Differential inhibition of two proteolytic activities in bovine lens neutral-proteinase preparations

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Hydrolysis of carbobenzoxy-Leu-Leu-Glu 2-naphthylamide by bovine lens neutral-proteinase preparations is not affected by the esterase inhibitor di-isopropyl fluorophosphate, whereas hydrolysis of carbobenzoxy-Gly-Gly-Leu *p*-nitroanilide is completely inhibited. Hydrolysis of α -crystallin, a lens structural protein, can be inhibited by only 50% after prolonged treatment with di-isopropyl fluorophosphate. These data suggest that the lens neutral-proteinase preparation contains at least two enzymes, one of which may be a serine proteinase. This may account, in part, for the previously observed complex response of the preparation to inhibitors.

Ocular-lens proteolytic enzymes are of considerable interest because of their potential effects on the crystallins, structural proteins needed for lens transparency. When transparency is lost, changes are observed in these structural proteins, notably an increase in low-*M*_r polypeptides and free amino acids, which could be due to proteolytic enzymes (Barber, 1973; van Heyningen, 1976; Horwitz, 1983). Autolysis of bovine lens extracts has been shown to occur *in vitro* at neutral pH and with a high temperature optimum. A neutral proteinase, active under similar conditions, has been partially purified from bovine lens (Waley & van Heyningen, 1962; Blow *et al.*, 1975). It is distinct from other lens proteinases with activity at neutral pH (Srivastava & Ortwerth, 1983; Yoshida *et al.*, 1984). Hydrolysis *in vitro* of a lens structural protein by the lens neutral-proteinase preparation is inhibited by three types of proteolytic-enzyme inhibitors, including iPr₂P-F, EDTA and *p*-chloromercuribenzoate (Blow *et al.*, 1975). Consequently the enzyme has been considered to be (i) a serine proteinase with an unusual requirement for Ca²⁺ or Mg²⁺ and with a thiol group necessary for activity (Blow *et al.*, 1975; van Heyningen, 1978), (ii) a metalloendopeptidase (Blow, 1977), or (iii) similar to Ca²⁺-dependent cysteine proteinases, but not yet classified (Barrett & McDonald, 1980).

Abbreviations used: iPr₂P-F, di-isopropyl fluorophosphate; *p*-NA, *p*-nitroanilide; Cbz-, carbobenzoxy; -2-NNp, 2-naphthylamide.

The possibility of more than one enzyme in the preparation has not been previously proposed as a reason for the complex response of the partially purified enzyme preparation to inhibitors. We have used synthetic endopeptidase substrates in conjunction with the serine-esterase inhibitor iPr₂P-F to demonstrate the presence of at least two distinct endopeptidase activities in the bovine lens neutral-proteinase preparation.

Experimental

Lenses were dissected from bovine (1.5 years), Sprague-Dawley-rat (6 weeks) and human (17 years) eyes and stored at -70°C before use. Crystallin was prepared from bovine lenses as previously described (Blow *et al.*, 1975; Wagner *et al.*, 1982). Cbz-Gly-Gly-Leu-*p*-NA was obtained from the Peptide Institute, Osaka, Japan. Cbz-Leu-Leu-Glu-2-NNp was synthesized by the method of Wilk & Orlowski (1980) from Cbz-Leu-Leu-*N*-hydroxysuccinimide ester and α -L-Glu-2-NNp (Bachem, Torrance, CA, U.S.A.). All chemicals were reagent grade.

Preparation of neutral-proteinase activities from mammalian lenses

To prepare cortical extracts, individual lenses were stirred in 5–8 vol. of 10 mM-Hepes (pH 7.5)/5 mM-MgCl₂ (Hepes buffer) until the outer 60% (w/w of total lens) was dispersed. The dispersion was centrifuged at 9600g for 25 min at

5°C and the supernatant fluid collected. The pellet was washed once with Hepes buffer and the supernatant fractions were combined. The method of Blow *et al.* (1975) was used to prepare partially purified (250-fold) neutral proteinase as previously described (Wagner *et al.*, 1982). Enzyme fractions were dialysed against Hepes buffer and stored in portions at -70°C.

Assay methods

Hydrolysis of α -crystallin was measured as previously described (Wagner *et al.*, 1982) by detection of the appearance of new *N*-termini with fluorescamine (Sigma Chemical Co., St. Louis, MO, U.S.A.). Hydrolyses of synthetic endopeptidase substrates (1 mM-Cbz-Gly-Gly-Leu-*p*-NA or 0.2 mM-Cbz-Leu-Leu-Glu-2-NNp) by partially purified neutral proteinase (0.2 mg·ml⁻¹) or soluble lens extract (5–6 mg·ml⁻¹) were assayed as previously described (Wagner *et al.*, 1984). Substrates and enzyme were incubated in 13 mM-Hepes (pH 7.5)/5 mM-MgCl₂ at 37°C for 1 h. By using the appropriate standard curves, liberation of *p*-nitroaniline was calculated from the increase in *A*₄₁₀ after adjustment to pH 7 with 3 M-Trizma base, to maximize colour, and liberation of 2-naphtylamine was determined after diazotization (Goldberg & Rutenburg, 1958) by measuring the increase in *A*₅₈₀. Protein was measured by *A*₂₈₀ and by the Bradford method (Bio-Rad Protein Assay Kit; Bio-Rad, Richmond, CA, U.S.A.), with bovine serum albumin as a standard.

Effects of *iPr*₂*P*-F on neutral-proteinase activities

Bovine lens neutral proteinase and bovine, rat and human lens-cortical extracts were each supplemented with 250 mM-Hepes buffer, pH 7.5, to obtain a 13 mM final concentration for increased buffering. The enzyme preparations were treated for 0–24 h at room temperature with (1) 7 mM-*iPr*₂*P*-F in propan-2-ol, (2) an equivalent volume of propan-2-ol or (3) 7 mM-*iPr*₂*P*-F that had been hydrolysed in 0.5 M-KOH and neutralized. At the time intervals indicated in the Results and discussion section, the variously treated neutral-proteinase preparations were assayed for ability to hydrolyse α -crystallin, Cbz-Gly-Gly-Leu-*p*-NA and Cbz-Leu-Leu-Glu-2-NNp. In some experiments, portions of each of the treated preparations were dialysed against Hepes buffer before the assay. Stock *iPr*₂*P*-F was prepared by diluting 1 g to 1 M in anhydrous propan-2-ol that had been dried over molecular sieves (Type 4A). The stock solution was stored at 0°C. All operations with *iPr*₂*P*-F were carried out in a fume hood, and materials that came in contact with *iPr*₂*P*-F were decontaminated by soaking in 0.5 M-KOH.

Results and discussion

Pre-incubation of partially purified bovine lens neutral proteinase with *iPr*₂*P*-F differentially affects the hydrolysis of synthetic substrates and α -crystallin. In Fig. 1 the percentage of activity remaining in *iPr*₂*P*-F-treated preparations is plotted on a logarithmic scale versus time, the samples treated with propan-2-ol for the same time period being used as controls. The hydrolysis of Cbz-Gly-Gly-Leu-*p*-NA was 50% inhibited after 0.5 h and 95% inhibited after 2.5 h pre-incubation of the preparation with *iPr*₂*P*-F, whereas the ability to hydrolyse Cbz-Leu-Leu-Glu-2-NNp was not affected (Fig. 1). The effect of *iPr*₂*P*-F on the hydrolysis of α -crystallin, an endogenous lens protein, by bovine lens neutral-proteinase preparations is more complex. No more than 50% of the control level of activity hydrolysing α -crystallin can be inhibited by *iPr*₂*P*-F, even after 25 h pre-incubation with the inhibitor (Fig. 1). A rapid

Fig. 1. Effect of preincubation with *iPr*₂*P*-F on neutral-proteinase activity

The substrates used were: $\circ$, α -crystallin; $\bullet$, Cbz-Leu-Leu-Glu-2-NNp; $\blacktriangle$, Cbz-Gly-Gly-Leu-*p*-NA. Control values, obtained by preincubation with propan-2-ol, at zero time and after 2.5 h were respectively 634 and 583 nmol of leucine equivalents·h⁻¹·mg⁻¹ (α -crystallin); 298 and 353 nmol of 2-NNp·mg⁻¹ (Cbz-Leu-Leu-Glu-2-NNp); and 770 and 645 nmol of *p*-nitroaniline·h⁻¹·mg⁻¹ (Cbz-Gly-Gly-Leu-*p*-NA). In other experiments 100% inhibition of Cbz-Gly-Gly-Leu-*p*-NA hydrolysis was achieved by extending the preincubation time to 3 h. Details of the procedure used are given in the Experimental section.

initial loss of approx. 20% of enzyme activity hydrolysing both α -crystallin and Cbz-Gly-Gly-Leu-*p*-NA is observed. To determine the cause of this initial loss, the conductivity and pH of incubation mixtures were determined before and after incubation. Control experiments were conducted in which neutral-proteinase preparations were pre-incubated with fluoride ion or with hydrolysed iPr_2P -F. The rapid initial loss of activity cannot be attributed to an inhibitory effect of fluoride ions, pH change, conductivity change or hydrolysis products. It is possible that a subpopulation of enzyme molecules reacts more rapidly with iPr_2P -F or that another enzyme species is present in the population. In a typical experiment, after 2.5h at room temperature, the activity of control preparations remained unchanged, or changed by less than 20%, as described in the legend to Fig. 1. The iPr_2P -F inhibition is not reversible by dialysis, consistent with the idea of covalent modification of the enzyme. These data suggest the presence of both iPr_2P -F-sensitive and -insensitive components in the preparation.

The effect of iPr_2P -F on the hydrolyses of synthetic substrates by unfractionated lens-cortical soluble protein parallels the results with partially purified preparations. Hydrolysis of Cbz-Gly-Gly-Leu-*p*-NA by bovine, rat and human lens extracts is 50% inhibited by 7mM- iPr_2P -F after approx. 0.5h, whereas hydrolysis of Cbz-Leu-Leu-Glu-2-NNp is not affected. This is consistent with hydrolysis of the synthetic substrates by similar enzymes in all three species.

These experiments demonstrate that the previously observed inhibition of the lens neutral-

proteinase preparation by inhibitors of serine, metallo- and cysteine proteinases is in part due to the presence of at least two enzymes, one of which is iPr_2P -F-inhibitable and thus may be a serine proteinase.

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