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Action of cathepsin D on fructose-1,6-bisphosphate aldolase

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Cathepsin D inactivated aldolase at pH values between 4.2 and 5.2; the chloride, sulphate or iodide, but not citrate or acetate, salts of sodium or potassium accelerated the rate of inactivation. Cathepsin D cleaved numerous peptide bonds in the *C*-terminus of aldolase, but the major site of cleavage in this region was Leu^{354}–Phe^{355}. The most prominent peptide products of hydrolysis were Phe-Ile-Ser-Asn-His-Ala-Tyr and Phe-Ile-Ser-Asn-His. Up to 20 amino acids were removed from the *C*-terminus of aldolase, but no further degradation of native aldolase was observed. By contrast, extensive degradation of the 40000-M_r subunit was observed after aldolase was denatured. The cathepsin D-inactivated aldolase cross-reacted with antibodies prepared against native aldolase and had the same thermodynamic stability as native aldolase, demonstrated by differential scanning calorimetry and fluorescence quenching of tryptophan residues. Furthermore, the cathepsin-modified and native forms of aldolase were both resistant to extensive proteolysis by other purified cellular proteinases and lysosomal extracts at pH values of 4.8–8.0.

The specificity of proteinases is generally defined in terms of their action on small peptides or on medium-sized peptides, such as the insulin B-chain, that have little or no secondary or tertiary structure (Barrett, 1977). The results of such studies give little information as to how the proteinase will act on a native protein. For example, it is well known that trypsin acting on a native protein will not cleave all of the potentially susceptible Arg–Xaa and Lys–Xaa bonds and may not cleave any of them (Mihalyi, 1972). So it is clear that higher orders of structure of the substrate protein place major constraints on the action of the proteinase. Very little is known about the nature of these constraints, but because it is probable that the early rate-limiting stages of intracellular protein degradation involve the action of cellular proteinases on native protein substrates, they will be important to an understanding of the control of intracellular protein turnover.

As an approach to a better understanding of the effects of native structure on the susceptibility of proteins to the action of proteinases, we have investigated the degradation of rabbit muscle fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) by cathepsin D (EC 3.4.23.5). Aspects we have considered include the effect of degradation on aldolase specificity, structure and thermodynamic stability, which must affect the course of further degradation.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Rabbit muscle aldolase (specific activity 11 μmol of fructose 1,6-bisphosphate cleaved $\cdot$ min^{-1} $\cdot$ mg^{-1}) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human liver cathepsins D and B, prepared by methods described by Barrett (1973), were a gift from Dr. Alan J. Barrett (Strangeways Laboratory, Cambridge, U.K.). Pepstatin was obtained from the Peptide Research Institute (Osaka, Japan). All other chemicals were obtained from Sigma Chemical Co., except where noted.

Aldolase concentration was determined by the A_{280}, assuming $A^{0.1\%}_{1\,cm}$ to be 0.91 (Grazi & Blanzieri, 1977). Minor protein contaminants were removed by cellulose phosphate chromatography with 10mM-Tris/Mops (4-morpholinopropanesulphonic acid) buffer, pH 7.0, containing 10mM-EDTA; the enzyme was eluted with 20mM-KCl and 2mM-fructose 1,6-bisphosphate. Eluted fractions with aldolase activity were pooled, concentrated on an Amicon concentrator with a PM10 membrane

(Amicon Corp., Lexington, MA, U.S.A.), and dialysed against 100mM-Tris/HCl, pH7.0, to remove substrate. Aldolase activity was measured as previously described (Bond & Barrett, 1980).

Antiserum to rabbit muscle aldolase was prepared in roosters. Roosters were injected subcutaneously with 0.2mg of aldolase in a solution containing 0.14mg·ml^{-1} emulsified with complete Freund's adjuvant (1:1, v/v) (Colorado Serum Co., Denver, CO, U.S.A.); additional aldolase (0.6mg) emulsified in incomplete Freund's adjuvant was injected every 2 weeks. After 4–8 weeks, single precipitin lines were observed on Ouchterlony plates when the antisera were tested against aldolase preparations. After precipitin lines were formed, the Ouchterlony plates were washed with 4% NaCl in 10mM-Tris/HCl buffer, pH7.0, and stained for protein with Coomassie Brilliant Blue, or for aldolase activity [10mM-fructose 1,6-bisphosphate/glyceraldehyde-3-phosphate dehydrogenase (0.12mg·ml^{-1})/10mM-NAD^+/10mM-sodium arsenate/Nitroblue Tetrazolium (0.4mg·ml^{-1})/phenazine methosulphate (0.024mg·ml^{-1}); 37°C; 20min]. The bands stained for protein and activity were coincident.

Calorimetric measurements were made on a Microcal MC-1 scanning adiabatic calorimeter (Microcal, Amherst, MA, U.S.A.) at a rate of 1°C·min^{-1} between 20 and 80°C. The differential heat capacity of the sample as a function of temperature was measured. The transition temperature, T_m, was the temperature at the midpoint of the transition from native to denatured aldolase. The area under the transition curve was integrated and normalized for protein concentration to yield the specific transition enthalpy, Δh_d(mJ·mg^{-1}). Aldolase (1.5–2mg·ml^{-1}) was in 5mM-sodium phosphate/5mM-sodium acetate/50mM-sodium sulphate, pH5–8. Calorimetric procedures were as previously described (Chlebowski & Mabrey, 1977).

Samples were subjected to high-resolution SDS/polyacrylamide-gel electrophoresis on slab gels as described by O'Farrell (1975). Samples were diluted with an equal volume of buffer containing 0.125M-Tris/HCl, 4% (w/v) SDS, 20% (v/v) glycerol and 1% (v/v) 2-mercaptoethanol, pH6.5, and boiled for 5min. Gels, 10% (w/v) polyacrylamide, were run at 25mA/gel for about 3h and were calibrated with phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soya-bean trypsin inhibitor (M_r 21000) and lysozyme (M_r 14300) (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Aldolase (1mg·ml^{-1}) in 0.1M-sodium acetate buffer, pH4.8, containing 0.1M-NaCl was incubated with cathepsin D (33 μg·ml^{-1}) at 23°C for 3h. Under these conditions aldolase was inactivated 85–95%. These conditions were used unless otherwise indicated.

Aldolase samples which were inactivated with cathepsin D were dried in a Savant vacuum concentrator (Savant Instruments, Hicksville, NY, U.S.A.) and then dissolved in 100μl of 0.1% H_3PO_4. The protein solution (75μl) was applied to a C-18 reverse-phase high-pressure-liquid-chromatography column (Waters Associates, Milford, MA, U.S.A.) equilibrated with 0.1% H_3PO_4. The protein and polypeptides were eluted with a linear gradient of 0.1–0.05% H_3PO_4 and 0–50% acetonitrile as the second solvent at 2ml·min^{-1} over 60min. Absorbance was monitored at 215nm. Fractions were collected every minute. Fractions that contained peptides were dried in a Savant vacuum concentrator and were hydrolysed *in vacuo* with 0.1ml of 6M (constant-boiling) HCl at 110°C for 24h. Samples were then dried and dissolved in 100μl of 0.2M-sodium citrate, pH2.2, and applied to a Durrum amino acid analyser (Durrum Instrument, Palo Alto, CA, U.S.A.) equipped with a Varian CDS111 integrator. Peptide products were characterized by comparison with the known primary structure of aldolase.

Fluorescence intensity was measured with an SLM series 4800 spectrophotometer (SLM Instruments, Urbana, IL, U.S.A.) equipped with a Hewlett–Packard 9815 A calculator. Excitation was at 295nm. Filter 0-54 was used to eliminate emission wavelengths less than 300nm. Aldolase was dissolved in 0.01M-Tris/HCl, pH7.5, with A_{295} less than 0.1. Fluorescence was quenched by the addition of increasing concentrations of acrylamide, a constant aldolase concentration being maintained.

Results

When aldolase was incubated with cathepsin D at pH4.8, activity towards fructose 1,6-bisphosphate was lost, whereas there was a slight increase in activity towards fructose 1-phosphate (results not shown). The inactivation generally followed first-order kinetics until 80–90% of activity was lost. About 2–5% residual activity of aldolase remained even after prolonged incubations with high concentrations of cathepsin D (6–12h and up to 100μg of cathepsin D·ml^{-1}) or with additions of fresh cathepsin D after 98% inactivation was achieved. The inactivation could be completely prevented by pepstatin, a potent inhibitor of cathepsin D. The addition of the substrate fructose 1,6-bisphosphate, at 4.1 and 9.1mM, to the incubation mixture had no effect on the inactivation of aldolase.

Cathepsin D inactivation of aldolase was observed between pH4.2 and 5.2; the proteinase had no effect on aldolase activity above pH5.6, and aldolase lost activity rapidly, in the absence of proteinases, below pH4. Rate constants for inactivation ranged

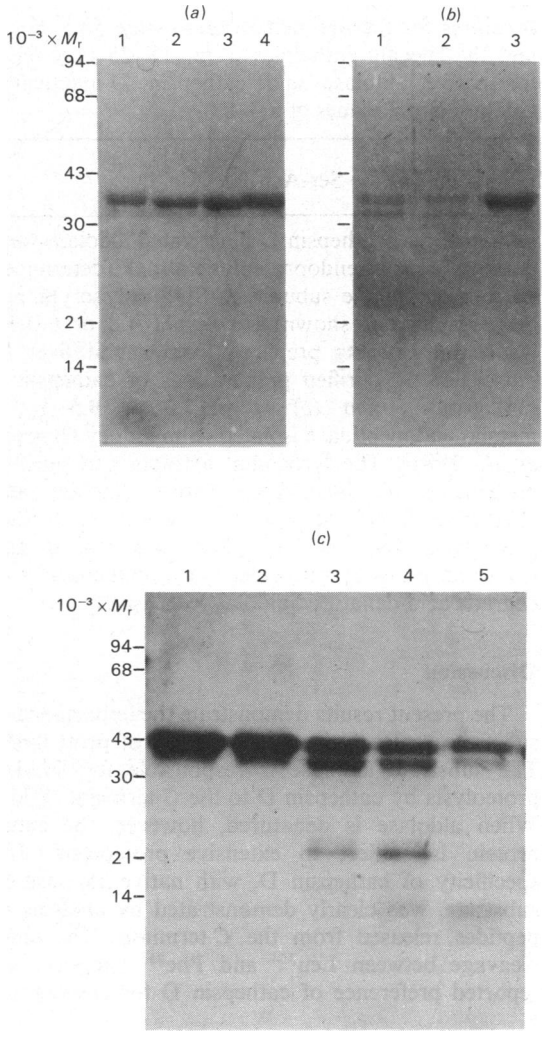

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of native and acid-denatured aldolase before and after incubation with cathepsin D*

Aldolase (0.7 mg · ml^{-1}) in 10 mM-sodium acetate, at pH 4.8 or 3.2, containing 0.1 M-NaCl was incubated at 22°C with or without 40 μg of cathepsin D · ml^{-1}. (*a*) Native aldolase, incubated at pH 4.8 with cathepsin D, was 95% inactivated by 2 h; 20 μl samples were applied to the gels; tracks 1, 2 and 3 contained aldolase incubated with cathepsin D for 2, 4 and 8 h respectively; track 4, aldolase incubated 8 h in the absence of cathepsin D. (*b*) A solution of aldolase was adjusted to pH 3.2 with HCl; no aldolase activity could be detected; the acid-inactivated aldolase was incubated with cathepsin D at pH 3.2; 20 μl samples were applied to gels; tracks 1 and 2 contained aldolase incubated with cathepsin D for 1 and 3 h respectively; track 3, aldolase incubated without cathepsin D for 3 h. (*c*) Aldolase that was acid-denatured at pH 3.2 was subsequently dialysed for 18 h against 10 mM-sodium acetate, pH 4.8, at 4°C; no aldolase activity was recovered; 40 μl samples were applied to gels; track 1, acid-inactivated aldolase incubated at pH 4.8 without cathepsin D for 5 h; tracks 2–5, aldolase incubated with cathepsin D for 0.08, 1, 3 and 22 h respectively.

from 0.046 min^{-1} at pH 4.2 to 0.018 min^{-1} at pH 5.2 in the presence of 33 μg of cathepsin D · ml^{-1} in 66 mM-sodium acetate/330 mM-NaCl at 23°C.

Salts had a marked effect on the inactivation of aldolase by cathepsin D. The first-order rate constants for inactivation in 50 mM-pyridine acetate, pH 4.8, varied from 0.20 min^{-1} · (mg of cathepsin D)$^{-1}$ in the presence of 0.01 M-NaCl to 1.1 min^{-1} · (mg of cathepsin D)$^{-1}$ in the presence of 0.5 M-NaCl. The anionic component of salts appeared to be the component which affected the inactivation of aldolase by cathepsin D. Chloride, iodide and sulphate ions appeared to be equally effective in accelerating inactivation; citrate and acetate ions were not as effective. The rate of hydrolysis of denatured haemoglobin by cathepsin D, assayed by the method of Anson (1938), was decreased 20% in the presence of 0.1 M-NaCl, 36% with 0.2 M-NaCl and 44% with 0.5 M-NaCl compared with the rate of hydrolysis with no added NaCl. Thus salts do not accelerate cathepsin D activity towards all substrates and more likely affect the protein substrates rather than the proteinase.

Cathepsin D degradation of native aldolase resulted in a slight decrease in the subunit molecular weight of aldolase as indicated by the mobility on SDS/polyacrylamide gels (Fig. 1*a*). To show that the tertiary and/or quaternary structure of aldolase was responsible for the resistance of the major portion of the protein to proteolysis, aldolase was denatured before exposure to cathepsin D. Difference spectroscopy indicated that aldolase underwent extensive conformational alteration at pH 3.9 and below, whereas no difference in the conformation of the enzyme was detected at pH 4.7 compared with 7.5 (results not shown). When aldolase was denatured at pH 3.2 and then exposed to cathepsin D at pH 3.2 or 4.8, extensive proteolysis of the 40000-M_r subunit was observed (Figs 1*b* and 1*c*). The observation that proteolysis was more rapid at pH 3.2 than 4.8 may be due to greater activity of cathepsin D at pH 3.2 or to partial renaturation of aldolase at pH 4.8.

The nine peptide products that resulted from the hydrolysis of native aldolase by cathepsin D were all present near the *C*-terminus of aldolase (Table 1); there was no indication that hydrolysis of peptide bonds occurred before Pro^{342}. The most prominent products were two peptides resulting from hydrolysis of the Leu^{354}–Phe^{355} bond. The other peptide products were generally the result of cleavage of

peptide bonds with at least one hydrophobic residue. The *C*-terminal 20 amino acid residues of aldolase and the major (⇓) and minor (↓) sites of cleavage were:

Pro↓Ser↓Gly-Gln↓Ala-Gly↓Ala-Ala↓Ala-Ser-Glu-Ser↓Leu⇓Phe↓Ile-Ser-Asn-His↓Ala↓Tyr

The cathepsin D-inactivated enzyme showed no tendency to dissociate into subunits; both native and cathepsin-inactivated aldolase sedimented as the 160000-M_r (approx.) tetramer when subjected to sucrose density centrifugation by the method of Martin & Ames (1961). In addition, the cathepsin D-inactivated enzyme gave a single line of identity with native enzyme on Ouchterlony plates with antibody prepared against native rabbit muscle aldolase.

To determine whether the cathepsin D-inactivated aldolase was more easily unfolded by denaturing agents than was native aldolase, both forms were exposed to urea and conformational changes in the protein were monitored by fluorescence quenching of tryptophan residues (Fig. 2). Acrylamide efficiently quenches tryptophan fluorescence, and this reflects the degree of exposure of tryptophan in proteins (Eftink & Ghiron, 1976). Stern–Volmer plots of F_0/F (see Fig. 2) against acrylamide concentration revealed identical quench curves for native and cathepsin D-inactivated aldolase, indicating that both forms had similar resistance to urea denaturation.

The resistance of native and cathepsin D-inactivated aldolase to thermal denaturation, as indicated by differential scanning calorimetry, was also similar, if not identical. The transition temperatures for thermal denaturation were 58.5–60°C and the specific enthalpies were (18–25 mJ)·mg^{-1} for native aldolase and cathepsin D-inactivated aldolase at pH values of 5.0–8.0.

Native and cathepsin D-inactivated aldolase were both resistant to endoproteolytic attack (determined by mobility of the subunit on SDS/polyacrylamide gels; results not shown) (*a*) at pH 4.8 or 6.0 by lysosomal extracts prepared from rabbit liver or muscle or by purified preparations of cathepsin B (35 μg·ml^{-1}), and (*b*) at pH 7.0 or 9.5 by a metallo-endopeptidase isolated from kidney (Beynon *et al.*, 1981). The lysosomal extracts and purified proteinases all inactivated native aldolase and effected a limited proteolysis; however, no further proteolysis was evident. The proteinases and lysosomal extracts were able, as was cathepsin D, to degrade acid-denatured aldolase extensively.

Discussion

The present results demonstrate the importance of substrate structure in the regulation of proteolysis. The substrate aldolase is responsible for limiting proteolysis by cathepsin D to the *C*-terminal region. When aldolase is denatured, however, the entire protein is subject to extensive proteolysis. The specificity of cathepsin D, with native aldolase as substrate, was clearly demonstrated by analysis of peptides released from the *C*-terminus. The main cleavage between Leu^{354} and Phe^{355} supports the reported preference of cathepsin D for cleavage of

Table 1. *Amino acid composition of peptides released from native aldolase by cathepsin D*

Aldolase (18 nmol of the subunit) was incubated with cathepsin D (0.1 nmol) in a total volume of 0.8 ml, containing 80 mM-pyridine acetate, pH 4.8, and 0.2 M-NaCl at 22°C for 70 min. The aldolase/cathepsin D ratio was 184:1 (w/w). The sample was prepared and peptides separated using high-pressure liquid chromatography as described in the Experimental section. Separated peptides were acid-hydrolysed and the amino acid composition of each peptide was determined. Peptides, deduced from the known primary structure of aldolase, are listed in order of amount recovered. The nmol ratio is the measured amount (in nmol) of an amino acid in a peptide divided by the nanomolar amount of the first amino acid in the peptide that eluted from the amino acid analyser. The *C*-terminus of rabbit muscle aldolase is (Lai, 1975):

-Thr^{341}-Pro-Ser-Gly-Gln^{345}-Ala-Gly-Ala-Ala-Ala^{350}-Ser-Glu-Ser-Leu-Phe^{355}-Ile-Ser-Asn-His-Ala^{360}-Tyr

Amino acid analysis (nmol ratios)	Recovery (nmol)	Deduced peptide
Asp (1.0); Ser (1.2); Ala (1.1); Ile (0.9); Tyr (1.0); Phe (1.0); His (1.1)	4.2	Phe^{355}...Tyr^{361}
Asp (1.0); Ser (1.0); Ile (0.7); Phe (0.7); His (0.9)	4.0	Phe^{355}...His^{359}
Asp (1.0); Ser (1.0); Ala (0.9); Ile (0.8); Tyr (0.5); His (3.5)	1.0	Ile^{356}...Tyr^{361}
Ser (1.0); Glu (0.9); Gly (1.4)	1.0	Ser^{343}...Gln^{345}
Glu (1.0); Gly (1.9); Ala (1.1)	1.0	Gly^{344}...Gly^{347}
Asp (1.0); Ser (1.2); Ala (1.2); Ile (0.7); His (0.8)	0.7	Ile^{356}...Ala^{360}
Ser (1.0); Glu (0.6); Ala (0.6); Leu (0.5)	0.7	Ala^{350}...Leu^{354}
Ser (1.0); Glu (0.7); Ala (0.5)	0.7	Ala^{350}...Ser^{353}
Ser (1.0); Glu (0.7); Gly (2.0); Ala (0.7)	0.6	Ser^{343}...Gly^{347}

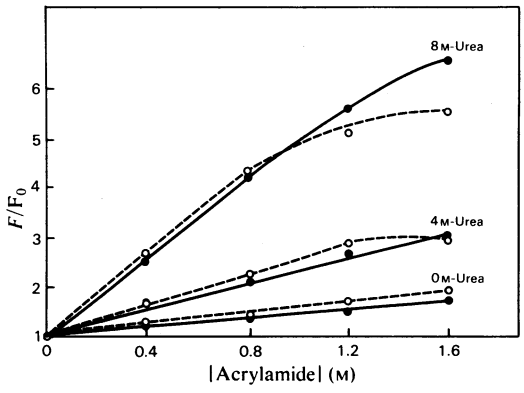

Fig. 2. *Fluorescence quenching of tryptophan residues in native and cathepsin D-inactivated aldolase*

Native or cathepsin D-inactivated aldolase was dissolved in 0.01 M-Tris, pH 7.5 at a concentration of 0.76–1.53 mg · ml^{-1}. The A_{295} of each sample was less than 0.1. Samples were incubated with either 0 M-, 4 M- or 8 M-urea before mixing with acrylamide. Samples were diluted 1:1 with various concentrations of acrylamide and fluorescence was then measured as described in the Experimental section. Stern–Volmer plots of F_0/F against acrylamide concentration were done for native aldolase (●) and cathepsin D-inactivated aldolase (○), where F_0 was the fluorescence of unquenched sample and F was the fluorescence of sample quenched with acrylamide. Acrylamide concentration was expressed as molarity after dilution with the sample.

peptide bonds between amino acids with large hydrophobic side chains (Barrett, 1977). The mode of action of two other proteinases has also been demonstrated with native aldolase as substrate; chymotrypsin cleaves only a hexapeptide from the *C*-terminus by cleaving the Phe^{355}–Ile^{356} bond (Midelfort & Mehler, 1972) and cathepsin B removes sequential dipeptides from the *C*-terminus (Bond & Barrett, 1980). The peptidyl dipeptidase activity of cathepsin B has only been observed on native polypeptide structures of aldolase and glucagon (Aronson & Barrett, 1978); there is no evidence of this type of exopeptidase activity on peptides with no tertiary structure, such as insulin B chain (Barrett, 1977; M. J. McKay, M. K. Offermann, A. J. Barrett & J. S. Bond, unpublished work). This again emphasizes the importance of the substrate structure on proteinase action.

Although the present work and work of others has established that the *C*-terminus of aldolase is quite susceptible to attack by proteinases, the present study is the first on the structural properties of the portion of native aldolase that is resistant to proteolysis. The partially degraded aldolase by all criteria tested is as stable as the native enzyme. Cathepsin D-treated aldolase retains antigenic sites, quaternary structure, resistance to urea and thermal denaturation comparable with that of native aldolase and resistance to further proteolytic attack. Because the *C*-terminus is so susceptible to proteolysis, it is reasonable to suggest that neutral or acidic proteinases *in vivo* attack the *C*-terminal region of native aldolase initially, but our work indicates that this would not lead to the total degradation of the enzyme. We propose that total degradation of aldolase *in vivo* [the enzyme has a half-life of 1–2 days in muscle (Dolken & Pette, 1974; MacDonald *et al.*, 1979)] requires (*a*) the very high concentrations of lysosomal enzymes (e.g. 25–40 mg · ml^{-1}) that are thought to be present in lysosomes (Dean & Barrett, 1976), (*b*) a unique proteinase capable of initiating extensive degradation of the enzyme, or (*c*) a non-proteolytic modification that alters aldolase protein conformation, rendering the molecule susceptible to complete proteolysis. Studies indicate that disulphides, such as oxidized glutathione, destabilize aldolase and render it more susceptible to extensive degradation by lysosomal and non-lysosomal proteinases (Bond & Offermann, 1981) and more susceptible to endocytosis and degradation by perfused tissues (J. S. Bond & N. N. Aronson, Jr., unpublished work). Thus thiol-exchange reactions that result in unstable protein conformations may be important in initiating the degradation of muscle aldolase to amino acids and peptides.

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