Biochem. J. (1980) **189**, 17–25
Printed in Great Britain

Degradation of Fructose-1,6-bisphosphate Aldolase by Cathepsin B

A FURTHER EXAMPLE OF PEPTIDYLDIPEPTIDASE ACTIVITY OF THIS PROTEINASE

Judith S. BOND* and Alan J. BARRETT
Department of Biochemistry, Medical College of Virginia, Virgina Commonwealth University, Richmond, VA 23298, U.S.A. and Biochemistry Department, Strangeways Laboratory, Worts' Causeway, Cambridge CB1 4RN, U.K.

(*Received 27 November 1979*)

The mechanism of degradation of fructose-1,6-bisphosphate aldolase from rabbit muscle by the lysosomal proteinase cathepsin B was determined. Treatment of aldolase with cathepsin B destroys up to 90% of activity with fructose 1,6-bisphosphate as substrate, but activity with fructose 1-phosphate is slightly increased. Cathepsin L, another lysosomal thiol proteinase, and papain are also potent inactivators of aldolase, whereas inactivation is not caused by cathepsins D or H even at high concentrations, or by cathepsin B inhibited by leupeptin or iodoacetate. The cathepsin-B-treated aldolase shows no detectable change in subunit molecular weight, oligomer molecular weight or subunit interactions. Cathepsin B cleaves dipeptides from the *C*-terminus of the aldolase subunits. Four dipeptides are released sequentially: Ala-Tyr, Asn-His, Ile-Ser and Leu-Phe, and a maximum of five additional dipeptides may be released. There are indications that this peptidyldipeptidase activity of cathepsin B may be an important aspect of its action on protein substrates generally.

Lysosomal proteases†, and particularly the thiol proteinases, appear to be involved in the degradation of intracellular proteins *in vivo* (Huisman *et al.*, 1974; Dean, 1975; Ballard, 1977; Bohley *et al.*, 1978). The best characterized of these enzymes are cathepsin B (Barrett, 1973) (EC 3.4.22.1), cathepsin H (Kirschke *et al.*, 1977*a*) (EC 3.4.22.–), and cathepsin L (Kirschke *et al.*, 1977*b*) (EC 3.4.22.15), but much remains to be learned about the interaction between cellular proteinases and their cellular protein substrates, and the mechanisms involved in degradation of cellular enzymes. Cathepsin B has well-established endopeptidase activity against proteins such as haemoglobin, casein, cartilage proteoglycan, collagen, myofibrillar proteins and immunoglobulin G (Barrett, 1977; Schwartz & Bird, 1977). It is also reported that cathepsin B inactivates three intracellular enzymes, glucokinase, pyruvate kinase and fructose-bisphosphate aldolase (EC 4.1.2.13) (Otto, 1971; Davidson & Poole, 1975), whereas many other intracellular enzymes are not inactivated (Otto, 1971). Nakai *et al.* (1978) in a brief report, identified a single dipeptide, Ala-Tyr, released during digestion of aldolase by cathepsin B, which caused aldolase inactivation. We report here that cathepsin B acts as a peptidyldipeptidase with aldolase as substrate, i.e., there is a sequential cleavage of dipeptides from the *C*-terminus of the aldolase subunit. Cathepsin B cleaves up to nine dipeptides from the *C*-terminus and does not readily degrade the native protein further. The present work, taken together with the finding that cathepsin B also degrades glucagon by a peptidyldipeptidase action (Aronson & Barrett, 1978), indicates that this type of limited degradation may be an important general aspect of the action of cathepsin B on protein substrates.

Abbreviation used: dansyl, 5-dimethylaminonaphthalene sulphonyl.

* To whom correspondence should be addressed at the Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298, U.S.A.

† The terms 'protease' and 'proteinase' are here used in the senses originally defined by Grassmann & Dyckerhoff (1928), 'protease' being the broader term including both exopeptidases and endopeptidases, whereas 'proteinase' is synonymous with 'endopeptidase'.

Experimental

Rabbit muscle aldolase was purchased from

0306-3275/80/070017-09$01.50/1

Sigma Chemical Co., St. Louis, MO, U.S.A. The enzyme, in $(NH_4)_2SO_4$ suspension, was precipitated by centrifugation, dissolved (at 10mg/ml) in, and dialysed against, either 0.1M-sodium phosphate buffer, pH6.0, containing 1mM-EDTA and 1mM-dithioerythritol, or 0.1M-pyridine acetate buffer, pH6.0, containing 1mM-dithioerythritol. The aldolase (sp.act. 16.2U/mg) was assayed according to Johnson & Velick (1972) with 2mM-fructose 1,6-bisphosphate and approximately 2μg of aldolase in the cuvette. Where noted, assays were also done with fructose 1-phosphate (10mM) as substrate, and approximately 200μg of aldolase.

Human liver cathepsins B and D were purified by Dr. William N. Schwartz (Strangeways Laboratory, Cambridge, U.K.), by methods based on those of Barrett (1973). Cathepsins H and L from rat liver were the gift of Dr. Heidrun Kirschke (see Kirschke *et al.*, 1977*a*,*b*). The specific activities and purities of the four lysosomal proteinases were as described previously (Barrett, 1973; Kirschke *et al.*, 1977*a*,*b*). Dipeptide standards were purchased from Sigma or from Chemalog (South Plainfield, NJ, U.S.A). Leupeptin-Pr (the *N*-propionyl form of leupeptin) was the gift of Prof. H. Umezawa, Tokyo, Japan. Gel chromatography was done with Ultrogel AcA 34 acrylamide–agarose beads (LKB Instruments, London, U.K.).

Cathepsin B was stored as the reversibly inactivated tetrathionate derivative (W. N. Schwartz & A. J. Barrett, unpublished work). Immediately before use the enzyme was activated by incubation at pH6 with 2mM-cysteine or dithioerythritol for 5min at 18–23°C. Conditions for incubation of proteinases with aldolase are given in the legends to the Figures and Tables.

Polyacrylamide slab gel electrophoresis was in the buffer system of Wyckoff *et al.* (1977). Usually the thiol proteinases were inactivated with iodoacetate (5mM), before the samples were boiled with an equal volume of 2% (w/v) sodium dodecyl sulphate containing 2-mercaptoethanol (1%, v/v), glycerol (40%, v/v) and Bromophenol Blue (0.01%). After electrophoresis, gels were stained with 0.1% Coomassie Brilliant Blue G (Barrett *et al.*, 1979). Gels were calibrated with phosphorylase *a* (mol.wt. 100000), transferrin (mol.wt. 78000), bovine serum albumin (mol.wt. 68000), immunoglobulin G (mol.wt. 50000 and 25000), carbonic anhydrase (mol.wt. 29000), soya bean trypsin inhibitor (mol.wt. 21000), cytochrome *c* (mol.wt. 12750), aprotinin (mol.wt. 6500) and insulin B chain (mol.wt. 3000).

Ninhydrin-positive material was measured by the method of Rosen (1957). The amino-acid compositions of acid-hydrolysed peptides were determined on an amino-acid analyser (Locarte Instruments, London, U.K. or Durrum MBF Detector, Sunnyvale, CA, U.S.A.). Peptides were dansylated according to the procedures recommended by Gray (1972). Dansyl peptides and amino acids were extracted from reaction mixtures with ethyl acetate, dried and then dissolved in small quantities of 0.1M-pyridine acetate, pH5.0, for electrophoresis or chromatography. Dansyl derivatives were subjected to high-voltage electrophoresis on Whatman no. 1 paper (42cm × 20cm) in pyridine/acetic acid/water (7:8:1000, by vol.), pH4.5, for 20min at 66V/cm. The high-voltage electrophoresis apparatus was from CAMAG (Muttenz, Switzerland). Dansyl amino acids were also analysed by two-dimensional thin-layer chromatography (Metrione, 1978). Polyamide sheets (Cheng Chin Trading Co., Taiwan) were cut into 4.5cm × 4.5cm sections. Samples were chromatographed first with 90% formic acid/water (3:200, v/v). The sheet was dried, turned through 90° and then chromatographed in ethyl acetate/ethanol/conc. aq. NH_3 (20:5:1, by vol.). The sheets were examined by u.v. light and the fluorescent spots were compared to those of dansyl amino acid standards (BDH Chemicals, Poole, Dorset, U.K.).

Peptides were separated by chromatography on Amberlite AG50W (X2: 200–400 mesh, from Bio-Rad, Watford, Herts, U.K.). The column (1cm × 18cm) was equilibrated with pyridine acetate (0.2M, pH3.1). The peptides were eluted with a gradient of 110ml of 2M-pyridine acetate, pH5, flowing into a mixing chamber with 60ml of 0.2M-pyridine acetate, pH3.1 (Schroeder, 1972). Eluted fractions contained 1.6ml/tube; the flow rate was 12ml/h. Peptides were also separated by ascending paper chromatography. Samples were spotted on Whatman No. 1 paper, 1cm from the bottom of a sheet 25cm high × 57cm wide and chromatographed with n-butanol/acetic acid/water (40:6:15, by vol.). After air drying, the chromatograph was sprayed lightly with ninhydrin [1% (w/v) in acetone containing 2% (v/v) acetic acid and 0.1% cadmium acetate] or with fluorescamine (20mg/100ml of acetone containing 0.1% trimethylamine; fluorescamine was from Pierce, Rockford, IL, U.S.A.). Peptides were eluted from the papers with 50% aq. pyridine and dried *in vacuo*. Eluted peptides were hydrolyzed with 6M-HCl containing 1% phenol for 24h at 105°C and were analysed for amino acid content.

Results

Aldolase was rapidly inactivated by cathepsin B. Inactivation was prevented when cathepsin B was inhibited by leupeptin (Fig. 1) or inactivated by carboxymethylation with iodoacetic acid. Thus active cathepsin B was necessary for inactivation of aldolase.

Cathepsin L and papain, other thiol proteinases,

were also effective in inactivating aldolase whereas cathepsin H (also a thiol proteinase) and cathepsin D (a carboxyl proteinase) had no effect (Fig. 2). Thus proteolytic inactivation of aldolase is not unique to cathepsin B, but not all cellular proteinases are capable of causing inactivation under the conditions used.

Although cathepsin B treatment of aldolase led to 80–90% inactivation with fructose 1,6-bisphosphate as substrate, the cleavage of fructose 1-phosphate was slightly enhanced (Table 1). The ratio of activities of untreated aldolase towards fructose bisphosphate and fructose 1-phosphate is in reasonable agreement with published data and the effect of cathepsin B is to decrease the ratio. Carboxypeptidase A has a very similar effect (Drechsler *et al.*, 1959).

The subunit molecular weight of aldolase was decreased only slightly, if at all, by cathepsins B or L (Fig. 3). In addition, no significant fragmentation of aldolase subunits was detected when the concentration of cathepsin B was increased 60-fold, the

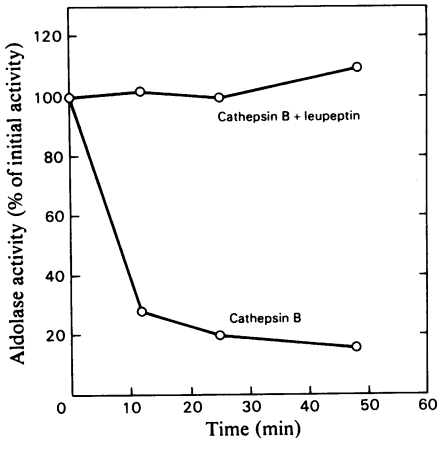

Fig. 1. *Inactivation of aldolase by cathepsin B*
Cathepsin B (5 μg) was activated by incubation with 2 mM-cysteine at 22°C for 5 min in 0.5 ml of sodium phosphate buffer (0.1 M) containing 1 mM-EDTA, pH 6.1. The incubation was continued for another 5 min in the presence or absence of leupeptin (5 μg). Aldolase (100 μg) was then added to the mixture and incubation continued at 22°C. The total volume of the incubation mixture was 0.51 ml. Portions of the incubation mixture (20 μl) were removed at the times indicated and assayed for aldolase activity.

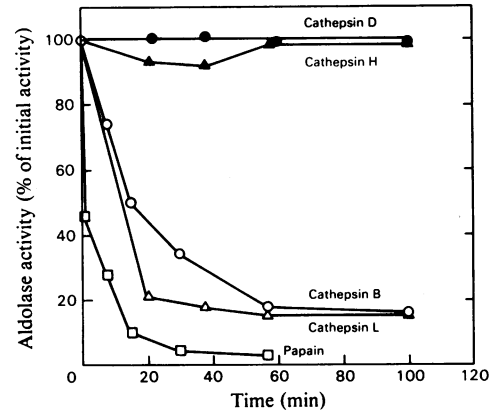

Fig. 2. *Effects of several proteinases on aldolase activity*
The thiol proteinases (cathepsins B, L, H and papain) were activated by incubating with 2 mM-cysteine as described in the legend to Fig. 1. Cathepsin B was present in the incubation mixture at 3 μg/ml, cathepsin L at 14 μg/ml, cathepsin H at 20 μg/ml, papain at 1 μg/ml and cathepsin D at 40 μg/ml. Aldolase (100 μg) was then added to the incubation mixture (total volume, 0.51 ml; temperature 22°C). Portions (20 μl) were removed at the times indicated and assayed for aldolase activity. Initial activity, measured 10 s after addition of aldolase, was not affected by any of the proteinases except papain. For mixtures containing papain, initial activity was taken as that in incubation mixtures containing carboxymethyl-inactivated papain.

Table 1. *Activities of aldolase and cathepsin B-degraded aldolase with fructose 1,6-bisphosphate and fructose 1-phosphate*
Aldolase (1 mg) previously dialysed against 0.1 M-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA was incubated alone or with cathepsin B (0.03 mg, activated with 2 mM-cysteine) in a volume of 200 μl at 23°C. After 20 min, 10 μl was removed, diluted 1:20, and 10 μl of this diluted sample was assayed for activity with fructose 1,6-bisphosphate; for activity with fructose 1-phosphate as substrate, 40 μl of the incubation mixture was added directly to the assay cuvette.

Incubation	Specific activity (μmol of substrate cleaved/min per mg of protein) with: Fructose 1,6-bisphosphate	Fructose 1-phosphate
Aldolase alone	16.2	0.075
Aldolase + cathepsin B	3.2	0.095

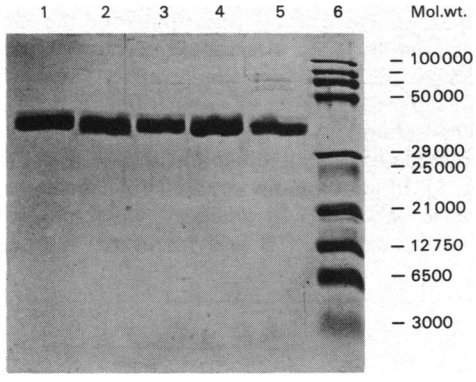

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of active and proteinase-inactivated aldolase*

Cathepsin B (1.5 μg; track 2), cathepsin L (7 μg; track 4), and papain (5 μg; track 5) were activated with 2 mM-cysteine in 0.5 ml of 0.1 M-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA. Aldolase (100 μg in 10 μl of buffer) was then added to each tube and the mixture was allowed to stand at room temperature (23°C) for 2 h. Controls included: aldolase incubated with no proteinase (track 1) and aldolase incubated with carboxymethylated cathepsin B (track 3). After incubation of aldolase alone or aldolase plus an active proteinase, 20 μl portions were removed, and 5 μl of 25 mM-iodoacetate was added to stop the reaction. Samples were boiled for 10 min with an equal volume of 2% (w/v) sodium dodecyl sulphate containing 1% mercaptoethanol and 40% (v/v) glycerol and were then applied to 12.5% (w/v) polyacrylamide gels containing sodium dodecyl sulphate at pH 7.4. Track 6 contains standards of the molecular weight indicated.

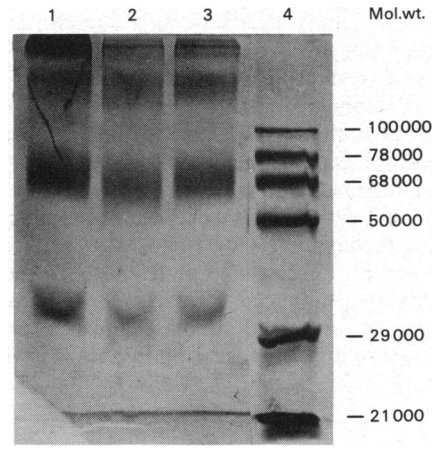

Fig. 4. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of aldolase and cathepsin B-degraded aldolase after glutaraldehyde treatment*

Track 1, aldolase; track 2, aldolase degraded by cathepsin B; track 3, aldolase incubated with carboxymethylated cathepsin B. The cross-linked proteins were boiled with sodium dodecyl sulphate and mercaptoethanol as described in the Experimental section and applied to a 7% (w/v) polyacrylamide gel containing sodium dodecyl sulphate at pH 7.4. Standards (track 4) include phosphorylase *a*, transferrin, bovine serum albumin, immunoglobulin G, carbonic anhydrase and soya bean trypsin inhibitor.

concentration of aldolase increased 15-fold and the time of incubation increased 10-fold over that described in the legend to Fig. 3. With papain, there was either no apparent change or partial conversion of aldolase subunits to a form with a fractionally decreased molecular weight (the latter is not obvious in Fig. 3). Extensive degradation of aldolase subunits did occur in mixtures containing papain if iodoacetate was omitted before treatment of the samples with sodium dodecyl sulphate (data not shown). In the experiments of Fig. 3, aldolase had been inactivated by 80–90% by cathepsins B and L and by 97% by papain when samples were taken for electrophoresis.

To determine whether cathepsin B was dissociating aldolase subunits, aldolase (10 mg) was incubated with activated cathepsin B or carboxymethylated cathepsin B (0.3 mg) for 3 h in 0.1 M-pyridine acetate buffer, (pH 6.0) containing 2 mM-dithioerythritol, in a total volume of 1.1 ml. Samples were assayed for aldolase activity; the mixture containing active cathepsin B had 16% of the aldolase activity compared to the control. The incubation mixtures (1 ml) were applied to an Ultrogel AcA 34 column equilibrated with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.3 M-NaCl and calibrated with Blue Dextran (mol.wt. 2×10^6), sheep immunoglobulin G (mol.wt. 150000) and $K_3Fe(CN)_6$ (mol.wt. 329); the A_{280} of the effluent was monitored. The elution profiles of active and inactivated aldolase were superimposable, indicating that each had a mol.wt. of about 160000 and that the action of cathepsin B had not resulted in the separation of subunits.

In a further experiment, the subunit interactions of aldolase were examined by treatment of the inactivated sample, and active control, with 10 mM-glutaraldehyde, pH 8.5, for 60 min at 20°C. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 4) indicated no difference in the pattern of cross-linking for aldolase and cathepsin-B-degraded aldolase.

Although no change in molecular weight or

subunit interactions of cathepsin-degraded aldolase was detected, there was an increase in ninhydrin-reactive material during incubation of aldolase with cathepsin B, indicating the appearance of new α-amino groups. To determine whether small peptides were being released, a mixture of aldolase and cathepsin B was incubated, and then chromatographed on a Sephadex G-25 column to separate proteins from small peptides and amino acids. The fractions containing peptides and amino acids were combined, hydrolysed and analysed for amino-acid content. Control experiments in which aldolase was incubated with carboxymethylated cathepsin B and then chromatographed on Sephadex G-25 revealed traces of several amino acids, including aspartate, serine, glutamate, glycine and alanine. The values for amino acids released from aldolase by active cathepsin B (Table 2) were corrected for the small quantities of amino acids found in control incubation mixtures containing inactive cathepsin B. The 10 amino acids found (Table 2) are those comprising the *C*-terminal 19 residues of rabbit muscle aldolase A (Lai, 1975). The last 20 residues are:

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

In several experiments proline, the twentieth amino acid from the *C*-terminal end, was never found in peptides released from aldolase. This is also significant in that proline is the *N*-terminal amino acid of the aldolase 40000-mol.wt. subunit, so it is clear that no fragments had been released from the *N*-terminus.

Several lines of evidence indicated that dipeptides were released by the action of cathepsin B. One indication of this came from an experiment in which ninhydrin-reactive material was measured before and after acid hydrolysis of the peptides (Table 3). The ninhydrin colour almost doubled after hydrolysis. Since peptides and amino acid differ somewhat in their colour yield with ninhydrin, an exact doubling was not expected, but the results were in good agreement with the idea that the amino acid residues were in dipeptides.

Peptides were dansylated, acid-hydrolysed and the dansyl amino acids were identified by comparison to standards after two-dimensional chromatography on polyamide sheets, or after high-voltage electrophoresis. Control experiments in which aldolase was incubated with carboxymethylated cathepsin B revealed no detectable release of peptides or dansyl amino acids. The dansyl derivatives of alanine, aspartate and/or glutamate, and isoleucine and/or leucine were found after incubation of aldolase with activated cathepsin B (Fig. 5). Neither chromatography nor electrophoresis gave a clear-enough separation of aspartate and glutamate, or isoleucine and leucine to show beyond doubt that all four amino acids were present as their dansylated derivatives. All five of the dansyl amino acids tentatively identified would be expected if dipeptides were released from the *C*-terminus of aldolase (the numbers represent the expected dipeptides):

```
                ↓   9   ↓   8   ↓
-Pro-Ser-Gly-Gln-Ala-Gly-Ala

7   ↓   6   ↓   5   ↓   4
-Ala-Ala-Ser-Glu-Ser-Leu-Phe

↓   3   ↓   2   ↓   1
-Ile-Ser-Asn-His-Ala-Tyr
```

In an attempt to obtain more conclusive evidence as to the formation of dipeptides, the peptides were partially separated on an Amberlite AG50W column

Table 2. *Amino acids in peptides released from aldolase by cathepsin B*

Aldolase (10mg), previously dialysed against 0.1M-pyridine acetate pH6.0, and cathepsin B (0.3mg), activated with 2mM-dithioerythritol, were incubated in 0.1M-pyridine acetate buffer, pH6.0, for 2h at 23°C in a total volume of 1ml. The mixture was then passed through a Sephadex G-25 column (10ml bed volume) equilibrated with 0.1M-pyridine acetate buffer, pH7.0. The A_{280} of eluted fractions indicated that proteins were eluted at 30–36% of the bed volume and another peak of smaller molecules was eluted at 65–80% of the bed volume. Portions of the eluted fractions were also analysed for ninhydrin-positive material. Ninhydrin-positive material was found in tubes at 55–80% of the bed volume; the contents of tubes in this region were pooled and dried in a rotary evaporator. The dried material was hydrolysed in 6M-HCl containing 1% phenol at 105°C for 24h and was analysed for amino acid content. The results are expressed as nmol of amino acid released from the equivalent of 4mg (or 100nmol) of aldolase subunits.

Amino acid	Position from *C*-terminus	Amount released (nmol)	Amount released (% of total)
Tyr	1	43	13
Ala	2, 12, 13, 14, 16	91	26
His	3	27	8
Asx	4	31	9
Ser	5, 9, 11	43	12
Ile	6	13	4
Phe	7	17	6
Leu	8	27	8
Glx	10	25	7
Gly	15	14	4

Table 3. *Ninhydrin reaction of peptide fraction before and after acid hydrolysis*

Aldolase and activated cathepsin B were incubated and the peptide fraction was collected as described in the legend to Table 2. The peptide fraction was divided into halves and dried. One-half was hydrolysed with 6 M-HCl containing 1% phenol at 105°C for 48 h and was subsequently dried *in vacuo*. The hydrolysed and unhydrolysed peptides were then dissolved in 0.1 M-pyridine acetate buffer, pH 5, and assayed for ninhydrin-positive material.

Peptide fraction	Ninhydrin-positive material (nmol leucine equiv./ 5 mg of aldolase)
Before hydrolysis	330
After hydrolysis	602

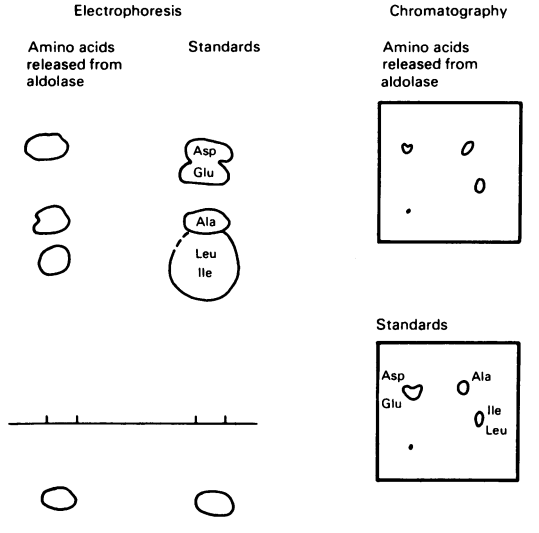

Fig. 5. *Identification of dansylated amino acids from peptides released from aldolase by cathepsin B*

Peptides released from aldolase were reacted with dansyl chloride and hydrolysed to amino acids prior to high-voltage electrophoresis or thin-layer chromatography. Dansylated amino acids were identified by comparisons with standards. Chromatography was in 90% formic acid/water (3:200, v/v) in the first (vertical) dimension and in ethyl acetate/ethanol/conc. aq. NH_3 (20:5:1, by vol.) in the second (horizontal) dimension on 4.5 cm × 4.5 cm polyamide sheets.

and then subjected to paper chromatography (Fig. 6 and Table 4). Three distinct peaks of ninhydrin-positive material were eluted, and samples A–E were taken for further analysis. The samples were

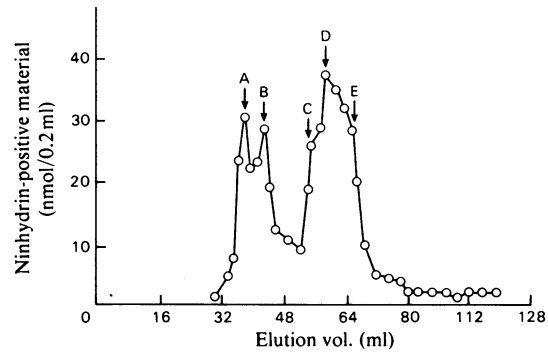

Fig. 6. *Chromatogrpahy on an AG50W column of peptides released from aldolase*

Peptides produced by cathepsin B (44 μg/ml) degradation of aldolase (60 mg in a total volume of 5.0 ml for 3 h at 22°C) were concentrated, dissolved in 1 ml of 0.2 M-pyridine acetate, pH 3.1, and applied to a Bio-Rad AG50W (X2: 200–400 mesh) column. Ninhydrin-positive material of eluted fractions is expressed as nmol of leucine equiv./0.2 ml of the sample. Arrows indicate the fractions further analysed by paper chromatography (Table 4) for peptides.

analysed in two ways: (*a*) portions were acid-hydrolysed and analysed for amino-acid content directly, and (*b*) portions were chromatographed on paper and peptides eluted from the paper were hydrolysed for amino-acid analysis (Table 4). Samples C and D were the simplest in that only alanine and tyrosine in equimolar quantities were present, and only one spot was present on paper before hydrolysis. Ala-Tyr is dipeptide 1 at the *C*-terminus of aldolase. Sample E contained aspartate, histidine, leucine, and phenylalanine, and after paper chromatography, two spots were evident: the fastest moving spot was Leu-Phe (dipeptide 4) and the slow moving spot was Asn-His (dipeptide 2). Sample B contained alanine, serine, isoleucine, glutamate and glycine, and upon paper chromatography two spots were observed. One spot contained only alanine, and moved exactly as Ala-Ala, dipeptide 7. The faster-moving and more diffuse ninhydrin-reactive spot from sample B contained alanine, serine, isoleucine, glutamate and glycine. This is a mixture of peptides probably containing Ile-Ser, dipeptide 3, and possibly Ala-Ala and Gly-Gln (peptides 7 and 9). Sample A contained alanine, serine and glutamate, and after paper chromatography, one major spot appeared. One possible explanation is that the spot was an unresolved mixture of Ala-Ser (peptide 6) and Glu-Ser (peptide 5). In summary, the peptide column chromatography and subsequent paper chromato-

Table 4. *Amino acid composition of fractions A–E eluted from an AG50W column: total amino acid content of fractions and amino acid content of peptides eluted after paper chromatography of fractions*

Fractions containing ninhydrin-positive material and indicated by arrows in Fig. 6 (samples A–E) were divided in two portions. One portion was dried *in vacuo*, hydrolysed with 6M-HCl containing 1% phenol at 105°C for 48h, and analysed for amino acid content. The other portion was rotary evaporated and the content was dissolved in 20μl of pyridine acetate, pH 5, 0.2M. Samples (10μl) of these tubes were spotted on paper and chromatographed. Ninhydrin-positive spots were cut out, eluted from the paper, hydrolysed, and analysed for amino acid content. The peptide sequences were deduced from the ratios of amino acids in eluted spots and the known structure of the aldolase subunit. Four standards (Ala-Tyr, Leu-Phe, Ala-Ser, Ala-Ala; 20nmol of each) were chromatographed concomitantly with samples eluted from the AG50W column.

		After paper chromatography:				
Fraction	Amino-acid and content (nmol/100nmol of amino acid in fraction)	Number of ninhydrin-positive spots	R_F of peptides	Amino-acid content of eluted peptides (nmol/100nmol of amino acid in fraction)	Deduced identity of peptides (position of dipeptide from *C*-terminus of aldolase)	R_F of standard peptides
A	Ser (48) Ala (38) Glu (13)	1	0.14	Ser (45) Ala (29) Glu (15)	Ala-Ser (6) Glu-Ser (5)	0.18
B	Ala (60) Ser (16) Ile (13) Glu (5) Gly (4)	2	0.32 0.42	Ala (98) Ser (37) Ile (23) Ala (16) Gly (14) Glu (10)	Ala-Ala (7) Ile-Ser (3) Ala-Ala (7) Gly-Gln (9)	0.31
C	Ala (52) Tyr (49)	1	0.45	Ala (45) Tyr (55)	Ala-Tyr (1)	0.45
D	Ala (49) Tyr (51)	1	0.42	Ala (48) Tyr (52)	Ala-Tyr (1)	0.45
E	Asp (34) His (35) Leu (15) Phe (17)	2	0.04 0.76	Asp (45) His (55) Leu (46) Phe (54)	Asn-His (2) Leu-Phe (4)	 0.78

graphy allowed positive identification of Ala-Tyr, Asn-His, Leu-Phe, and Ala-Ala, dipeptides 1, 2, 4 and 7 from the *C*-terminus of aldolase.

To determine whether dipeptides were released sequentially, aldolase was incubated with cathepsin B, portions of the incubation mixture were removed at fixed times and the rate of release of dipeptides was determined (Fig. 7). It is clear that there is a sequential release of the first four dipeptides. No attempt was made to quantify the rate of release of the other peptides released because of the small amounts released and the fact that, other than dipeptide 7 (Ala-Ala), dipeptides 5, 6, 8 and 9 were not clearly resolved in the one-dimensional chromatographic system.

Purified preparations of two other intracellular enzymes were also tested as substrates for cathepsin B: rabbit liver fructose 1,6-bisphosphatase (EC 3.1.3.11) and mitochondrial and cytosolic forms of pig heart glutamate–aspartate transaminase (EC 2.6.1.1). Cathepsin B had no effect on the catalytic activities or subunit molecular weights of the phosphatase or transaminases and did not cause release of dipeptides.

Discussion

We have found that human cathepsin B degrades aldolase by a peptidyldipeptidase action, cleaving dipeptides sequentially from the *C*-terminus. This is the first report of such activity of cathepsin B on a protein substrate. Taken together with evidence for such action on the polypeptide glucagon (Aronson & Barrett, 1978), the results suggest that peptidyldipeptidase action may be a major aspect of the way in which cathepsin B degrades specific proteins.

The specificity of cathepsin B for the cleavage of

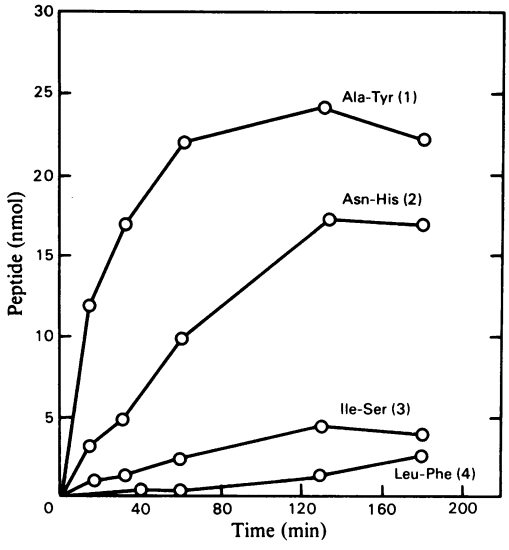

Fig. 7. *Release of dipeptides from aldolase*
Aldolase (60mg) was incubated with cathepsin B (140μg) in 0.1M-pyridine acetate buffer, pH6.0, containing 2mM-dithioerythritol, at 22°C, in a total volume of 5ml. Portions of the incubation mixture (0.9ml) were removed at the times indicated and the reaction was stopped by the addition of 0.1ml of 50mM-iodoacetate. Peptides released were separated from proteins on a Sephadex G-25 column and the peptide fraction for each time point was concentrated and subsequently chromatographed on paper with n-butanol/acetic acid/water (40:6:15, by vol.). Fluorescamine treatment of the paper revealed a maximum of five fluorescent spots. The peptides were eluted from the paper, acid-hydrolysed and analysed for amino-acid content. The mobilities of, and amino acid residues found in, the fluorescamine-positive spots were: $R_F = 0.06$, Asp, His; $R_F = 0.18$, Ser, Ala, Glu, Gly; $R_F = 0.35$, Ala; $R_F = 0.44$, Ala, Tyr, Ile, Ser; $R_F = 0.81$, Leu, Phe. The amounts of amino acids found in eluted spots with R_F values of 0.06, 0.44 and 0.81 are shown in the Figure and the dipeptide sequences were deduced from the known aldolase sequence. Numbers in parentheses indicate the position of the dipeptide from the *C*-terminus of aldolase.

bonds on the carboxyl side of pairs of arginine residues that is evident with low-molecular-weight synthetic substrates was first reported for the bovine enzyme (McDonald & Ellis, 1975), and has since been confirmed for human cathepsin B (C. G. Knight, W. N. Schwartz & A. J. Barrett, unpublished work). This specificity raised the possibility that cathepsin B was responsible for the cleavage at pairs of basic amino acid residues that is so characteristic of processing of proteins and generation of polypeptide hormones (Geisow, 1978). In order to determine whether cathepsin B would also cleave at dibasic sequences in polypeptides, Aronson & Barrett (1978) examined the action of the enzyme on glucagon. There was no detectable cleavage at the Arg(17)–Arg(18) sequence, but instead cathepsin B cleaved dipeptides sequentially from the *C*-terminus. This action had been quite unexpected, and it was not clear to what extent it was dictated by the structure of the substrate, although it seemed significant that the related proteinase, papain, was known to degrade glucagon quite differently. It was confirmed that both bovine and rat cathepsin B acted similarly to the human enzyme.

Studying the action of carboxypeptidase A on aldolase, Drechsler *et al.* (1959) showed that removal of the *C*-terminal tyrosine residue resulted in 93% loss of activity against fructose 1,6-bisphosphate, but a slight increase in activity against fructose 1-phosphate. Our own findings for the effect of cathepsin B on the catalytic activity of aldolase are closely similar, and it seems very probable that the removal of the first *C*-terminal dipeptide, Ala-Tyr, accounts for the change in activity. In fact, during the course of our work, Nakai *et al.* (1978) published a brief report attributing the inactivation of aldolase by rabbit cathepsin B to removal of the *C*-terminal dipeptide. The results of the Japanese workers indicate that only the first dipeptide was released by the rabbit enzyme, however, and this is in complete contrast to our own findings with human cathepsin B.

The endopeptidase activity of cathepsin B is, of course, well established (Barrett, 1977), but this may be dependent on the substrate, and has normally been observed in work with much higher concentrations of cathepsin B than are required to show peptidyldipeptidase activity. Since cathepsin B exists in lysosomes in very high concentration (approximately 1mM; Dean & Barrett, 1976) the enzyme would be expected to show potent endopeptidase activity in that location, whereas trace concentrations of the enzyme elsewhere in the cell might show only peptidyldipeptidase activity.

In addition, cathepsin B may bring about highly selective cleavages at certain dibasic sequences during the post-translational processing of some proteins (Quinn & Judah, 1978). Geisow (1978) has shown that the sensitivity of such bonds to cleavage is correlated with location outside α-helical regions, often near reverse turns, in the secondary structure of the polypeptides.

Cathepsin B exists in lysosomes together with the related thiol proteinases, cathepsins H and L. Cathepsin H did not inactivate or affect the sodium dodecyl sulphate/polyacrylamide-gel pattern of aldolase even at 10-fold higher concentrations than were used for cathepsin B. In contrast, cathepsin L

seemed to act on aldolase in a very similar way to cathepsin B (Kirschke *et al.*, 1977*a*, and the present work). It has become common to use the inactivation of aldolase as a criterion for the identification of cathepsin B. However, it is important to recognize that cathepsin L shares this activity.

The discovery that cathepsins B and L readily inactivate aldolase by very limited proteolysis may necessitate re-examination of some earlier work on the occurrence of inactive forms of aldolase in tissues of senescent animals (e.g. Gershon & Gershon, 1973; Anderson, 1974). In such work, tissues were homogenized in media containing EDTA and mercaptoethanol, in which the thiol proteinases could have been active, producing catalytically inactive molecules retaining immunological cross-reactivity and much of the normal primary structure. Such forms may be formed *in vivo* or *in vitro*, but would clearly be quite different from the 'error proteins' sometimes supposed to accumulate in aging. In fact, Petell & Lebherz (1979) have evidence indicating that 'defective' liver aldolase molecules are produced during storage of livers from old mice rather than being produced in liver cells *in vivo*. The 'defective' or inactive forms of aldolase did not have *C*-terminal tyrosine residues, and the 'non-defective' enzyme does, and inactivation could be completely eliminated by addition of phenylmethylsulphonyl fluoride and leupeptin during tissue homogenization. Our work would indicate that cathepsins B or L might very well be responsible for the limited proteolysis associated with 'defective' aldolase molecules.

The authors are indebted to Dr. W. N. Schwartz for providing human liver cathepsins B and D, to Dr. H. Kirschke for rat liver cathepsins L and H, to Dr. B. L. Horecker for rabbit liver fructose 1,6-bisphosphatase, to Dr. M. Martinez-Carrion for pig heart glutamate aspartate transaminase, and to Dr. G. M. Herring and Dr. Darrell Peterson for performing the amino-acid analyses. Most of this research was done while J. S. B. was on educational leave from the Medical College of Virginia, Virginia Commonwealth University. J. S. B. is a recipient of a Research Career Development Award from the National Institutes of Arthritis, Metabolism and Digestive Diseases. This work was supported in part by Grant AM 19691 from the National Institutes of Arthritis, Metabolism and Digestive Diseases.

References

Anderson, P. J. (1974) *Biochem. J.* **140**, 341–343

Aronson, N. N., Jr. & Barrett, A. J. (1978) *Biochem. J.* **171**, 759–765

Ballard, F. J. (1977) *Essays Biochem.* **13**, 1–37

Barrett, A. J. (1973) *Biochem. J.* **131**, 809–822

Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 181–208, North-Holland, Amsterdam

Barrett, A. J., Brown, M. A. & Sayers, C. A. (1979) *Biochem. J.* **181**, 401–418

Bohley, P., Kirschke, H., Langner, J., Riemann, S., Wiederanders, B., Ansorge, S. & Hanson, H. (1978) in *Protein Turnover and Lysosomal Function* (Segal, H. L. & Doyle, D., eds.), pp. 379–391, Academic Press, New York

Davidson, E. & Poole, B. (1975) *Biochim. Biophys. Acta* **397**, 437–442

Dean, R. T. (1975) *Nature (London)* **257**, 414–416

Dean, R. T. & Barrett, A. J. (1976) *Essays Biochem.* **12**, 1–40

Drechsler, E. R., Boyer, P. D. & Kowalsky, A. G. (1959) *J. Biol. Chem.* **234**, 2627–2634

Geisow, M. J. (1978) *FEBS Lett.* **87**, 111–114

Gershon, H. & Gershon, D. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 909–913

Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138

Grassmann, W. & Dyckerhoff, H. (1928) *Hoppe-Seyler's Z. Physiol. Chem.* **179**, 41–78

Huisman, W., Lanting, L., Doddema, H. J., Bouma, J. M. W. & Gruber, M. (1974) *Biochim. Biophys. Acta* **370**, 297–307

Johnson, L. W. & Velick, S. F. (1972) *J. Biol. Chem.* **247**, 4138–4143

Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S., Bohley, P. & Hanson, H. (1977*a*) *Acta Biol. Med. Germ.* **36**, 185–199

Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S. & Bohley, P. (1977*b*) *Eur. J. Biochem.* **74**, 293–301

Lai, C. Y. (1975) *Arch. Biochem. Biophys.* **166**, 358–368

McDonald, J. K. & Ellis, S. (1975) *Life Sci.* **17**, 1269–1276

Metrione, R. M. (1978) *J. Chromatogr.* **154**, 247–250

Nakai, N., Wada, K., Kobashi, K. & Hase, J. (1978) *Biochem. Biophys. Res. Commun.* **83**, 881–885

Otto, K. (1971) in *Tissue Proteinases* (Barrett, A. J. & Dingle, J. T., eds.), pp. 1–28, North-Holland, Amsterdam

Petell, J. K. & Lebherz, H. G. (1979) *J. Biol. Chem.* **254**, 8179–8184

Quinn, P. S. & Judah, J. D. (1978) *Biochem. J.* **172**, 301–309

Rosen, H. (1957) *Arch. Biochem. Biophys.* **67**, 10–15

Schroeder, W. A. (1972) *Methods Enzymol.* **25**, 203–213

Schwartz, W. N. & Bird, J. W. C. (1977) *Biochem. J.* **167**, 811–820

Wyckoff, M., Rodbard, D. & Chrambach, A. (1977) *Anal. Biochem.* **78**, 459–482