation without disturbing the tertiary structure of the molecule, suggesting that these methionines might reside near a solvent-exposed region of the protein. This assumption was verified by the recently reported crystal structure of the human leptin Ala100Glu analog (18). The protein structure consists of four antiparallel α-helices (A, B, C, and D) similar to that of the long chain helical cytokine family (18). The N-terminus of the molecule where Met^{1} was introduced in recombinant human leptin in this study is highly flexible and exposed. Met^{69} is near the end of the B helix as part of a very short loop connecting the B and C helices. Met^{69} might be more structurally hindered or less exposed than Met^{1} but still remains accessible to oxidation. Oxidation of these two residues was relatively fast and showed little effect on leptin molecular structure. The non-cooperative kinetic model obtained from TBHP oxidation of leptin, clearly suggests that these two residues may be located in different regions of the protein. This observation can be confirmed by the crystal structure of leptin where Met^{69} is located at the opposite end of the four-helix bundle from Met^{1}.

In the H_2O_2 oxidation studies, the Met^{1}, Met^{69} di-oxidized leptin was the major soluble oxidized product. Further oxidation at Met^{55} and Met^{137} resulted in generation of tri-, tetra-, and highly oxidized leptins that readily formed precipitate. There was only a small amount of tri-, and tetra-oxidized leptins detected in solution, and the precipitate contained mostly tetra- and highly oxidized leptin, and no tri-oxidized species. These results suggest that tri-oxidized leptins were transiently present during oxidation and quickly transformed to tetra- and other oxidized species. These observations clearly indicate that oxidation of Met^{55} and Met^{137} was detrimental to the molecular stability of leptin with possible reasons described below. The oxidation rates of these two methionines are relatively slow, which may be due to that they reside near a solvent-inaccessible region of leptin; probably in the interior of the protein. In the crystal structure of human leptin (18), Met^{55} and Met^{137} are parts of the B and D helices, respectively, where the two side chains folds together near the leptin core structure of the four helical bundle. The oxidation of Met^{55} and Met^{137} residues would occur concurrently with the disruption of the tertiary

structure which then led to the formation of precipitate. The likelihood of these two residues being oxidized upon protein unfolding would probably be the same, and therefore the rates of oxidation for Met^{55} and Met^{137} should be comparable. Oxidation-induced structural perturbation of leptin appears to account for the very unstable nature of the two tri-oxidized leptins.

The bioactivity of Met^{1} mono-oxidized leptin remained intact while a significant decrease in activity occurred for the Met^{1}, Met^{69} di-oxidized leptin. Therefore Met^{69} might be critical for maintaining leptin in its biologically active form, or could itself play a critical role in the function of the protein, especially in the binding of leptin to the receptor. It is also possible that oxidation of Met^{69} destabilizes the protein structure (slightly lower thermostability, see above) and the lower activity of the Met^{1}, Met^{69} di-oxidized leptin might also reflect protein denaturation occurring at 37 °C during the 3 day incubation period required for *in vitro* biological assay. This has been observed during the *in vitro* oxidation of other cytokines (our unpublished data).

Our results showed that the four methionines in leptin possessed different degrees of reactivity to chemical oxidation which was shown in the order of $Met^{1} > Met^{69} >> Met^{55} \approx Met^{137}$. On the other hand, the importance of the methionines for maintaining leptin structural integrity was reversed, i.e., $Met^{55} \approx Met^{137} >> Met^{69} \approx Met^{1}$. It appeared, however, that an intact Met^{69}, but not Met^{1}, was essential for optimum biological activity of leptin. Leptin found in human serum contains only three methionines, Met^{55}, Met^{69}, and Met^{137}, and the integrity of all of these three methionines would be required for the protein to have both full potency and a stable tertiary structure.

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