Oxidative Degradation of Antiflammin 2

Jennifer M. Ye and Janet L. Wolfe[1,2]

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Purpose. To study the oxidation of the methionine residue of antiflammin 2 (HDMNKVLDL, 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 (RPHPLC) 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 acceleration of the rates 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 oxiding 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 A_2 (PLA_2) 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 (HDMNKVLDL, AF2), a synthetic nonapeptide derived from the active region of lipocortin I (residues 246–254 of lipocortin I), has potent PLA_2 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 PLA_2 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 PLA_2-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 autofraction 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), 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, pH 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.

[1] Department of Pharmaceutical Sciences, University of Tennessee, 26 South Dunlap Street, Memphis, Tennessee 38163.
[2] To whom correspondence should be addressed.

Oxidation 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} mM), 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 H_2O_2: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 oxidation 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 min/ml; 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 (V_R: 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.

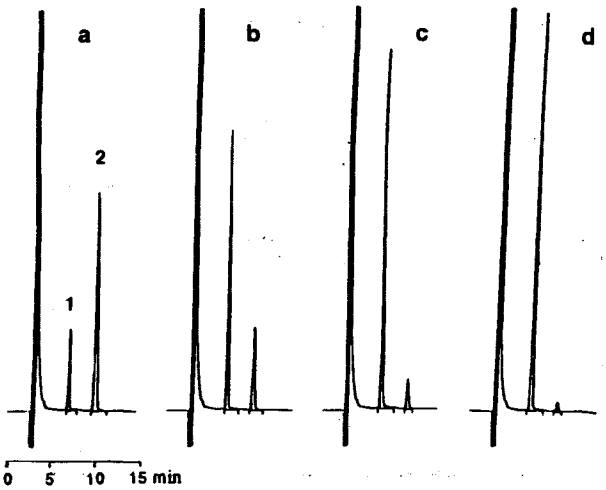

Fig. 1. HPLC chromatogram of AF2 in 115 mM hydrogen peroxide at pH 5.0 (0.1 M acetate, μ = 0.1 M) and 20°C. Aliquots were removed from the reaction mixture and injected onto the HPLC system at a, 2; b, 17; c, 32; and d, 47 min, as shown in the above chromatograms.

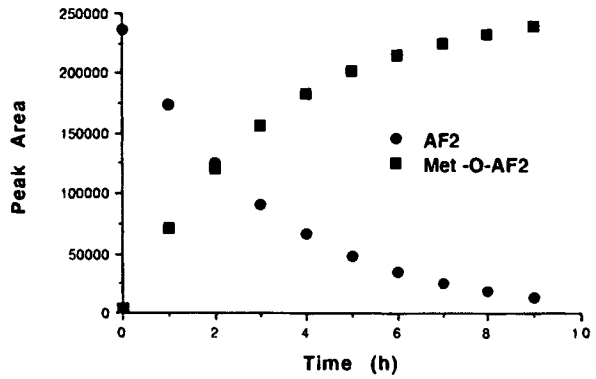

Fig. 2. A plot of the loss of AF2 and the formation of Met(O)-3-AF2 at pH 5.0 (0.01M acetate, μ = 0.1M) in 11.5 mM hydrogen peroxide as a function of time. Each point was obtained by injecting an aliquot of the reaction mixture onto the HPLC system.

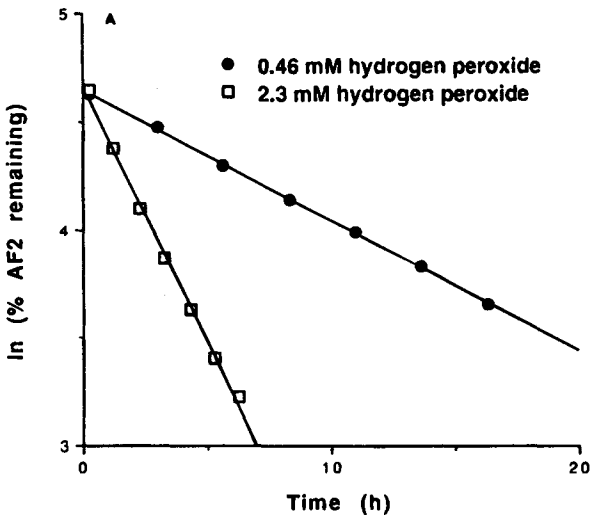

Fig. 3A. The loss of AF2 as a function of time is shown at pH 5.0 (0.01M acetate buffer, μ = 0.1 M) for different hydrogen peroxide concentrations. Each data point was obtained by loading an aliquot of the reaction mixture onto the HPLC system.

This particular reaction was performed using pH 5.0 sodium acetate buffer (0.01 M) with an ionic strength of 0.1 M at 20°C. The initial concentration of AF2 was 50 μg/ml and the initial concentration of hydrogem peroxide was 11.5 mM. As seen from the figure, the product Met(O)-3-AF2 readily formed as AF2 disappeared, and the reaction can be described by the following equation:

$$\text{AF2(A)} \xrightarrow{H_2O_2} \text{Met(O)-3-AF2(B)} \qquad (1)$$

Kinetic analysis of the data from all conducted experiments was based on the pseudo-first-order kinetic model derived from the above equation, because the peroxide used in the present study was in large excess compared with the amount of AF2. The differential equations describing the rates of AF2 loss and Met(O)-3-AF2 formation are expressed as

$$d[A]/dt = -k[A] \quad \text{and} \quad d[B]/dt = k[A],$$

respectively, where k is the pseudo-first-order rate constant. The corresponding analytical solutions are

$$A = A_0e^{-kt} \quad \text{and} \quad B = A_0(1 - e^{-kt}),$$

respectively. Since k is proportional to the concentration of H_2O_2, the second order rate constant k′ was obtained from the slope of the following equation:

$$k = k'[H_2O_2]$$

Table I summarizes the pseudo-first-order rate constants (from both measurements of AF2 loss and Met(O)-3-AF2 formation) and the corresponding second-order rate constants of the oxidation at 20°C in pH 3.0, 5.0, 7.0, and 9.0 buffer solutions (0.01 M and 0.1 M ionic strength). The standard deviations were less than 5%. Figures 3A and B show the representative linear plots of AF2 oxidation as a function of time at different peroxide concentrations in pH 5.0 buffer (0.01 M with 0.1 M ionic strength). The correlation coefficients are greater than 0.99.

The influence of pH on the oxidation rates of AF2 was studied at pH 3.0 (citrate), 5.0 (acetate), 7.0 (phosphate), 8.0 (phosphate), 8.5 (phosphate), 9.0 (borate), and 10.0 (carbonate), respectively, at 0.01 M buffer concentration and with 0.1 M

Table I. Observed Rate Constants for Oxidation of AF2 by Hydrogen Peroxide at Different pHs[a]

	Psuedo-first-order rate constants × 10_2 (h^{-1}) for the formation of Met(O)-3-AF2			Second-order rate constants
pH\[H_2O_2]	0.46 mM	11.5 mM	115 mM	($M^{-1}h^{-1}$)
3.0	1.380 ± 0.58	33.91 ± 0.30	373.7 ± 0.6	32.64
	1.387 ± 0.59[b]	34.83 ± 0.62[b]	370.4 ± 8.7[b]	
5.0	1.096 ± 0.49	33.86 ± 0.96	387.8 ± 6.8	33.94
	1.080 ± 0.47[b]	33.88 ± 0.40[b]	357.3 ± 8.6[b]	
7.0	1.284 ± 0.59	33.22 ± 2.30	373.4 ± 1.3	32.64
	1.300 ± 0.90[b]	32.70 ± 0.33[b]	383.4 ± 6.2[b]	
9.0	3.247 ± 0.57	81.14 ± 4.06	171.1 ± 1.2[c]	74.51
	3.198 ± 1.02[b]	80.24 ± 0.96[b]	167.0 ± 15.9[b,c]	

[a] Reactions were performed at 20°C with 0.01 M buffer concentration and 0.1 M ionic strength. Rates were calculated from the loss of AF2 except where indicated. Data were reported as mean ± standard deviation.

[b] Psuedo-first-order rate constants were calculated from the formation of Met(O)3-AF2.

[c] Hydrogen peroxide concentation was 23 mM.

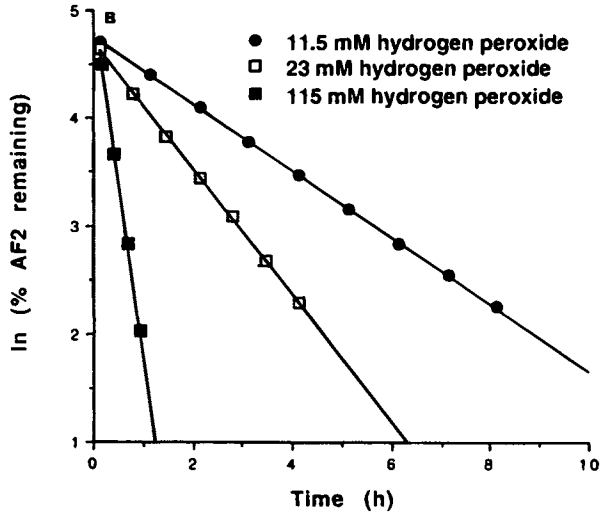

Fig. 3B. The loss of AF2 is shown at pH 5.0 (0.01M acetate, μ = 0.1M) for the indicated hydrogen peroxide concentrations. Each data point was obtained by injecting an aliquot of the reaction mixture onto the HPLC system.

ionic strength. At neutral to acidic pH range (pH 3.0–7.0), the rates differed only within experimental error, and the oxidation was essentially not affected by the change of pH in this region, as shown in Figure 4. However, the oxidation was accelerated in alkaline reaction medium and the rate increased significantly at pH 9.0 and 10.0 (data for pH 10.0 is not shown in Figure 4).

The oxidation was also studied in two buffer concentrations (0.0005 M and 0.0025 M) at pH 9.0 (borate) and 10.0 (carbonate), respectively. It was found that the rate of oxidation increases as a function of buffer concentration. Higher buffer concentrations at these pHs caused AF2 degradation by routes other than oxidation.

The effect of buffer concentration was also studied at pH 5.0 from 0.005 to 0.1 M (acetate, 0.1 M ionic strength),

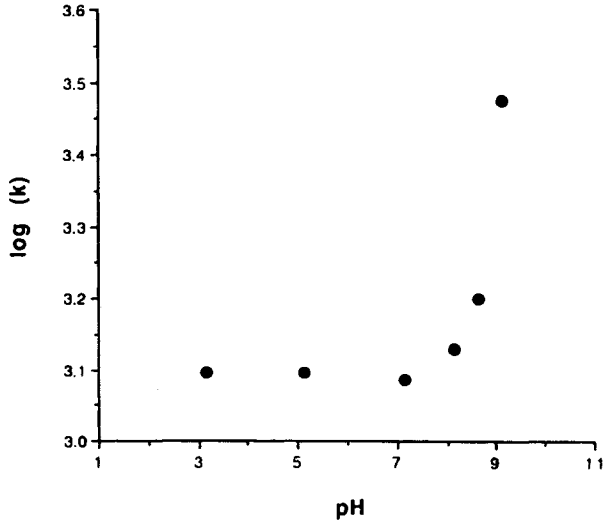

Fig. 4. pH-rate profile for the oxidation of AF2 in 11.5 mM hydrogen peroxide. The reaction was performed at 20°C and the ionic strength was maintained at 0.1M.

respectively, at 20°C. The results are presented in Figure 5a and showed the rate was not affected by the change of acetate concentration at peroxide concentrations studied.

The effect of ionic strength on the oxidation of AF2 was studied since ionic strength may contribute to peptide degradation. The results are shown in Figure 5b. At low hydrogen peroxide concentrations (0.46 and 11.5 mM), no apparent variations on the oxidation rates were observed when the ionic strengths changed from 0.01 to 0.3 M (0.01 M buffer concentration). As the concentration of the oxidant increased to 115 mM, the oxidation rates increased slightly over the range of the ionic strengths studied. Therefore, this oxidation was essentially insensitive to the variation of ionic strength at pH 5.0.

In the early 1960s, the oxidation of organic sulfides by hydrogen peroxide was studied extensively by Bannard et al. (14). They concluded that the reaction rate is affected by the nature and composition of solvent media, and the reaction is specific acid catalyzed. Nguyen et al. (15) reported on the oxidation of methionine residues in relaxin, and they found the oxidation rate is fairly independent of the pH over the range studied (from 3.0 to 8.0), and the specific acid catalysis is relatively weak and is masked by water catalysis at moderate acidity. The data presented in our study are consistent with these literature results in the pH range of 3.0 to 7.0.

However, the oxidation rate of AF2 increases significantly at pH 9.0 (borate) and 10.0 (carbonate). It was reported that the oxidation of methionine could be catalyzed by metal ions, such as iron and copper (16, 17), in the presence of ascorbate. The buffer salts utilized in this study contained significantly lower levels of these metals than were used in the literature reports (16, 17). Therefore, the accelerated degradation observed at alkaline pHs is probably not due to catalysis by these species. In addition, it has been reported that a neighboring His residue catalyszes the oxidation of methionine in the presence of Fe^{2+} (18). However, the observed increase of oxidation rate of AF2 only at pH 9.0 and 10.0 should not be catalyzed by histidine. In the present study, there is no indication of histidine-catalyzed oxidation of methionine at other pHs. In addition, at pH 9.0 and 10.0, free methionine was found to oxidize at rates comparable to those of the oxidation of AF2 (data not shown).

Literature reports have shown buffer catalysis in the iodine oxidation of N-acetylmethionine methyl ester (19, 20, 21). This buffer catalysis reaction involves the nucleophilic attack of the buffer species at the sulfonium sulfur formed by addition of I^+ to the sulfur center. This reaction is strongly catalyzed by carboxylate, phosphate, and carbonate anions. The oxidation of AF2 by hydrogen peroxide may be similarly influenced by buffer catalysis since, as previously mentioned, the oxidation rate is dependent on the concentrations of buffer species at pH 9.0 (borate) and pH 10.0 (carbonate).

Previous research on AF2 degradation in aqueous solutions (9) has shown extensive degradation at alkaline pHs. Small peaks were observed adjacent to the sulfoxide at pH 9.0 and 10.0 in the oxidation of AF2. It is possible that AF2 degradation at high pHs is due to several competing mechanisms, including oxidation of methionine.

The thermodynamic study of AF2 oxidation was performed by measuring the reaction rates at 10, 20, 30, and 40°C, respectively, in 0.01 M acetate buffer solution at pH 5.0 with ionic strength of 0.1 M maintained with NaCl. An Arrhenius

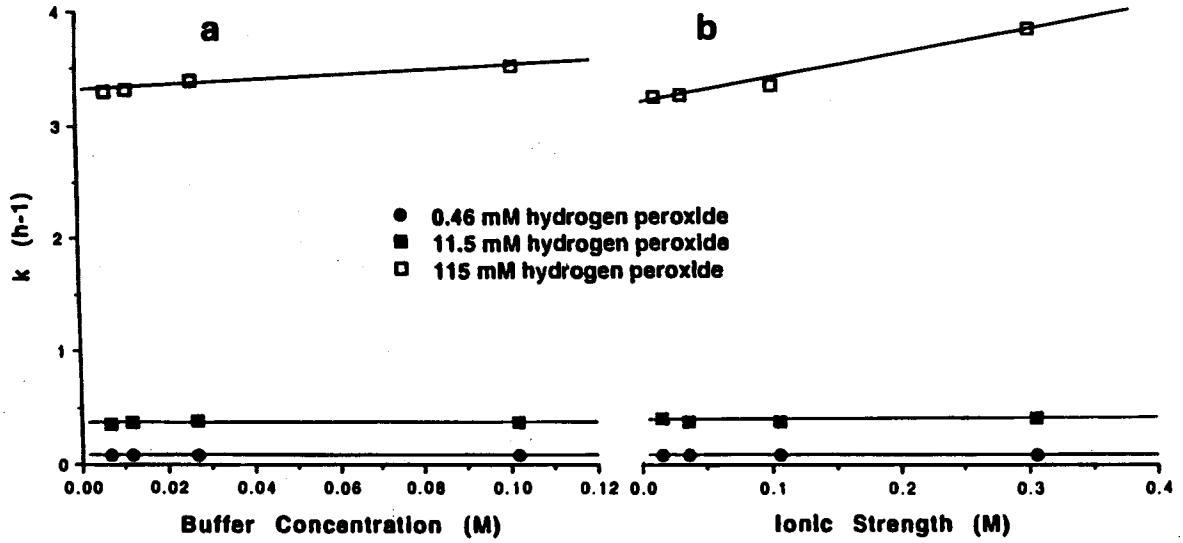

Fig. 5. a, AF2 oxidation rates as a function of buffer concentrations. b, AF2 oxidation rates as a function of ionic strengths. The reactions were carried out at pH 5.0 and 20°C.

plot is shown in Figure 6. The activation energy of the oxidation was estimated from the slope of the straight line, which was 12.22 kcal/mol.

CONCLUSION

The oxidation of AF2 was studied as a function of pH, buffer concentration, ionic strength, and temperature. The half-life of the oxidation at neutral to acidic conditions and at 20°C is 60 h with a molar ratio of 1:10 of AF2/H_2O_2. We conclude that the methionine residue in AF2 is not easily oxidized in the absence an oxidant, such as hydrogen peroxide, since the oxidation of AF2 in normal buffer solutions is very slow (9). In addition, buffer concentration and ionic strength have little effect on the oxidation rates of AF2 by hydrogen peroxide at acidic to neutral pHs. Therefore, the loss of biological activity observed by other laboratories is not due to oxidation of the methionine residue.

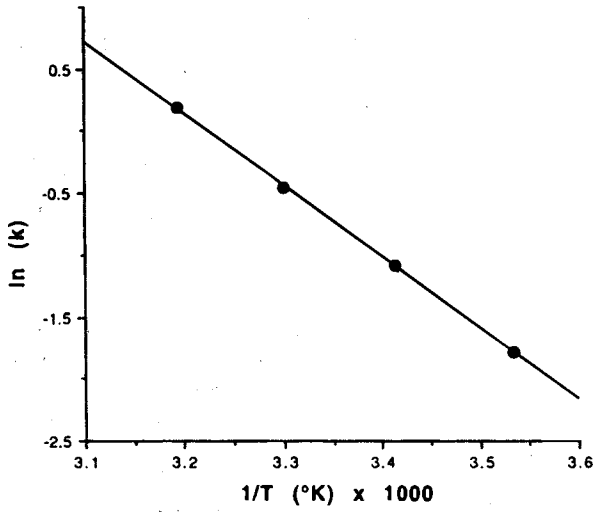

Fig. 6. Arrhenius plot for the loss of AF2. Reactions were performed in 11.5 mM hydrogen peroxide at pH 5.0 (μ = 0.1M, 0.01M acetate).

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