THE MOLECULAR STOICHIOMETRY OF TRYPSIN INHIBITION BY HUMAN ALPHA-1-PROTEINASE INHIBITOR

David A. Johnson, Ralph N. Pannell, and James Travis*

Department of Biochemistry
University of Georgia
Athens, Georgia 30602

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Summary: The stoichiometry of interaction of human alpha-1-proteinase inhibitor with porcine trypsin has been determined using a highly purified preparation of inhibitor. In contrast to the reports of others, one mole of alpha-1-proteinase inhibitor was found to inhibit two moles of trypsin. Disc gel electrophoresis indicates that the 2:1 complex is preferentially formed even when free alpha-1-proteinase inhibitor is still present.

INTRODUCTION

Human α-1-proteinase inhibitor, previously referred to as α-1-antitrypsin, is responsible for more than 70% of the inhibitory activity of plasma. This protein has always been assumed to interact with proteinases at a 1:1 ratio on a molar basis (1-4). Recently, however, two preparations of the inhibitor, which were isolated by substantially different procedures (5,6), have been found to have an increased inhibitory activity towards trypsin, (greater than 1:1 binding) suggesting that one molecule of α-1-PI may have multiple inhibitory sites. In this laboratory we have obtained highly purified α-1-PI (7) with a specific activity 30-60% greater than the best previously reported (6). For this reason, we have re-investigated the stoichiometry of interaction of the inhibitor with trypsin. This report summarizes the results obtained using two different preparations of α-1-PI.

Abbreviations: α-1-PI, alpha-1-proteinase inhibitor; BAEE, N-benzoyl-L-arginine ethyl ester.

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MATERIALS AND METHODS

Bovine trypsin (2x crystallized) was purchased from Worthington Biochemical Co. BAEE was obtained from Schwartz/Mann and p-nitrophenyl-p'-guanidinobenzoate was a product of Cyclo Chemical.

Analytical polyacrylamide disc electrophoresis was performed according to Brewer and Ashworth (8) in 7.5% gels at pH 8.3. Proteins were located by staining with amidoschwartz.

Porcine trypsin (M.W. 23,400) was prepared according to Travis and Liener (9), and treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone to rule out any possibility of interference from chymotrypsin. The extinction coefficient used for a 1% solution at 280 nm was 13.5. The porcine trypsin was found to be 82% active by the active site titration method of Chase and Shaw (10). Porcine trypsin was used in preference to bovine trypsin because it is more stable, particularly at the alkaline pH used for pre-incubations with α-1-PI. We have found bovine trypsin to be 65% active and others have reported it to be only 55% active (11).

Trypsin, esterase activity was measured at pH 8.0 (0.1 M Tris-HCl) in the presence of 50 mM Ca^{+2} by the technique of Schwert and Takenaka (12) as described by Mallory and Travis (13). α-1-PI inhibitory activity was determined as a loss of trypsin esterase activity. A given amount of inhibitor was incubated with a standard sample of trypsin for ten minutes at pH 8.0, followed by the addition of substrate under the usual assay conditions. One unit of inhibitory activity was defined as being equivalent to the loss of one unit of trypsin esterase activity. Specific activities are reported as inhibitory units per absorbance unit 280 nm.

The purification of α-1-PI has been described elsewhere (7); however a brief account of the scheme seems appropriate. Albumin free plasma, prepared by chromatography on sepharose blue dextran as described by Travis and Pannell (14), was fractionated by ammonium sulfate precipitation taking the precipitate between 65% and 80% saturation. This fraction was chromatographed on

DEAE-cellulose at both pH 8.8 and pH 6.5. The pH 6.5 chromatography was required to separate α-1-PI from α-1-acid glycoprotein (orosomucoid), a contaminant which migrates slightly ahead of α-1-PI on polyacrylamide disc gel electrophoresis and stains poorly with amidoschwartz. A molecular weight of 58,000 g/mole and an extinction coefficient of 6.88 for a 1% solution at 280 nm were used in the current experiments (7).

RESULTS

The stoichiometry of the inhibition of porcine trypsin by α-1-PI was determined by pre-incubating various amounts of α-1-PI with a constant amount of trypsin for 10 minutes followed by measurement of esterase activity as described in Methods. Increasing the pre-incubation time to as long as one hour had no effect. Assays were performed in duplicate, with the average value reported. Percent activity is the esterase activity remaining relative to a control pre-incubated at pH 8.0 for the same length of time. The results of these determinations are shown in Figure 1. The ratio, mole α-1-PI/mole porcine trypsin, refers to the amounts of each in the pre-incubation mixture. The moles of trypsin in these mixtures were calculated from the 280 nm absorbance of the solution used based on a molecular weight of 23,400 and corrected for 82% active sites.

These determinations were performed on two separate occasions. In the first experiment (A) freshly prepared α-1-PI with a specific activity of 20.0 was used. In experiment B a different α-1-PI preparation with a specific activity of 16.0 was used. The preparation with the lower specific activity had been stored for several days at 4°C and pH 8.8.

The linear plots obtained intersect the mole α-1-PI/mole porcine trypsin axis on each side of 0.5. A value of 0.5 corresponds to the inhibition of two moles of trypsin per mole of α-1-PI. The difference in the two experimental results is probably due to differences in the specific activities of the α-1-PI preparations used. Experiment A implies that α-1-PI inhibits slightly more

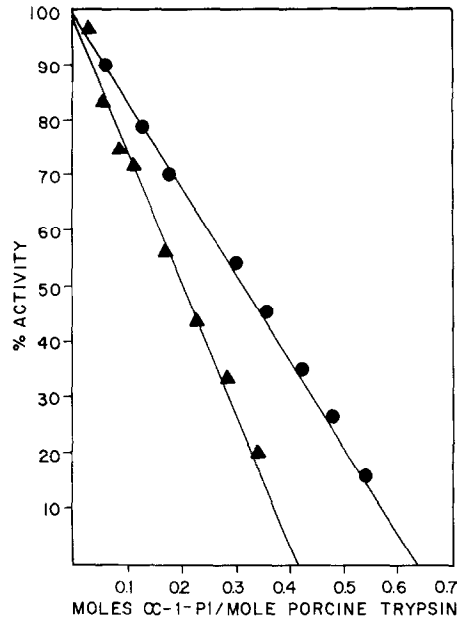

Fig. 1. Percentage of esterase activity remaining at various molar ratios of α-1-PI to porcine trypsin. Experiment A, ▲—▲, α-1-PI with a specific activity of 20. Experiment B, ●—●, α-1-PI with a specific activity of 16.

than two moles of trypsin per mole. This additional loss of esterase activity is possibly due to the involvement of uncomplexed trypsin in the degradation of the trypsin-α-1-PI complex.

The results obtained in the above experiment were corroborated by polyacrylamide disc gel electrophoresis of mixtures of α-1-PI and porcine trypsin. The mixtures were prepared at pH 8.0 and electrophoresis was begun within 30 minutes. As can be seen from the patterns, (Figure 2) the α-1-PI band does not disappear until two moles of trypsin per mole of α-1-PI have been added. At a 0.5:1.0 molar ratio of trypsin to α-1-PI only one complex band is seen, while at 1:1, 1.5:1 and 2:1 (trypsin:α-1-PI) two bands representing complex are seen. The fact that all of the α-1-PI band does not disappear until a 2:1 molar ratio is reached, suggests to us that two molecules of trypsin are being bound simultaneously to each molecule of α-1-PI. Therefore a 1:1 complex probably does not even exist, except as a possible short lived intermediate. At present we do not know the meaning of the double complex bands, but the

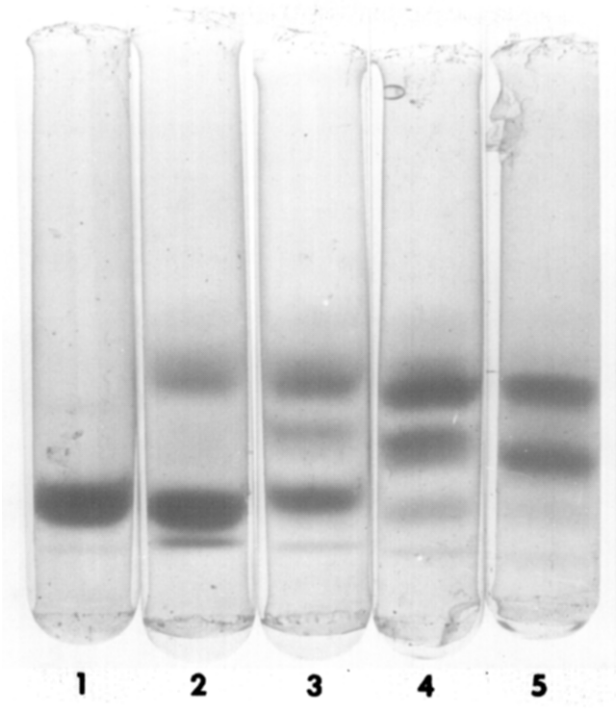

Fig. 2. Polyacrylamide disc gel electrophoresis of mixtures of α-1-PI and porcine trypsin at various molar ratios (porcine trypsin:α-1-PI) (1) 0:1, (2) 0.5:1, (3) 1:1, (4) 1.5:1, (5) 2:1.

faster band, not seen at 0.5:1, may be a complex from which some basic peptide has been lost.

DISCUSSION

From the results obtained in Figures 1 and 2 it can be assumed that the stoichiometry of inhibition of trypsin by α-1-PI is 2:1. This finding has some important implications. The amount of α-1-PI in normal plasma has long been reported to be 210-500 mg per 100 ml of plasma (15), but this value was determined from the trypsin inhibitory capacity of plasma assuming 1:1 stoichiometry. Clearly, the level of α-1-PI in normal plasma is only half the above value. Indeed we recover 53% of the antitrypsin activity of whole plasma in our product but the protein yield is only 47 mg per 100 ml of plasma (7). This indicates that the normal level is about 100 mg per 100 ml of plasma.

Measurement of the stoichiometry of interaction of bovine trypsin with α-1-PI has been made in several laboratories (1-4). Unfortunately, each of these groups assumed that the preparations of trypsin used were 100% active when, in all probability, a value of 65% was more realistic. Thus, meaningful comparisons of the inhibitory activity of α-1-PI preparations from different laboratories cannot be made.

Recently, Fasco and Fenton reported methods for titrating the active sites of thrombin, plasmin, and chymotrypsin (11). Thus, it should now be possible to measure exact inhibitory capacities of preparations of proteinase inhibitors with activities directed toward the above enzymes, and trypsin.

The mechanism by which α-1-PI inhibits proteolytic action is unknown. Furthermore, no results have as yet been reported as to whether any bond cleavage takes place in the inhibitor during the interaction. Studies in this direction are in progress as well as experiments to determine whether proteinases of higher molecular weight, such as plasmin, are inhibited in the same stoichiometric ratio as reported here for trypsin.

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