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EFFECT OF OXIDATION OF METHIONINE IN A PEPTIDE SUBSTRATE FOR HUMAN ELASTASES: A MODEL FOR INACTIVATION OF α_1-PROTEASE INHIBITOR*

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Summary: Human α_1-protease inhibitor which is an important plasma protein, contains a methionine residue at its reactive site. A model synthetic peptide substrate, succinyl-L-alanyl-L-alanyl-L-prolyl-L-methionine p-nitroanilide, has been employed to study the effect of oxidation of methionine on the rate of hydrolysis of this substrate by human elastases. The methionine sulfoxide derivative obtained by mild oxidation of this substrate is hydrolyzed by pancreatic elastase 2 and leukocyte elastase at rates that are 5% and 0.3% of the rates measured for hydrolysis of the parent compound by the respective enzymes. These results suggest that oxidation of the active site methionine residue of human α_1-protease inhibitor may decrease the rate of reaction of pancreatic or leukocyte elastase with this inhibitor.

INTRODUCTION

It is now widely accepted that the degradation of elastin associated with emphysema probably results from a local imbalance of elastolytic enzymes and the naturally occuring tissue and plasma protease inhibitors (1). In this regard, a genetic deficiency of the major serum protease inhibitor, α_1-PI[1], has been shown to be associated with the premature development of pulmonary emphysema (2). Human α_1-PI, which accounts for 90 percent of the trypsin inhibitory capacity in plasma, rapidly inhibits human pancreatic (3) and leukocyte (4) elastases. Recently, Johnson and Travis (5) reported that leukocyte elastase, as well as pancreatic chymotrypsin and trypsin, interact with a unique methionine residue in α_1-PI during complex formation. These

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[1] α_1-PI, α_1-protease inhibitor; SAAPM-NA, succinyl-L-alanyl-L-alanyl-L-prolyl-L-methionine p-nitroanilide; SAAPMS-NA, succinyl-L-alanyl-L-alanyl-L-prolyl-L-methionine sulfoxide p-nitroanilide.

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authors suggested that oxidation of this methionine residue could decrease the rate of reaction of proteases with α_1-PI, presumably by reducing the affinity of the enzyme for the oxidized methionine. Since enzymatic hydrolysis is also dependent on the affinity of the enzyme for a specific configuration of a substrate, it appeared that this hypothesis could be tested using a synthetic, methionine containing peptide. During the course of a study on the substrate specificity of human pancreatic elastase 2, we synthesized a tetrapeptide p-nitroanilide (SAAPM-NA) with a methionine in the P1 position. The present study reports the effect of oxidation of this methionine residue on the rate of hydrolysis of this compound by human pancreatic and leukocyte elastases.

MATERIALS AND METHODS

Human pancreatic elastase 2 was purified as described previously (6). Human leukocyte elastase was a generous gift of Dr. I.P. Crawford, La Jolla, California. SAAPM-NA was synthesized using standard peptide synthesis techniques as part of a series of compounds designed to investigate the substrate specificity of human pancreatic elastase 2, the details of which will be reported in a future communication. The synthetic approach employed was as follows: 1) Pro-t-butyl ester was acylated with CBZ-ala-p-nitroanilide in methylene chloride in the presence of triethylamine; 2) The CBZ group was removed by catalytic hydrogenation and the resulting oil was acylated with CBZ-ala-p-nitroanilide; 3) Crystalline CBZ-ala-ala-pro-t-butyl ester was treated with 50% trifluoroacetic acid in methylene chloride to remove the t-butyl group; 4) CBZ-methionine was coupled to p-nitroanilide using the phospho-azo procedure; 5) The CBZ group was removed from CBZ-met-p-nitroanilide by treatment with 16% HBr in acetic acid for one hour in the presence of a 5-fold molar excess of methionine; 6) CBZ-ala-ala-pro was coupled to met-p-nitroanilide using EDAC in methylene chloride in the presence of 1-hydroxybenzatriazole and triethylamine; 7) the CBZ group was removed with HBr/HOAc in the presence of excess methionine as described above; and 8) the amine was reacted with succinic anhydride.

SAAPM-NA was a single spot (R_f = 0.67) by thin layer chromatography, and gave a consistent amino acid analysis. The ϵ_{315} for SAAPM-NA was 13,500.

SAAPMS-NA was prepared from SAAPM-NA by oxidation with DMSO/HCl/acetic acid (10:50:100) as described by Savige and Fontana (7). This procedure yields only the sulfoxide derivative. The product contained one mole of methionine sulfoxide, and no methionine sulfone, following hydrolysis in 3 M p-toluenesulfonic acid and amino acid analysis. Methionine sulfoxide was resolved from methionine sulfone under the conditions employed. SAAPMS-NA gave a major spot (R_f = 0.47) and a minor impurity spot on TLC, and yielded a consistent amino acid composition. The ϵ_{315} for SAAPMS-NA = 12,000.

The kinetic analyses of the hydrolysis of SAAPM-NA or SAAPMS-NA by elastase 2 were performed in 0.2 M Tris.HCl (pH 8.0) at 25° at enzyme concentrations of 0.66 µg/ml and 2.5 µg/ml, respectively. The absorbance of the p-nitroaniline produced was measured continuously on a Gilford spectrophotometer at 410 nm. Two K_m determinations were made for each compound with duplicate determinations

at eight substrate levels between 0.4 and 10 mM. The kinetic analysis of the hydrolysis of SAAPM-NA by human leukocyte elastase was performed at an enzyme concentration of 5.7 µg/ml. A K_m curve was determined using four substrate levels between 0.2 and 5 mM. The K_m and k_{cat} were determined from the data following a least squares analysis (8). The relative rates of hydrolysis of SAAPM-NA and SAAPMS-NA by leukocyte elastase were determined at 2.5 mM substrate concentration.

RESULTS AND DISCUSSION

The kinetic parameters for the hydrolysis of SAAPM-NA and SAAPMS-NA by human pancreatic elastase 2 and leukocyte elastase are shown in Table I. SAAPM-NA is a significantly better substrate for human pancreatic elastase 2 than for the leukocyte enzyme. Pancreatic elastase 2 hydrolyzes this compound, with a methionine in the P1 position, at a rate which approaches that of the best substrates for this enzyme[2]. In contrast, human leukocyte elastase has been shown to preferentially hydrolyze substrates in which valine occupies the P1 position (9,10).

The oxidation of the methionine residue in SAAPM-NA to the sulfoxide yields a compound which is a poorer substrate for elastase 2 in terms of both K_m and k_{cat}. The ratio k_{cat}/K_m for hydrolysis of SAAPMS-NA by human pancreatic elastase 2 *versus* SAAPM-NA is decreased by approximately 30-fold. The rate of hydrolysis of SAAPMS-NA by leukocyte elastase is so low that it was not possible to obtain accurate kinetic data. However, the data obtained indicate that oxidation of the methionine residue results in at least a 300-fold decrease in the rate of hydrolysis of this peptide by human leukocyte elastase.

The observation that the human pancreatic and leukocyte elastases are very sensitive to oxidative changes in substrates in which methionine occupies the P1 position, coupled with the demonstration of a methionine in the corresponding P1 position in α_1-PI (5), suggests that these enzymes might react much more slowly with α_1-PI that contains a methionine sulfoxide residue in this position. However, trypsin is also rapidly bound by α_1-PI despite the

[2] Del Mar, E.G., Largman, C., Brodrick, J.W., and Geokas, M.C., unpublished observations

Table I

KINETICS FOR HYDROLYSIS OF SAAPM-NA AND SAAPMS-NA BY HUMAN ELASTASES

Enzyme	Substrate	K_m(M)	$k_{cat}(sec^{-1})$	$k_{cat}/K_m(sec^{-1}M^{-1})$
Pancreatic Elastase 2	SAAPM-NA	0.0013	6.2	4770
Pancreatic Elastase 2	SAAPMS-NA	0.0035	0.56	160
Leukocyte Elastase	SAAPM-NA	0.0044	0.32	72
Leukocyte Elastase [a]	SAAPMS-NA	-	-	-

[a] The rate of hydrolysis of SAAPMS-NA was so low that accurate K_m and k_{cat} values could not be determined for hydrolysis of this substrate by leukocyte elastase. The rate of hydrolysis of this compound was less than 0.3% that observed for hydrolysis of SAAPM-NA by leukocyte elastase.

lack of a basic residue at the P1 site, suggesting that an extended binding site may contribute to the stability of α_1-PI-protease complexes, as has been demonstrated for complexes of trypsin with bovine pancreatic trypsin inhibitor (11). Thus, the extent to which oxidation of the P1 methionine residue in α_1-PI destabilizes protease binding will depend upon the relative contribution of interactions remote from the "active site" of the inhibitor.

We have previously reported that immunoreactive forms of human pancreatic elastase 2 are present in normal serum (12). The possible role of pancreatic elastase 2 in the pathogenesis of degenerative diseases which involve elastic tissue, such as emphysema or atherosclerosis, remains unclear. However, the balance between circulating pancreatic elastase 2 or leukocyte elastase and α_1-PI should be important in the mechanism of tissue injury. Consequently, any process which results in the reduction of the binding capacity of this inhibitor might be of significance. Since methionine is relatively easily oxidized to the sulfoxide derivative (7), it is possible that agents present in cigarette smoke might oxidize the active site methionine in α_1-PI. In this regard, Janoff and Carp have reported that cigarette smoke condensate appears

to inactivate α_1-PI *in vitro* with respect to inhibition of porcine pancreatic or leukocyte elastase (13). Furthermore, Travis *et al* (14) have recently stated that α_1-PI is rapidly inactivated by treatment with N-chlorosuccinimide, a specific oxidant for methionine.

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