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The Specificity of Cathepsin B

HYDROLYSIS OF GLUCAGON AT THE *C*-TERMINUS BY A PEPTIDYLDIPEPTIDASE MECHANISM

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The manner in which human liver cathepsin B (EC 3.4.22.1) digests glucagon was determined. After reaction of the proteinase with the substrate for 24h, more than 15 products were formed. During the first 7h of reaction, eight products were formed; seven of these were dipeptides that originated from the *C*-terminal portion of the glucagon molecule, whereas the eighth peptide was the remaining large fragment of the hormone, consisting of residues 1–19. Measurement of the rate of formation of the products showed that cathepsin B degraded glucagon by a sequential cleavage of dipeptides from the *C*-terminal end of the molecule. Cathepsin B from both rat liver and bovine spleen was shown to hydrolyse glucagon by the same mechanism.

The thiol proteinase cathepsin B (EC 3.4.22.1) (see review by Barrett, 1977) may be responsible for most of the protein digestion in the lysosomal system. This suggested importance has been argued on the basis of studies that showed that inhibitors of cathepsin B caused a striking inhibition of protein degradation in each experimental system that was investigated, including leupeptin used *in vitro* (Huisman *et al.*, 1974), in tissue culture (Hopgood *et al.*, 1977) and *in vivo* (Dunn & Aronson, 1978) and chloroquine used in tissue culture (Wibo & Poole, 1974). Indeed, cathepsin B is capable of digesting many important proteins of the body, such as the soluble cytoplasmic proteins from liver (Dean, 1975), haemoglobin (Otto, 1971), collagen (Burleigh *et al.*, 1974; Etherington, 1974), immunoglobulin G (E. Davies & W. Schwartz, unpublished work), myosin (Schwartz & Bird, 1977) and cartilage proteoglycans (Morrison *et al.*, 1973; Roughley & Barrett, 1977).

Cathepsin B may also cleave certain precursor proteins, generating their active forms. Such proteolytic conversions commonly require limited proteolysis on the *C*-terminal side of a pair of basic amino acid residues. This is true of the formation of insulin from proinsulin (Chance, 1972), albumin from proalbumin (Russell & Geller, 1975) and parathyrin from proparathyrin (Hamilton *et al.*, 1974). These processes take place in Golgi vesicles, but which enzymes are involved in this limited proteolysis is unknown. There have been preliminary reports that suggest that cathepsin B from either bovine spleen (McDonald & Ellis, 1975) or rat liver (Friedrich *et al.*, 1978) converted proinsulin into insulin *in vitro*, and J. D. Judah (personal communication) found that treatment of proalbumin with cathepsin B from bovine liver resulted in the formation of albumin.

Cathepsin B is able to act at a pH near neutrality (pH6–7) and is well able to hydrolyse synthetic substrates containing arginine residues. The substrate most commonly used to measure the enzyme experimentally is benzoylarginine 2-naphthylamide (Barrett, 1972, 1976). If this substrate is replaced by one that contains a diarginine sequence, the enzyme shows almost a 100-fold greater efficiency (as measured by the ratio $k_{cat.}/K_m$) when catalysing the hydrolysis (McDonald & Ellis, 1975; C. G. Knight, personal communication). Therefore the ability of purified human liver cathepsin B to perform a specific cleavage at diarginine residues in protein substrates was investigated. The protein used for the present study is glucagon, since this 29-amino acid peptide hormone contains one diarginine pair (Arg^{17}–Arg^{18}) in its amino acid sequence (Fig. 1).

Experimental

Materials

Cathepsin B was purified from human liver by the procedure of Barrett (1973). Cathepsin B from rat liver [purified by methods based on those of Schwartz & Bird (1977) and Kirschke *et al.* (1976, 1977)] was supplied by Dr. W. N. Schwartz, of this

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Laboratory, and Professor K. Otto (Institute of Physiological Chemistry, Bonn, German Federal Republic) provided cathepsin B from bovine spleen. Glucagon and 2,3-dimercaptopropanol (British Anti-Lewisite, BAL) were purchased from Sigma (London) Chemical Co., Kingston upon Thames KT2 7BH, Surrey, U.K. The amino acid composition of the glucagon was determined experimentally and was consistent with the published sequence (Fig. 1). Mercaptoethanesulphonic acid was obtained from Pierce and Warriner, Chester CH1 4EF, U.K.

Methods

Digestion of glucagon with cathepsin B was in 0.3ml of 0.067–0.4M-acetic acid (approx. pH3) containing 1mM-dimercaptopropanol and 3mg of glucagon/ml. Exact reaction conditions are given in the legends to the Figures. The glucagon was dissolved in 1M-acetic acid at a concentration of approx. 1% of the weight of glucagon, and the digestions were performed at room temperature (approx. 22°C). Such reactions were stopped by addition of iodoacetic acid to a final concentration of 0.1mM. Samples (25μl) of digests that initially contained 100μg of glucagon were applied to Whatman no. 1 sheets (42cm×20cm) as 1cm streaks, and subjected to electrophoresis at 83V/cm for 20min in pH6.0 buffer (pyridine/acetic acid/water, 10:1:89, by vol.). The high-voltage electrophoresis apparatus was from CAMAG (4132 Muttenz, Switzerland). Peptide products were sometimes analysed also by ascending chromatography perpendicular to the direction of electrophoresis, in butan-1-ol/acetic acid/water (40:6:15, by vol.). Peptides were detected on the dried paper by dipping in a cadmium/ninhydrin reagent (Barrolier *et al.*, 1957). In one experiment (a 7h digest) the peptide products were initially separated by ion-exchange chromatography on Dowex 50 (X2) resin in a linear gradient of pyridine acetate buffers (Schroeder, 1972). The eight peptides produced during this reaction were further purified by preparative electrophoresis at pH6 and additionally some were chromatographed in the butanol/acetic acid/water solvent. The peptides were then eluted from the paper with 1M-acetic acid.

These purified peptides were hydrolysed for 24h at 110°C either with 6M-HCl under reduced pressure or with 3M-mercaptoethanesulphonic acid (Penke *et al.*, 1974). The amino acid compositions of the hydrolysates were determined on an amino acid analyser (Locarte Instruments, London W12 9RT, U.K.) by use of a single-column system recommended by the manufacturer. On the basis of the known amino acid sequence of glucagon (Fig. 1), the sequence of each peptide product (dipeptide) was unequivocally deducible from its amino acid composition. The peptides had approximately integral molar ratios of constituent amino acids after acid hydrolysis (Table 1) and were homogeneous by both high-voltage electrophoresis at pH6 and chromatography in butan-1-ol/acetic acid/water (40:6:15, by vol.).

The hydrolysis of glucagon by purified cathepsin B from both rat liver and bovine spleen was also investigated. To 0.5mg of glucagon in 0.13ml of 0.5M-acetic acid that contained 3.5mM-dimercaptopropanol was added 5μl of cathepsin B from either human liver, rat liver or bovine spleen. Each reaction was for 18h at 22°C and contained an amount of enzyme that catalysed equivalent hydrolysis of azocasein. This latter activity was determined at 40°C in a pH6 buffer containing 75mM-sodium–potassium phosphate, 1mM-EDTA, 2mM-cysteine and 1.5% (w/v) azocasein. After 1h the hydrolysis of azocasein was stopped by adding 2.5ml of 3% (v/v) trichloroacetic acid. After filtration the A_{366} of the filtrate was measured. On the basis of this azocasein assay, each glucagon reaction contained enzyme that had catalysed an increase of 3.35 in the A_{366}.

Results

Products after 24h reaction

The progress of glucagon digestion was followed by high-voltage electrophoresis of samples taken at various times during a 24h period (Fig. 2). It was evident that human liver cathepsin B did not catalyse only a limited hydrolysis of the polypeptide hormone (as, for example, at the Arg[18]–Ala[19] bond). Thus electrophoresis and ascending chromatography

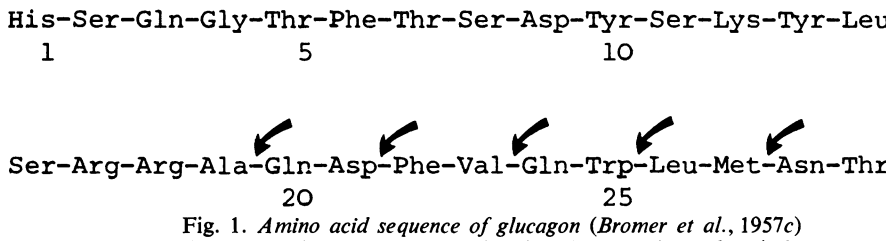

Fig. 1. *Amino acid sequence of glucagon (Bromer et al., 1957c)*
The arrows show the pattern of major cleavages by cathepsin B.

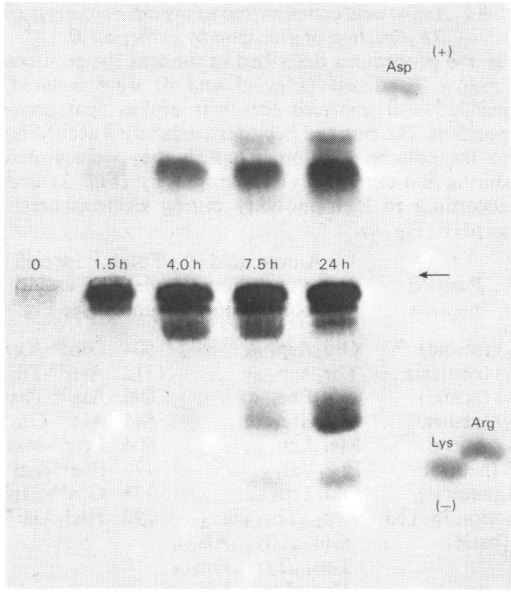

Fig. 2. *Electrophoretic separation of products from the digestion of glucagon by cathepsin B*

A sample (1.51 mg) of glucagon was digested at 22°C with 20 μg of human liver cathepsin B in 3 ml of 67 mM-acetic acid containing 1 mM-dimercaptopropanol. At the indicated times, 0.55 ml portions of the mixture were removed, frozen in small vials, and freeze-dried. To each dried sample was added 0.1 ml of water, and half of the resulting solution was immediately applied to Whatman no. 1 paper. The zero-time sample was taken before the addition of enzyme. Aspartic acid, lysine and arginine (each 10 nmol) were used as standards and the paper was subjected to high-voltage electrophoresis at pH 6 and stained with ninhydrin as described in the text. The origin is indicated by the arrow and the anode is at the top.

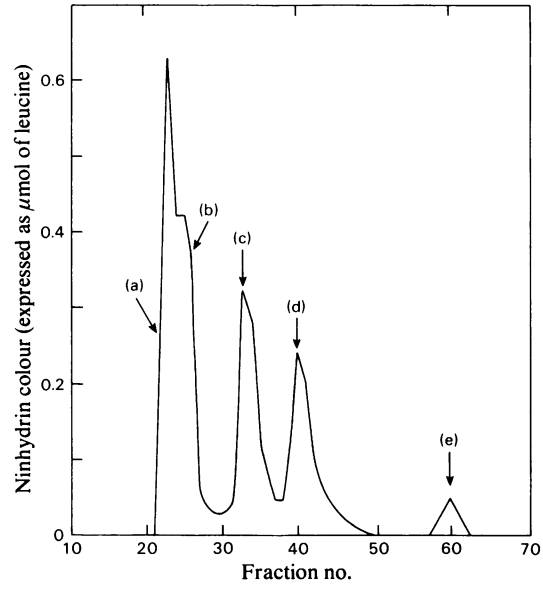

Fig. 3. *Ion-exchange chromatography of 7h digest of glucagon*

A sample (9.6 mg) of glucagon was digested for 7 h at 22°C with 40 μg of human liver cathepsin B in 0.93 ml of 0.4 M-acetic acid containing 3.2 mM-dimercaptopropanol. The reaction was stopped by adding 25 μl of 10 mM-iodoacetic acid. The solution was adjusted to approx. pH 3.1 by adding 1.6 μl of 6 M-HCl and this material was applied to the top of a column (18 cm × 1 cm) of Bio-Rad AG 50W (X2; 200–400 mesh) cation-exchange resin. The column was eluted at 38°C by the method of Schroeder (1972) with a gradient of pyridine/acetic acid buffers made from 116 ml of pH 5 buffer flowing into a mixing chamber with 60 ml of pH 3.1 buffer (0.2 M in pyridine). Fractions (1.6 ml) were collected every 10 min and this flow rate was maintained by pumping. Samples (0.1 ml) were analysed with ninhydrin by the method of Spies (1957), with leucine as standard. The most basic component that was eluted from the column was not detectable in the fractions with ninhydrin, but this material was later found to be in the combined and concentrated fractions 78–110. Tubes 22 (a), 26 (b), 33 (c), 40 (d), and 59–60 (e) were analysed separately as described in the legend for Fig. 4.

of the sample at 24 h showed at least 15 products. However, one of the peptides showed greater mobility towards the cathode than either free arginine or lysine (see Fig. 2). This material was eluted from the paper after preparative electrophoresis, and on amino acid analysis it was found to contain only arginine. We concluded this to be the dipeptide Arg^{17}–Arg^{18}.

The pattern of products seen on electrophoresis of the 4 h sample (Fig. 2) showed four distinct components, two being almost immobile in electrophoresis (neutral substances) and one each that migrated respectively towards the anode (an acidic substance) and cathode (a basic substance). Comparison of this separation with that for the 1.5 h sample shows that the first acidic peptide product(s) were detected only after 4 h of reaction, at which time the first basic peptide(s) had also been released by cathepsin B.

Ion-exchange chromatography of products

Digestion of glucagon was for 7 h and the products were separated by cation-exchange chromatography (Fig. 3), followed by further purification by high-voltage electrophoresis and chromatography on paper. The initial separation of the products by

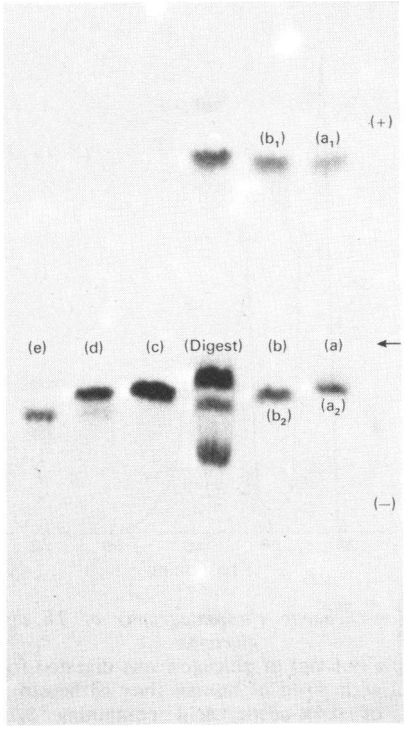

Fig. 4. *Peptide products from glucagon after 7h digestion*

Fractions (a), (b), (c), (d) and (e) from the Bio-Rad AG 50W-X2 column (see Fig. 3) were freeze-dried and redissolved in 0.2ml of water [fractions (a) and (b)], 0.1ml of water [fractions (c) and (d)] or 0.15ml of 0.34M-acetic acid [fraction (e)]. Portions of these concentrated samples, 10μl of (a), 10μl of (b), 15μl of (c), 10μl of (d) and 15μl of (e) plus a sample (10μl) of the original 7h digest, were subjected to high-voltage electrophoresis and stained with ninhydrin as described in the text. The amino acid composition of each peptide and its sequence as it occurs in glucagon are shown in Table 1. The arrow indicates the origin and the anode is at the top.

Table 1. *Amino acid compositions of peptides obtained after 7h digestion of glucagon by cathepsin B*

By the procedures described in the text the peptides from a 7h digest (Figs. 3 and 4) were isolated, purified and analysed for their amino acid composition. The purified peptides are labelled according to the column fraction in which they were eluted during ion-exchange chromatography (Fig. 3) and according to their mobility during electrophoresis at pH6 (Fig. 4).

Purified peptide	Amino acid composition (molar ratio)	Total yield (nmol)	Identification (see Fig. 1)
(a_1) (acidic)	$Glu_1Asp_{1.04}$	504	Gln^{20}-Asp^{21}
(a_2) (neutral)	$Thr_1Asp_{1.03}$	1312	Asn^{28}-Thr^{29}
(b_1) (acidic)	$Asp_1Phe_{1.01}$	696	Asp^{21}-Phe^{22}
(b_2) (neutral)	Ala_1Glu_1	545	Ala^{19}-Gln^{20}
(c) (neutral)	$Met_1Leu_{1.06}$	604	Leu^{26}-Met^{27}
(d) (neutral)	$Phe_1Val_{1.2}$	493	Phe^{22}-Val^{23}
(e) (neutral)	$Glu_1Trp_{0.71}$	128	Gln^{24}-Trp^{25}
fraction 78–110 (basic)	$Asp_{2.3}Thr_{1.8}Ser_{3.8}$ $Glu_{1.4}Gly_{1.1}Ala_{0.9}$ $Leu_{1.0}Tyr_{1.6}Phe_{0.8}$ $His_{0.8}Lys_{1.0}Arg_{2.5}$	192	His^1-Ala^{19}

ion-exchange chromatography was incomplete, but five ninhydrin-positive components were obtained, plus a sixth, more basic, substance that was not initially detected by its ninhydrin reactivity in the samples taken from the column effluent. Several of the column fractions were analysed by electrophoresis, and eight peptide products were observed (Fig. 4). These peptides were purified by preparative electrophoresis and paper chromatography, isolated, and their amino acid compositions determined (Table 1). Seven of the products were dipeptides that originated from the *C*-terminal portion of the glucagon molecule. The eighth peptide, the most basic product, consisted of the remainder of the glucagon molecule (residues 1–19). It should be noted that the overall electrophoretic pattern of products for this 7h reaction (Fig. 4) was the same as that for the 4h point of the previous experiment described above and in Fig. 2.

Order of release of dipeptides

The period of 4–7h required for the simultaneous appearance of both the first acidic and basic peptides could best be explained by removal of dipeptides in sequence from the *C*-terminal end of the glucagon molecule (see the Discussion section). This possible peptidyldipeptidase action of cathepsin B was verified by examining the rates of release of low-molecular-weight products from glucagon during the first 3h of reaction. Such peptides were separated from the large glucagon fragments by gel chromatography on Sephadex G-10. The low-molecular-weight fractions from each time point were combined, hydrolysed with acid and their total amino acid composition was determined (Fig. 5). Threonine and aspartic acid (from Asn^{28}) were released most rapidly at the same rate, followed by a slower release of leucine and methionine, again in parallel. The predicted sequential and quantitative pattern was not as obvious for the release of the four amino acids contained in the next two dipeptides at the *C*-terminal end of the glucagon molecule (Gln^{24}-Trp^{25} and Phe^{22}-Val^{23}). The rates of release of all four of these amino acids were about the same, which indicated that cathepsin B hydrolysed the Val^{23}-Gln^{24} bond much more

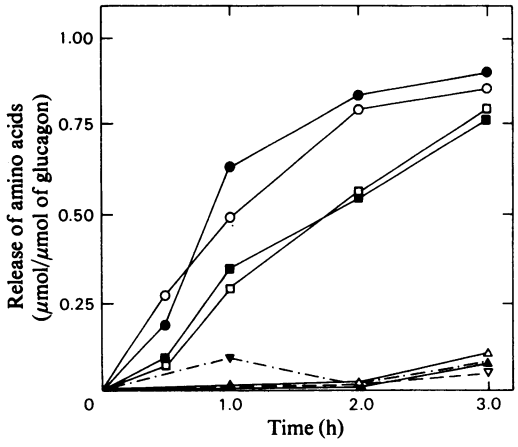

Fig. 5. *Rate of release of peptide amino acids from glucagon*
A sample (13.2mg) of glucagon was treated at 22°C with 70μg of human liver cathepsin B in 4.4ml of 0.4M-acetic acid containing 3.2mM-dimercaptopropanol. Portions (1.0ml) of the digest were removed at 0.5, 1, 2 and 3h, and the reaction was stopped by the addition of 10μl of 10mM-iodoacetic acid. Each sample was then separated into high- and low-molecular-weight fractions on a column (45cm × 1cm) of Sephadex G-10. The column was eluted with 0.2M-acetic acid and 0.8ml fractions were collected every 5min. Glucagon was eluted between fractions 12 and 16, whereas the low-molecular-weight peptide products (detected by ninhydrin reactivity) were eluted in fractions 20–25. These latter column fractions were combined, concentrated by evaporation at 40°C under reduced pressure and redissolved in 0.5–2ml water. Portions of each concentrated solution were then hydrolysed at 110°C and subjected to amino acid analysis by the procedures described in the text. ●, Asp; ○, Thr; ■, Met; □, Leu; △, Phe; ▲, Val; ·–▼–, Glu; –▽––, Trp.

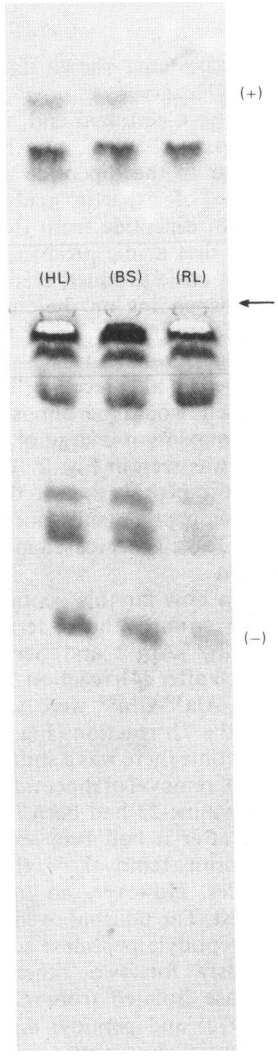

Fig. 6. *Digestion of glucagon by cathepsin B from different sources*
Glucagon was digested with human liver cathepsin B (HL), bovine spleen cathepsin B (BS) or rat liver cathepsin B (RL), under conditions described in the Experimental section. Samples (30μl) were analysed by high-voltage electrophoresis at pH6. The arrow indicates the origin and the anode is at the top.

slowly than the two bonds cleaved to give the first two dipeptide products.

Properties of the reaction between cathepsin B and glucagon

The reaction between glucagon and cathepsin B was further characterized to ensure that the unusual mechanism was not due to the action of a contaminating enzyme. The reaction was completely stopped in the presence of either 0.1mM-iodoacetic acid or 0.04mM-leupeptin, and an enzyme sample that had been exposed to pH8 for 1h did not degrade the hormone.

Glucagon also proved to be a very effective inhibitor of the hydrolysis of *N*-carbobenzoxy-arginylarginine 2-naphthylamide by the proteinase. This inhibition was not purely competitive in nature, but appeared to be of the mixed type when analysed by the graphic procedure of Cornish-Bowden (1974). The K_i was 30μM at pH4.5 (C. G. Knight, unpublished work). Finally, after causing glucagon to react for 24h, purified cathepsin B both from rat liver and bovine spleen produced exactly the same pattern of peptide fragments as did the human liver enzyme (Fig. 6).

Discussion

The present studies have shown that cathepsin B initially degrades glucagon by removing, in sequence, dipeptides from the *C*-terminal end of this peptide hormone. By this mechanism, no charged amino acid would appear in the dipeptide products until there was release of aspartic acid-21, which is present in the fifth dipeptide from the *C*-terminus. At that time the first acidic product, Gln^{20}-Asp^{21}, as well as the first basic product, His^{1}-Ala^{19}, would occur. At pH6, depending on the charged state of the imidazole nitrogen of His^{1}, this latter large fragment from glucagon would have a net charge of +1.5 to +2.0, whereas all preceding large products and glucagon itself would be almost neutral and would possess a maximum charge of +0.5 to +1.0. This exact result was seen in Fig. 2. The first acidic peptide(s) and basic peptide appeared after 4.0h of reaction. The basic peptide (subsequently shown to be residues 1–19; Table 1) decreased in concentration by 24h of reaction.

It is not known how far this peptidyldipeptidase action continued toward the *N*-terminus of the substrate, but Arg^{17}-Arg^{18} and Ser^{11}-Lys^{12} were among the products after 24h reaction. The dipeptides Asp^{21}-Phe^{22} and Ala^{19}-Gln^{20} were also significant products during the 7h reaction (Fig. 4). This indicated that at sometime there was a shift by one amino acid in the phase of removal of dipeptides. This would have occurred if valine-23 had been released as the free amino acid after it had been exposed on the *C*-terminus by prior removal of the first three terminal dipeptides. However, no free valine was found in this digest. The unusual overall mechanism of proteolysis, a peptidyldipeptidase action, has been reported previously for two other enzymes, a bacterial proteinase isolated from *Escherichia coli* (Yaron *et al.*, 1972) and peptidyl dipeptidase (EC 3.4.15.1, angiotensin I-converting enzyme) that removes the *C*-terminal His-Leu dipeptide from angiotensin I to form angiotensin II (see review by McDonald & Schwabe, 1977). However, the properties of these enzymes are very different from those of cathepsin B.

No other enzyme, including chymotrypsin and trypsin (Bromer *et al.*, 1957*a*,*b*), subtilisin (Sinn *et al.*, 1957), papain (Desmazeaud, 1972), cathepsin B2 (Afroz *et al.*, 1976) and cathepsin A (Matsuda, 1976), has been found to hydrolyse glucagon in this way. However, an important question still to be answered is what contribution does the substrate glucagon provide in regulating its own pattern of digestion by cathepsin B? The question arises because we have found no other protein substrate to be degraded in the same manner by human liver cathepsin B (N. N. Aronson, unpublished work). Many of the potential substrates that were tried were not hydrolysed at all under the conditions used for glucagon; these included the oxidized B-chain of bovine insulin, lysozyme, protamine sulphate and sperm-whale myoglobin. Cathepsin B is also known to degrade many proteins such as collagen (Burleigh *et al.*, 1974), proteoglycan (Roughley & Barrett, 1977) and myosin (Schwartz & Bird, 1977) by an endoproteolytic action.

Chou & Fasman (1975) have calculated that glucagon has a complex tertiary structure, especially at the portion of the molecule that is being attacked by cathepsin B. They have predicted that, depending on the environment, two different conformations exist: one in which residues Ala^{19}-Met^{27} are in an α-helix and another in which these same residues assume a β-sheet configuration. At the concentration of glucagon and acidic pH used in our studies it is known that the molecule can form fibrous gels (Staub *et al.*, 1955). However, reactions with cathepsin B performed at pH6 and in much more dilute solutions of substrate (0.18mg/ml) yielded the same products as digests at pH3 and high glucagon concentration, although the reaction was much slower (N. N. Aronson, unpublished work). The maximum rate for glucagon hydrolysis by cathepsin B (as measured by the increase in ninhydrin-reactive residues was between pH3 and 4, a result very similar to that reported for the release of acid-soluble radioactive products from ^{125}I-labelled glucagon by rat liver cathepsin B (Kirschke *et al.*, 1976).

Some low-molecular-weight peptides were tried as substrates for human liver cathepsin B. The two tripeptides, Gly-Gly-Val and Glu-Gly-Phe, were not hydrolysed, whereas *N*-carbobenzoxy-Phe-His-Leu, a substrate commonly used to measure angiotensin I-converting enzyme, was slowly cleaved to yield free leucine (N. N. Aronson, unpublished work).

Studies have shown that at least three thiol proteinases, cathepsins B, H and L (Kirschke *et al.*, 1976, 1977), are present in lysosomes from rat liver and that these enzymes have rather similar properties. We suggest that this novel reaction of cathepsin B with glucagon could be used as a definitive property of the enzyme to distinguish it from the other lysosomal proteolytic activities.

This research was done while N. N. A. was on leave from The Pennsylvania State University as a Research Fellow of the British Heart Foundation and the American Heart Association. We thank Dr. C. G. Knight for his contribution to kinetic studies, Dr. W. N. Schwartz for his help with studies on cathepsin B from other tissues and species, Professor K. Otto for generously supplying a sample of bovine spleen cathepsin B and Dr. G. M. Herring for performing the many amino acid analyses important to this research. This work was also supported by the Medical Research Council and grant AM-15465 from the National Institute of Arthritis, Metabolism and

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