The effect of synthetic analogues of chymostatin upon protein degradation in isolated skeletal muscle

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A series of peptides based on the structure of the proteinase inhibitor chymostatin were tested for their toxicity and ability to suppress protein degradation in the isolated mouse diaphragm. The inhibitory activities of the analogues were very similar, in marked contrast to their disparate abilities as inhibitors of chymotrypsin. Toxicity was determined by measurement of the rates of protein synthesis and of leakage of lactate dehydrogenase into the incubation medium. No significant toxicity was measurable at concentrations of inhibitor that were effective at suppressing proteolysis. The structural features of the chymostatin molecule may be less than optimal for suppression of proteolysis in muscle.

Included amongst the wide range of secondary metabolites that are produced by micro-organisms are a number of proteinase inhibitors (Umezawa, 1982; Umezawa & Aoyagi, 1977). These are a family of modified oligopeptides, individual members of which are active towards all four classes of endopeptidases (Umezawa, 1982). Serine proteinases are inhibited by peptide aldehydes such as leupeptin, elastatinal, antipain and chymostatin. Cysteine proteinases are also inhibited by these aldehydes, but are specifically affected by the oxirane inhibitor E64. The activity of aspartyl proteinases is decreased by pepstatin, and some metalloendopeptidases are inhibited by phosphoramidates such as talopectin and phosphoramidon (Bond & Beynon, 1985).

The broad spectrum of activity of this family of inhibitors, coupled with their relatively low toxicity (Seglen, 1984), has led to their use in a wide variety of biological systems. Proteinase inhibitors are introduced into such systems either to suppress a specific proteolytic activity or as diagnostic aids to identify the proteinases that mediate a particular biological event. However, studies using these inhibitors may be compromised by the lack of structural variants as, typically, only one member of each class of inhibitors has been isolated. It is

impossible therefore to identify antagonistic structural features such as the effect of an amino acid residue that diminishes binding of an otherwise suitable inhibitor.

We have adopted a rational approach to the design of a series of peptides related to chymostatin (Galpin *et al.*, 1983, 1984). Chymostatin is an inhibitor of the serine proteinase chymotrypsin (Tatsuta *et al.*, 1973), subtilisin (Galpin *et al.*, 1984), mast cell chymase (Barrett & McDonald, 1980) and to a lesser extent the cysteine proteinases cathepsins B, H and L (Seglen, 1984) and the calcium-activated proteinases calpain I and calpain II (Libby & Goldberg, 1980). The inhibitory activity of chymostatin is attributable to the C-terminal phenylalanine aldehyde (Fig. 1). We have prepared a series of di- and tripeptide phenylalanine aldehydes and their corresponding semicarbazones and have shown that the tripeptide aldehydes are comparable with chymostatin in their ability to inhibit the chymotrypsin-catalysed hydrolysis of *N*-glutarylphenylalanine 4-methylcoumarylamide (Galpin *et al.*, 1984). Further, the same inhibitors are equieffective in the suppression of lysosomal (methylamine-sensitive) and non-lysosomal proteolysis in isolated rat hepatocytes (Grinde *et al.*, 1983). Chymostatin and the new inhibitors are the most effective inhibitors of the non-lysosomal component of intracellular protein catabolism (Grinde & Seglen, 1980; Bates *et al.*, 1982; Seglen, 1984). In skeletal muscle incubated *in vitro*, chymostatin is an effective

Abbreviations used: Z-, benzyloxycarbonyl; PheH, phenylalaninal.

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Fig. 1. Structures of chymostatin and related analogues
(a), Chymostatin; (b), Z-Arg-Leu-PheH; (c), Z-Arg-Leu-PheH semicarbazone; (d), Z-Leu-PheH.

inhibitor of protein degradation (Libby & Goldberg, 1980, 1981). This effect is also seen in wasting muscle produced by denervation or muscular dystrophy and it has been suggested that this inhibitor may be of value in suppressing atrophy *in vivo* (Libby & Goldberg, 1980).

We have now investigated the ability of certain of our chymostatin analogues to suppress proteolysis in the isolated mouse diaphragm. Specifically, we have used the close analogue of chymostatin, Z-Arg-Leu-PheH, the same blocked tripeptide in which the aldehyde group is replaced by the corresponding semicarbazone and an analogue that is a blocked dipeptide lacking the basic arginine residue, Z-Leu-PheH (Fig. 1). Our data suggest that some of the structural features of chymostatin are not essential for effective inhibition of proteolysis in skeletal muscle.

Materials and methods

Materials

The synthetic chymostatin analogues were prepared as described previously (Galpin *et al.*, 1983,

1984). Chymostatin was purchased from the Peptide Research Institute, Osaka, Japan. Insulin B chain (oxidized) was obtained from Schwarz-Mann, Orangeburg, NY, U.S.A. *N*-Glutaryl-phenylalanine 4-methylcoumarylamide was purchased from Cambridge Research Biochemicals, Harston, Cambridge, U.K. Bovine α -chymotrypsin was purchased from Sigma, and carrier-free Na ^{125}I and L-[U- ^{14}C]tyrosine were obtained from Amersham International. Adult Swiss random-bred mice of either sex were obtained from the animal facility in the Department of Biochemistry. Animals were allowed free access to food and water and were maintained at 22°C on a 12h dark/12h light cycle.

Measurement of protein degradation in isolated mouse diaphragm

Intracellular protein degradation was assayed as the rate of tyrosine release from hemidiaphragms incubated at 37°C as described previously for rat diaphragm (Fulks *et al.*, 1975). We have found that comparison of hemidiaphragms eliminates some of the scatter in degradation rates that are a feature

of this system (Fulks *et al.*, 1975; Libby & Goldberg, 1980). The release of tyrosine into the incubation medium was determined by the fluorimetric assay of Waalkes & Udenfriend (1957). The coefficient of variation of this assay was less than 2.5%. No significant fluorescence is developed with blocked tyrosine residues such as *N*-acetyl-tyrosine ethyl ester, *N*-benzoyltyrosine ethyl ester or tyrosine residues within small peptides such as the insulin B chain. Rates of protein degradation are calculated as nmol of tyrosine released/h per g wet wt. (the tissue was weighed at the end of the incubation period).

When inhibitors were included in the incubation media they were added from stock solutions dissolved in dimethyl sulphoxide and compared with control incubations containing an equivalent concentration (0.5%) of solvent.

Measurement of protein synthesis

Hemidiaphragms were incubated in the medium described by Fulks *et al.* (1975) to which was added unlabelled tyrosine to a final concentration of 0.1 mM and [^{14}C]tyrosine (2.5 $\mu\text{Ci/ml}$). After an incubation period of 1 h the tissue was removed from the medium, rinsed and blotted before homogenization in 2.0 ml of 0.1 M-sodium phosphate buffer, pH 7.5, with a Polytron-type homogenizer (Ystral). A portion of the homogenate (0.05 ml) was diluted to 1.0 ml in the same buffer and 0.25 ml of trichloroacetic acid (25% w/v) was added. A sample of this mixture (0.2 ml) was applied to a Whatman GF/C disk which was washed with 5% (w/v) trichloroacetic acid (10 ml), followed by 10 ml of ethanol/diethyl ether (1:1, v/v). The trichloroacetic acid-insoluble radioactivity was determined by scintillation counting. The efficiency of measurement of incorporated radioactivity using this process was $83 \pm 2.6\%$ ($n = 10$). In experiments designed to assess the effect of the inhibitors of protein synthesis, one of the pair of hemidiaphragms was incubated in the presence of peptide, and the other was incubated in the presence of solvent alone.

Measurement of lactate dehydrogenase leakage

The viability of the incubated tissue was also assessed by determination of the rate of leakage of a soluble enzyme, lactate dehydrogenase, into the medium. At various times during the incubation of hemidiaphragms, samples of the medium were removed and assayed immediately for lactate dehydrogenase. The assay was conducted spectrophotometrically at 340 nm and 25°C and the reaction mixture consisted of 0.1 M-sodium phosphate buffer, pH 7.5, 3.6 mM-sodium pyruvate and 0.26 mM-NADH. At the end of the incubation period the diaphragm was homogenized in the

medium and the total lactate dehydrogenase was measured. The leakage of enzyme into the medium is expressed as the percentage of the total activity that leaked from the tissue in 1 h.

Proteinase assays

The hydrolysis of ^{125}I -insulin B chain was conducted as determined previously (Mulligan *et al.*, 1982; Bond *et al.*, 1983). Activity towards *N*-glutarylphenylalanine 4-methyl coumarylamide was assayed according to Galpin *et al.* (1984). In all cases, assays conducted in the presence of inhibitors were compared with controls that contained an equal concentration of solvent. Chymostatin is a 'slow' inhibitor (G. C. Macadam & R. J. Beynon, unpublished work) and enzyme:inhibitor mixtures were preincubated for 10 min prior to the addition of substrate. This interval is sufficient to allow complete equilibration of the mixture. Extracts of skeletal muscle were prepared by homogenization of freshly dissected diaphragms in 0.1 M-Tris buffer, pH 7.9, with a Polytron-type homogenizer (Ystral). The homogenates were used without further fractionation.

Treatment of data

Inhibition data were obtained for a range of inhibition from approx. 0 to 80%. Where appropriate, a hyperbolic curve was fitted to the data by using a non-linear curve fitting program written by one of us (Green *et al.*, 1982; Beynon, 1983). These data were used to obtain IC_{50} values, the concentration of inhibitor required to give half-maximal effect. Pooled data are expressed as means $\pm$ S.E.M. for n observations.

Results and discussion

The incubated diaphragm has been used extensively in studies of protein degradation in muscle. It is essential that this isolated tissue is maintained in a metabolic state that approximates as closely as possible that which exists *in vivo*. Our preliminary studies established that the degree of oxygenation was a critical factor in maintaining the metabolic integrity of this tissue. Hemidiaphragm preparations (average wt. 35 mg) were initially incubated in sealed test tubes (16 mm $\times$ 100 mm) after extensive flushing with O_2/CO_2 (19:1). The medium was equilibrated with the same gas mixture beforehand. However, under these conditions the rate of leakage of lactate dehydrogenase into the medium was high (19.2%/h). When a fine glass tube was inserted under the surface, such that the O_2/CO_2 mixture was bubbled through the medium throughout the incubation, the rate of lactate dehydrogenase release was reduced to 10.5%/h. This improvement in cellular integrity was con-

firmed by electron microscopy and was accompanied by a slight decrease in the rate of degradation from 178 nmol/h per g wet wt. to 158 nmol/h per g wet wt. This simple modification has been included in all incubations.

The rate of protein degradation, determined by the release of tyrosine into the medium, was subject to considerable scatter on a day-to-day basis (158 ± 7.2 nmol/h per g wet wt., $n = 189$). Similarly, the degree of inhibition of protein degradation by chymostatin at saturating concentrations is scattered (inhibition $39.5\% \pm 2.5\%$, $n = 12$). This is in keeping with the findings of others (Fulks *et al.*, 1975) but we do not have a satisfactory explanation for this observation.

The inhibition of protein degradation by chymostatin (Fig. 2) was similar to that reported previously (Libby & Goldberg, 1980) and resembled that obtained in hepatocytes in the presence of lysosomotropic agents (Grinde & Seglen, 1980). A similar effect was observed with the close analogue of chymostatin, Z-Arg-Leu-PheH. However, the two analogues Z-Leu-PheH and Z-Arg-Leu-PheH semicarbazone were almost as effective in suppressing this process. Non-linear curve fitting of a simple hyperbolic function to the inhibition data revealed the IC_{50} values to be in the

range 2–11 μ M. In all cases, the maximal inhibition attainable was similar: 46%, 52%, 52% and 54%, respectively. The response shown by Z-Leu-PheH is included for completeness, although the effect of this inhibitor may not be described best as a simple hyperbolic function (Fig. 2).

The 5-fold range in IC_{50} contrasts dramatically with their abilities as chymotrypsin inhibitors (Galpin *et al.*, 1983, 1984). We conclude that those structural features that make such compounds effective chymotrypsin inhibitors may not be essential to their action on protein degradation in skeletal muscle. The effectiveness of the inhibitors must also be considered in terms of their ability to exert toxic effects upon the tissue. One of the analogues of chymostatin, Z-Arg-Leu-PheH, has been demonstrated to be non-toxic to primary hepatocyte cultures at concentrations that inhibit protein degradation (Grinde *et al.*, 1983). However, we have extended our studies in order to assess the toxicity of chymostatin and the three analogues towards preparations of mouse diaphragm.

The effect of the inhibitors on rates of protein synthesis is shown in Table 1. Chymostatin, Z-Arg-Leu-PheH and Z-Arg-Leu-PheH semicarbazone were without effect upon the rate of

Fig. 2. Inhibition of muscle proteolysis by chymostatin and related analogues

Isolated mouse hemidiaphragms were incubated in the presence of a range of concentrations of chymostatin (a), Z-Arg-Leu-PheH (b), Z-Arg-Leu-PheH semicarbazone (c) or Z-Leu-PheH (d). The rate of release of tyrosine was compared with that of the paired control and is expressed as a percentage of the control rate. For all experiments, n is between 4 and 6.

Table 1. *Effect of chymostatin and analogues on protein synthesis in the isolated mouse diaphragm*

Isolated hemidiaphragms were incubated as described in the Materials and methods section and the rate of protein synthesis was determined in the presence or absence of the inhibitors. The protein synthesis inhibitor cycloheximide was included in one set of incubations as a positive control. For all experiments, $n = 6$ and the results were analysed by Student's t test for paired samples.

Inhibitor (concn.)	Protein synthesis (nmol/h per g wet wt.)		<i>P</i>
	– Inhibitor	+ Inhibitor	
Chymostatin (33 μ M)	61.5 $\pm$ 7.6	54.5 $\pm$ 4.8	0.46
Z-Arg-Leu-PheH (31 μ M)	51.5 $\pm$ 4.6	47.6 $\pm$ 7.1	0.35
Z-Arg-Leu-PheH semicarbazone (29 μ M)	64.1 $\pm$ 9.2	70.6 $\pm$ 8.1	0.39
Z-Leu-PheH (50 μ M)	89.3 $\pm$ 4.0	74.7 $\pm$ 3.6	0.02
Cycloheximide (0.5 mM)	55.6 $\pm$ 6.3	6.4 $\pm$ 0.1	0.006

Table 2. *Effect of chymostatin and analogues on the leakage of lactate dehydrogenase from isolated mouse diaphragm*

Isolated hemidiaphragms were incubated as described in the Materials and methods section. At various times, samples (0.02 ml) of medium were removed and assayed for activity of lactate dehydrogenase. At the end of the incubation period the tissue was homogenized in the medium and the leakage of lactate dehydrogenase was expressed as a percentage of the total activity in medium plus tissue. Incubations in the presence or absence of inhibitors were compared by Student's t test for unpaired samples ($n = 6$ in each group). The P values are relative to incubations in the absence of inhibitors.

Inhibitor (concn.)	Rate of leakage (% of total/h)	<i>P</i>
None	7.3 $\pm$ 0.77	–
Chymostatin (33 μ M)	5.9 $\pm$ 0.48	0.15
Z-Arg-Leu-PheH (31 μ M)	7.7 $\pm$ 1.24	0.32
Z-Arg-Leu-PheH semicarbazone (29 μ M)	8.2 $\pm$ 0.81	0.44
Z-Leu-PheH (50 μ M)	4.8 $\pm$ 0.85	0.016

protein synthesis, whereas Z-Leu-PheH caused a significant reduction in the rate of synthesis to 84% of control values. Finally, the dramatic effect of cycloheximide (89% inhibition) precludes misinterpretation of data due to artefacts.

A further test for toxic effects upon tissues is provided by measurement of the efflux of enzymes from the tissue into the medium (Jones *et al.*, 1983). Accordingly, diaphragms were incubated in the absence and presence of chymostatin and the analogues, and the rate of leakage of a sarco-plasmic enzyme was determined (Table 2). Curiously, only Z-Leu-PheH had any effect upon the rate of efflux of this enzyme, and the effect was a reduction in rate from 7.3%/h to 4.8%/h. All of the data that we have gathered suggest that Z-Leu-PheH may behave in a fashion that is subtly different to chymostatin and the tripeptides. Collectively, these data provide clear evidence for the lack of toxic effects of chymostatin and the analogues. In turn, this implies that they affect degradative processes that are operating in the tissue normally, albeit at reduced rates.

Homogenates of skeletal muscle contain hydrolases that are active towards chymotrypsin sub-

strates (Sanada *et al.*, 1978; Libby & Goldberg, 1980). Accordingly, the four inhibitors were tested for their ability to inhibit the hydrolysis of *N*-glutarylphenylalanine 4-methylcoumarylamide or 125 I-insulin B chain by α -chymotrypsin or crude preparations of diaphragm (Fig. 3). Both substrates were hydrolysed effectively by chymotrypsin and the activities were strongly inhibited by chymostatin and Z-Arg-Leu-PheH. However, Z-Arg-Leu-PheH semicarbazone was much less inhibitory, such that the IC_{50} values were one to two orders of magnitude higher than those for Z-Arg-Leu-PheH. The dipeptide aldehyde Z-Leu-PheH was slightly less inhibitory than the semicarbazone towards chymotryptic digestion of *N*-glutarylphenylalanine 4-methylcoumarylamide and exhibited no inhibition towards chymotryptic degradation of insulin B chain at concentrations up to 90 μ M.

Both substrates were also hydrolysed by diaphragm preparations. The radioiodinated B chain of insulin is a convenient substrate for endopeptidases, because the two tyrosine residues (16 and 26) and the presence of a proline residue distal to Tyr-26 make hydrolysis of this peptide by exo-

Fig. 3. Effect of chymostatin and analogues on the hydrolysis of *N*-glutarylphenylalanine 4-methylcoumarylamide and ^{125}I -insulin B chain by chymotrypsin and homogenates of mouse diaphragm

Assays were conducted as described in the Materials and methods section and the inhibition of the hydrolysis of *N*-glutarylphenylalanine 4-methylcoumarylamide (a, b) and ^{125}I -insulin B chain (c, d) were assessed using chymotrypsin (a, c) and homogenates of mouse diaphragm (b, d). The inhibitors are chymostatin (▲), Z-Arg-Leu-PheH (△), Z-Arg-Leu-PheH semicarbazone (○) and Z-Leu-PheH (□).

peptidases unlikely. Chymostatin and the three analogues showed very similar inhibitory potential towards degradation of these substrates by diaphragm preparations (Fig. 3), especially when compared with the differences observed with chymotryptic degradation of these substrates. Chymostatin and the analogues are much less effective inhibitors of the activities in diaphragm preparations than of chymotrypsin. The IC_{50} values for chymostatin and Z-Arg-Leu-PheH are between 50- and 100-fold higher when used as inhibitors of diaphragm preparations. These data imply that the target activity is not chymotrypsin-like. Interestingly, there appear to be very low levels of mast cell chymase in our preparations, as this enzyme would be sensitive to chymostatin at concentrations an order of magnitude lower (Libby & Goldberg, 1980).

The data presented in Fig. 3 have been drawn in such a way to emphasize that the difference between the good chymotrypsin inhibitors (chymostatin and Z-Arg-Leu-PheH) and the poor chymotrypsin inhibitors (Z-Arg-Leu-PheH semicarbazone and Z-Leu-PheH) are largely eliminated (a) in the inhibition of protein degradation (Fig. 2) and (b) in the suppression of proteolytic activity in

crude diaphragm preparations. Collectively, our data provide strong evidence that the target proteinase of chymostatin in the suppression of muscle protein catabolism is not a chymotrypsin-like serine proteinase, although the nature of this proteinase has not yet been investigated. Lysosomotropic amines have negligible effect on the release of tyrosine by these preparations (Table 3). This implies a low level of expression of the lysosomal system and thus, that lysosomal proteinases are unlikely to be the target of the inhibitors investigated here. Similarly, the effectiveness of the semicarbazone argues against inhibition of the calpains, at least by formation of a hemithioacetal. Leupeptin reduced the rate of tyrosine release by a similar extent to that obtained with chymostatin and the analogues. Leupeptin and chymostatin in combination gave no additional inhibition of degradation (Table 3). Further, chymostatin and the analogues are not additive in their effects (M. T. Mulligan, unpublished work). From this data we may infer that the same proteolytic system is suppressed by chymostatin, the new analogues and leupeptin. Further work is needed to identify this system.

In all systems that have been examined, chymo-

Table 3. *Inhibitory effects on protein degradation in the isolated mouse diaphragm*

Isolated hemidiaphragms were incubated as described in the Materials and methods section and the rate of protein degradation (release of tyrosine into the medium) was assessed in the presence or absence of putative inhibitors. For all experiments, *n* = 6 and the data were analysed by Student's *t* test for paired samples.

Inhibitor (concn.)	Tyrosine release (nmol/h per g wet wt.)		<i>P</i>
	– Inhibitor	+ Inhibitor (%)	
NH ₄ Cl (5 mM)	124 ± 7.6	136 ± 15.7 (110)	0.49
Methylamine (5 mM)	143 ± 15.9	131 ± 12.2 (92)	0.43
Leupeptin (47 μM)	116 ± 7.1	55 ± 4.9 (47)	< 0.001
Chymostatin (33 μM)	144 ± 17.0	87 ± 14.0 (60)	0.020
Leupeptin (47 μM) + chymostatin (33 μM)	163 ± 10.0	90 ± 6.4 (55)	0.013

statin is only able to inhibit non-lysosomal proteolysis by approx. 20–50% (the present study; Libby & Goldberg, 1980; Grinde & Seglen, 1980; Bates *et al.*, 1982). A satisfactory explanation for this finding has never been given. One possibility is that non-lysosomal proteolysis contains more than one component, only one of which is sensitive to this inhibitor. Alternatively, it is feasible that the non-lysosomal proteolytic pathway is sensitive to chymostatin in a non-competitive fashion. Our findings on the structural requirements for inhibition would argue that those structural features needed for competitive inhibition of chymotrypsin are of less importance in the inhibition of muscle proteolysis and thus lend support to this view. Although the mechanism of action of these inhibitors remains unclear, we suggest that inhibitors based on data obtained with the natural product may not be optimally active in the biological system under investigation. Such finding clearly have relevance to suggestions that proteinase inhibitors may have potential in the treatment of muscle disease.

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