Oxidative Degradation of Antiflammin 2

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Received August 10, 1995; accepted November 9, 1995

Purpose. To study the oxidation of the methionine residue of antiflammin 2 (HDMNKVLDDL, AF2) as a function of pH, buffer concentration, ionic strength, and temperature using different concentrations of hydrogen peroxide and to determine the accessibility of methionine residue to oxidation.

Methods. Reversed-phase high-performance liquid chromatography (RP-HPLC) was used as the main analytical method in determining the oxidation rates of AF2. Calibration curves for AF2 and the oxidation product, methionine sulfoxide of AF2 (Met(O)-3-AF2), were constructed for each measurement using standard materials. Fast Atom Bombardment Mass Spectroscopy (FABMS) was used to characterize the product.

Results. Met(O)-3-AF2 was the only oxidation product detected at pH 3.0 to 8.0. The oxidation rates were independent of buffer concentrations, ionic strength, and pH from 3.0 to 7.0. However, there was an accelerating rate at basic pHs, and small amounts of degradation products other than Met(O)-3-AF2 were observed in this alkaline region.

Conclusions. Oxidation of methionine in AF2 does not cause the biological inactivation reported by other laboratories since this drug is relatively stable under neutral conditions in the absence of oxidizing agent.

KEY WORDS: antiflammin 2; oxidation; stability; degradation; HPLC.

INTRODUCTION

Corticosteroids are profoundly effective drugs for treating inflammatory diseases, and it has been suggested that the antiinflammatory effects are partially mediated by inducing regulatory proteins (1). One regulatory protein, lipocortin I, is believed to inhibit phospholipase A2 (PLA2) activity, thereby preventing the release of arachidonic acid from membrane phospholipids, thus inhibiting the production of inflammatory lipid mediators. It has been shown that antiflammin 2 (HDMNKVLDDL, AF2), a synthetic nonapeptide derived from the active region of lipocortin I (residues 246–254 of lipocortin I), has potent PLA2 inhibitory activity in vitro and striking antiinflammatory effects in vivo while not possessing any known side effects of corticosteroids (2–9). However, several laboratories have reported that they could not detect any inhibitory activity on pancreatic PLA2 in vitro or antiinflammatory activity in vivo with AF2 (10–12). Because the methionine residue at position 3 is susceptible to oxidation, it has been suggested that the lack of AF2 activity is attributed to oxidation of the methionine residue (13). It has also been found that oxidation of methionine is the major degradative pathway when AF2 is formulated in a petrolatum base.

The sensitivity of the methionine residue to oxidation has not been investigated; therefore, the effect on PLA2 inhibitory and antiinflammatory activity is still not known. In this study, oxidation of AF2 was conducted using the simple oxidant, hydrogen peroxide, to determine the ease of oxidation of the methionine residue as a function of peroxide concentration, pH, buffer concentration, and temperature.

MATERIALS AND METHODS

Materials

All chemicals, except methionine sulfoxide of AF2, Met(O)-3-AF2, were analytical grade and used as received: AF2 was obtained from Bachem, Inc. (Torrance, CA). Hydrogen peroxide (50%) was obtained from EM Scientific and diluted freshly with reaction buffer solutions to the concentration desired for each experiment. HPLC-grade acetonitrile and water were obtained from J. T. Baker. Trifluoroacetic acid (TFA, HPLC grade) was purchased from Aldrich. Met(O)-3-AF2 was purchased from Chiron Mimetopes and purified by reversed-phase HPLC on C-18 column with a mobile phase composition of 22% acetonitrile in water with 0.1% TFA at 220 nm and 4 ml/min flow rate. The peptide was characterized by FABMS.

HPLC Equipment

The HPLC system consisted of Shimadzu LC-10AD pumps, a SCL-10A system controller, a CR501 Chromatopac integrator, a SPD-10AV UV-vis detector, a SIL-10A autoinjector, a FRC-10A auto fraction collector, and a sample cooler. A Beckman ODS column (10 × 250 mm diameter, 10-μm resin) was used for peptide purifications, and a Vydac ODS column (4.6 × 250 mm, 5-μm resin) was used for all other analytical measurements.

Buffer Solutions

The buffer solutions were prepared at the following pHs: pH 3.0 (0.01 M sodium formate), pH 5.0 (0.01 M sodium acetate), pH 7.0 (0.01 M sodium phosphate), pH 8.0 (0.01 M sodium phosphate), pH 9.0 (0.01 M sodium borate), and pH 10.0 (0.01 M sodium carbonate), to study AF2 oxidation at different pHs. The buffer solutions for this study were maintained at 0.1 M ionic strength with sodium chloride. For experiments in which the rate of the reaction was studied as a function of buffer concentration, buffers were prepared at 0.005 M, 0.025 M, and 0.1 M at pH 5.0, each having 0.1 M ionic strength, respectively. To study the effect of ionic strength, buffers were prepared at pH 5.0 (0.01 M buffer concentration) with ionic strengths of 0.01 M, 0.03 M and 0.3 M, respectively. For temperature studies, buffer 5.0 buffer solution (0.01 M sodium acetate and 0.1 M ionic strength) was used. Buffer solutions were also prepared at pH 9.0 (sodium borate) and 10.0 (sodium carbonate) at 0.0005 M and 0.0025 M buffer concentrations, respectively, having 0.1 M ionic strength. The pH readings were obtained from an ORION pH/ISE meter.

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Oxidative Degradation of Antiflammin 2: Kinetic Measurements

The oxidation of AF2 was first performed in pH 5.0 buffer solution (0.01 M sodium acetate and 0.1 M ionic strength maintained with NaCl) at 20°C. The initial AF2 concentration was 50 µg/ml (4.6 × 10⁻² M), and the initial concentrations of hydrogen peroxide were used at 0.46 mM, 2.3 mM, 11.5 mM, 23.1 mM, and 115.3 mM corresponding to H₂O₂:AF2 molar ratios of 10, 50, 250, 500, and 2500, respectively. The reaction was initiated by adding the peroxide solution to the peptide solution in a silanized vial. The reaction was monitored by HPLC after withdrawing aliquots at selected intervals and loading onto the HPLC column for analysis. Calibration curves for AF2 were constructed over the concentration range of 5–55 µg/ml. The standard material, Met(O)-3-AF2, was used to construct the calibration curve for the putative oxidative product.

To further study the reaction as a function of pH, the reaction was performed in pH 3.0, 7.0, 8.0, 8.5, and 9.0 (0.01 M buffer concentration, 0.1 M ionic strength) buffer solutions at 20°C, respectively. To study the effect of buffer concentration, the reaction was performed at buffer concentrations of 0.005 M, 0.025 M, and 0.1 M, respectively, in pH 5.0 sodium acetate buffer with 0.1 M ionic strength at 20°C. To study the effect of ionic strength, the reaction was performed in pH 5.0 buffer (0.01 M sodium acetate) with 0.01 M, 0.03 M, and 0.3 M ionic strength, respectively, at 20°C. To study the effect of temperature, the reaction was performed at 10, 20, 30, and 40°C, respectively, in pH 5.0 buffer (0.01 M sodium acetate with 0.1 M ionic strength). Three different hydrogen peroxide concentrations, 0.46 mM, 11.5 mM, and 115.3 mM, were used in the above studies, respectively. To study the effect of buffer concentration at alkaline pHs, the reaction was carried out in pH 9.0 and 10.0 at 0.0005 M and 0.0025 M buffer concentrations, respectively, with 0.1 M ionic strength. The peroxide concentration was 11.5 mM. Each reaction was performed in triplicate.

HPLC Analysis

The HPLC system was used as described above, and a Vydac C-18 column (4.6 × 250 mm, 5-µm resin) was used to monitor the reactions. The analysis method for AF2 was chosen as follows: mobile phase, 20% acetonitrile in water with 0.1% TFA; flow rate, 1 ml/min; UV detection wavelength, 220 nm. AF2 and the oxidation product were quantitated by measuring the individual peak area, respectively, and the concentration of each was calculated from the corresponding standard curve.

Identification and Characterization of Oxidation Product from AF2

The oxidation product was purified by HPLC, lyophilized, and characterized by FABMS. Met(O)-3-AF2 standard was synthesized by Chiron Mimetopes and analyzed as described above. The oxidation product coeluted with the standard.

Data Analysis

Each experimental data set was fitted into the pseudo-first-order kinetic model (see section below) using Microsoft Excel software, and the pseudo-first-order rate constants for the loss of AF2 and that for the formation of Met(O)-3-AF2 were generated using nonlinear regression. The corresponding second-order rate constants were then calculated from the slopes of linear plots of the pseudo-first-order rate constants for the disappearance of AF2 versus peroxide concentrations at different reaction conditions, respectively. Standard deviations of the observed pseudo-first-order and the second-order rate constants were calculated.

RESULTS AND DISCUSSION

Identification and Characterization of Oxidation Product from AF2

A representative chromatographic profile, obtained from the HPLC monitoring of an oxidation reaction of AF2, is shown in Figure 1, which demonstrates the progress of oxidation of AF2 over time. The area of Peak 1 (Vₖ: 7 mL) increases with time and resulted from the oxidation of AF2 (Peak 2). In the middle stage of the oxidation reaction, the eluent under Peak 1 was collected, lyophilized, and analyzed by FABMS. The protonated peptide under Peak 1 had a mass of 1101 Da, which is equal to the mass of protonated monooxidized methionine residue at position 3 in AF2 (1084 (mass of AF2) + 17). Peak 2 coeluted with AF2 standard. The chromatographic retention value of the monooxidized product was compared with that of the reference standard, Met(O)-3-AF2 (see experimental section), and their peaks were superimposable. Further oxidation of the sulfoxide to the corresponding sulfone was not observed under any experimental conditions. Degradation of AF2 by routes other than oxidation was not observed in the pH range of 3.0 to 8.0.

AF2 Oxidation as a Function of pH, Buffer Concentration, Ionic Strength, and Temperature

The kinetics of AF2 oxidation were followed by plotting the area under the peaks for the intact drug AF2 and for the oxidation product Met(O)-3-AF2, respectively, as a function of time. Figure 2 shows one reaction profile representing the time courses of loss of AF2 and the formation of Met(O)-3-AF2.