Biochem. J. (1989) 259, 905–907 (Printed in Great Britain)

Inhibition of aspartic proteinases by α_2-macroglobulin

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The effect of α_2-macroglobulin, one of the major antiproteinases in the plasma of vertebrates, on the action of the aspartic proteinases chymosin, cathepsin D and cathepsin E towards peptide and protein substrates at pH 6.2 was examined. Activities towards protein substrates were blocked, thus demonstrating that α_2-macroglobulin can inhibit aspartic proteinases, in addition to serine proteinases, cysteine proteinases and metalloproteinases.

INTRODUCTION

Proteinases are classified on the basis of their catalytic mechanism as belonging to one of four classes [1]. Each of these classes is regulated under (patho)physiological conditions by specific families of inhibitors, with the exception of aspartic proteinases, the activities of which are controlled primarily by other mechanisms [2]. Each proteinase inhibitor is thus very selective in recognizing only proteolytic enzymes within a particular class, e.g. serine proteinases.

The large plasma protein α_2-macroglobulin (M_r 725000) is something of an exception in that it can inhibit across classes, i.e. serine proteinases, cysteine proteinases and metalloproteinases. However, its ability to inhibit aspartic proteinases has been uncertain [3,4], since the efficacy of the inhibitor begins to diminish below about pH 6 whereas, reciprocally, the activity of aspartic proteinases (or acid proteinases as they were formerly termed) decreases rapidly as the pH is raised above about 5.5 [5]. Thus there is a scarcely overlapping pH range within which the proteins express their respective functions. However, the development of synthetic chromogenic peptides as substrates for aspartic proteinases [6] permits accurate kinetic measurements still be be made with some aspartic proteinases at pH values slightly above 6, so that it is now possible to re-examine the susceptibility of this class of proteinase to the important macromolecular inhibitor.

MATERIALS AND METHODS

Recombinant chymosin was obtained by activation of prochymosin expressed in *Escherichia coli* and purified as described in ref. [7]. It was generously supplied by Dr. R. Holdsworth, Boots–Celltech Diagnostics, Slough, Berks., U.K. Cathepsin D was purified to homogeneity from human spleen [8], and cathepsin E from human erythrocytes [9] was generously given by Dr. K. Yamamoto, Nagasaki University School of Dentistry, Nagasaki, Japan. Bovine α_2M was obtained from Boehringer Mannheim, Lewes, East Sussex, U.K.

The chromogenic peptide substrates Lys-Pro-Ile-Glu-Phe-Phe-(4-NO_2)-Arg-Leu and Pro-Pro-Thr-Ile-Phe-Phe-(4-NO_2)-Arg-Leu were kindly given by Dr. B. M. Dunn, University of Florida Medical School, Gainesville, FL, U.S.A. Their hydrolysis [at the Phe–Phe-(4-NO_2) bond] was monitored spectrophotometrically at 300 nm as described previously [5,6,9].

Milk-clotting assays were carried out at pH 6.2 but otherwise exactly as described by McPhie [10]. Digestion of haemoglobin was carried out as follows. A 2.5% (w/v) solution of haemoglobin in water (300 μl) was adjusted to pH 3.0 with HCl and preincubated for 15 min at 37 °C to denature the haemoglobin. Then 75 μl of 0.4 M-Mes buffer was added and the final pH was adjusted to 6.2 by the addition of 5 M-NaOH. Reaction was initiated by the addition of proteinase (2.5 μg in 5 μl of water). After various lengths of time at 37 °C, 300 μl of 4% (w/v) trichloroacetic acid was added and the precipitated material was removed by centrifugation. The absorbance of each supernatant was read at 280 nm. Time courses were usually run with samples taken at 0, 15, 30 and 60 min, to ensure linearity of reaction.

Active concentrations of the three aspartic proteinases were determined by active-site titration (at pH 3.1) with isovaleryl-pepstatin, the concentration of which had been previously determined by amino acid analysis (after acid hydrolysis). The active concentration of α_2M solutions was determined at pH 6.2 by reaction with trypsin, the concentration of which had been quantified in turn by titration against a standard solution of soya-bean trypsin inhibitor (based on $A_{280}^{0.1\%} = 0.91$ [11]).

RESULTS AND DISCUSSION

Since only a narrow pH range was operationally feasible, a value of 6.2 was selected. The effective concentration of solutions of α_2M was determined by reaction against a standardized solution of trypsin at this pH. These values were calculated on the basis that 1 mol of α_2M is able to inhibit 2 mol of trypsin [12].

The influence of α_2M on the activity of chymosin towards a protein substrate (i.e. its ability to clot milk) at pH 6.2 was examined, and a typical profile is shown in Fig. 1. Strong inhibition was observed, and from the equivalence point 0.028 nmol of α_2M was required to inhibit 0.061 nmol of chymosin. Thus it would appear that the stoichiometry of inhibition of aspartic proteinases by α_2M (approx. 2:1) is similar to that observed against the other classes of proteolytic enzymes [12]. The

Abbreviations used: α_2M, α_2-macroglobulin; Phe-(4-NO_2), nitrophenylalanine.

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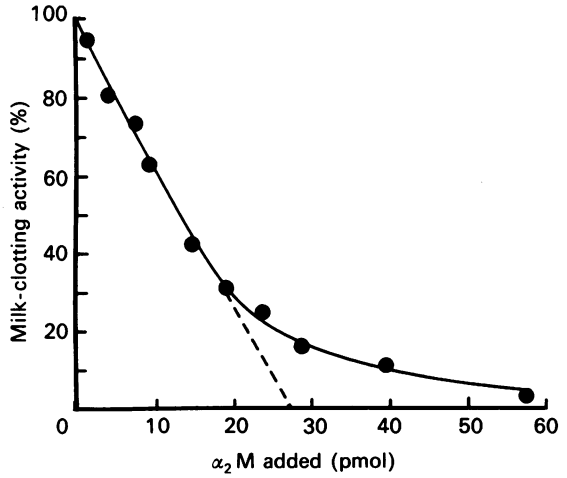

Fig. 1. Effect of α_2M on the milk-clotting activity of chymosin at pH 6.2

Chymosin (61 pmol) was preincubated for 1 min at 25 °C with various amounts of α_2M in 0.2 M-Mes buffer, pH 6.2, before the addition of 2 ml of a milk solution in the same buffer (prepared as in ref. [10]). The courses of the reactions were monitored spectrophotometrically at 510 nm, and clotting times were calculated as described in ref. [10]. Values are expressed as percentages of the uninhibited chymosin activity.

shape of the titration curve might be indicative of a reaction that is sufficiently slow for it not to have gone to completion at concentrations near the equivalence point.

Many previous investigations have shown that α_2M operates by entrapping the (other classes of) target proteolytic enzyme. These can be demonstrated still to be in an active form by the use of low-M_r peptide substrates, which can gain access by diffusion into the inhibitor–proteinase complex whereas (larger) protein substrates are hindered. Thus the influence of α_2M on the hydrolysis of Lys-Pro-Ile-Glu-Phe-Phe-(4-NO_2)-Arg-Leu by chymosin was examined (Table 1). The macromolecule appeared to be acting in a non-competitive manner, since the $k_{cat.}$ value was decreased by approximately half with no significant change in the K_m values obtained. This may be explained most readily by the peptide substrate not being permitted completely unobstructed access to the proteinase, probably because of its (relatively large) size. Aspartic proteinases require peptides of at least seven residues in length as substrates [5,6], whereas enzymes such as trypsin and chymotrypsin are readily active towards much smaller (essentially dipeptide) synthetic substrates.

To determine whether this inhibitory effect was applicable to other aspartic proteinases, human cathepsin D was incubated at pH 6.2 with haemoglobin as substrate (cathepsin D does not clot milk) in the presence and in the absence of α_2M. Under the conditions employed, cathepsin D alone (2.5 μg) generated an activity (as measured by change in A_{280}) of 0.12/30 min. Complete inhibition (ΔA_{280}/30 min = 0) of the cathepsin D activity (60 pmol) towards this substrate was observed with a very slight molar excess (32 pmol) of α_2M (assuming that 1 mol binds 2 mol of proteinase). It was not possible to assess the influence of α_2M towards cathepsin D acting on small substrates, since cathepsin D is unable to hydrolyse the chromogenic peptides at pH values above about 5.0 [13]. It has been shown previously [3] that cathepsin D binds α_2M, although the enzymic properties of the complex were not characterized.

Cathepsin E similarly is essentially inactive by itself at pH values above about 5.4 [9]. However, by the addition of ATP, the enzyme can be stabilized such that most of the activity is still expressed at pH values up to about 6.6 [13]. Thus kinetic parameters for the hydrolysis of a synthetic peptide substrate by this enzyme were determined (Table 2), and the influence of α_2M (of necessity in the presence of ATP) on these parameters was assessed. It can be seen (Table 2) that the macromolecule did not influence the cleavage of the peptide substrate by this aspartic proteinase. In this case, it was not possible, unfortunately, to evaluate the effect of α_2M on hydrolysis of protein substrates (either haemoglobin or milk) by cathepsin E. Haemoglobin is not digested significantly at this pH (even in the presence of ATP), and, although cathepsin E does clot milk at pH 6.2 (in the presence of ATP), interactions between the nucleotide and the Ca^{2+} of the milk produced very complicated reaction courses and times of clotting that were considered too slow in any case to be of practical value.

Nevertheless, from the data obtained it would appear that, just as with the other classes of proteinase, α_2M is an effective inhibitor of aspartic proteinases when protein substrates are utilized (chymosin on milk and cathepsin D on haemoglobin). Reciprocally, it has relatively little

Table 1. Effect of α_2M on the hydrolysis of Lys-Pro-Ile-Glu-Phe-Phe-(4-NO_2)-Arg-Leu by chymosin at pH 6.2

Chymosin (42 pmol) was added into a final volume of 800 μl of 0.1 M-Mes buffer, pH 6.2, at 37 °C in the absence or in the presence of α_2M (46 pmol). Substrate was added in various concentrations to initiate each reaction. The progress of each reaction was monitored by observing the decrease in A_{300}. The kinetic parameters were determined by computerized least-squares fitting of the measured initial velocities to those predicted by the Michaelis–Menten equation as described previously [9]. The values given are estimates ± S.E.

Addition	K_m (μM)	$k_{cat.}$ (s^{-1})
None	80±45	19±5
α_2M	80±40	9±3

Table 2. Effect of α_2M on the hydrolysis of Pro-Pro-Thr-Ile-Phe-Phe-(4-NO_2)-Arg-Leu by human cathepsin E at pH 6.2

Human erythrocyte membrane cathepsin E (18 pmol) was added into a final volume of 800 μl of 0.1 M-Mes buffer, pH 6.2, at 37 °C containing 6.25 mM-ATP and α_2M (44 pmol) when necessary. Other conditions were as described in the legend to Table 1.

Addition	K_m (μM)	$k_{cat.}$ (s^{-1})
None	5±1	10±1
α_2M	7±4	9±2

or no effect on the cleavage of small peptide substrates (by chymosin and cathepsin E). The pH of the entrapment of aspartic proteinase is probably crucial, in that at lower pH values, where aspartic proteinases are more active, α_2M is unstable and is likely to become itself a protein substrate, able to be digested by the aspartic proteinases. At pH values closer to neutrality, the inhibitor is stable and is able to function effectively to inhibit all four of the known classes of proteolytic enzymes.

This work was supported by an award (to J.K.) from Glaxo Group Research, Greenford, Middx., U.K. We are very grateful to our colleagues who generously supplied us with samples of their purified enzymes and substrates. It is also a pleasure to acknowledge valuable discussions with Dr. Peter Charlton, Dr. Ben Dunn, Dr. Alan Barrett and Dr. Peter Heinrich.

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Received 15 December 1988/20 February 1989; accepted 1 March 1989