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Skeletal muscle and liver contain a soluble ATP + ubiquitin-dependent proteolytic system

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Although protein breakdown in most cells seems to require metabolic energy, it has only been possible to establish a soluble ATP-dependent proteolytic system in extracts of reticulocytes and erythroleukaemia cells. We have now succeeded in demonstrating in soluble extracts and more purified preparations from rabbit skeletal muscle a 12-fold stimulation by ATP of breakdown of endogenous proteins and a 6-fold stimulation of ^{125}I-lysozyme degradation. However, it has still not been possible to demonstrate such large effects of ATP in similar preparations from liver. Nevertheless, after fractionation by DEAE-chromatography and gel filtration, we found that extracts from liver as well as muscle contain both the enzymes which conjugate ubiquitin to ^{125}I-lysozyme and an enzyme which specifically degrades the ubiquitin–protein conjugates. When this proteolytic activity was recombined with the conjugating enzymes, ATP + ubiquitin-dependent degradation of many proteins was observed. This proteinase is unusually large, approx. 1500 kDa, requires ATP hydrolysis for activity and resembles the ubiquitin–protein-conjugate degrading activity isolated from reticulocytes. Thus the ATP + ubiquitin-dependent pathway is likely to be present in all mammalian cells, although certain tissues may contain inhibitory factors.

INTRODUCTION

In mammalian and bacterial cells, blocking ATP production leads to a marked inhibition of intracellular protein breakdown (Simpson, 1953; Goldberg & St. John, 1976; Hershko & Ciechanover, 1982). Other studies have shown that ATP is essential not only for lysosomal proteolysis but also for soluble non-lysosomal pathways. An ATP-dependent pathway seems responsible for the degradation of short-lived enzymes (Hershko & Tomkins, 1971; Kulka & Cohen, 1973), long-lived proteins in growing fibroblasts (Gronostajski *et al.*, 1985), various proteins during maturation of reticulocytes into erythrocytes (Muller *et al.*, 1980; Boches & Goldberg, 1982; Speiser & Etlinger, 1982; Magnani *et al.*, 1986), and highly abnormal proteins in reticulocytes (Etlinger & Goldberg, 1977; Hershko *et al.*, 1978) and other cells (Ciechanover *et al.*, 1984).

Important progress in understanding this energy requirement was made possible by the development of a soluble reticulocyte preparation that shows a clear ATP requirement for proteolysis (Etlinger & Goldberg, 1977). In these preparations, ATP serves at least two distinct functions. It is essential both for the covalent attachment of ubiquitin (Ub) to amino groups on protein substrates (Ciechanover *et al.*, 1978; Hershko *et al.*, 1980; Hershko & Ciechanover, 1982) and also for the subsequent breakdown of the proteins conjugated to Ub (Hershko *et al.*, 1984). More recently, a proteinase has been partially purified from reticulocyte extracts that hydrolyses Ub–protein conjugates (Hough *et al.*, 1986; Waxman *et al.*, 1986, 1987). This enzyme is unusually large (M_r 1500000) (Waxman *et al.*, 1987) and requires ATP for function. The hydrolysis of some proteins that cannot be conjugated to Ub (Tanaka *et al.*, 1983, 1984; Katznelson & Kulka, 1985; Chin *et al.*, 1986) has also been reported to require ATP, but the enzymes involved in this process have not been identified.

ATP-dependent proteinases which directly utilize ATP but which do not require Ub have been isolated from bacteria (Chung & Goldberg, 1981; Charette *et al.*, 1981; Larimore *et al.*, 1982) and mammalian mitochondria (Desautels & Goldberg, 1982; Watabe & Kimura, 1985). Similar ATP-dependent proteinases have not been purified from the cytoplasm of mammalian cells, although murine erythroleukaemia cells seem to contain a similar Ub-independent activity (Waxman *et al.*, 1985). A variety of non-lysosomal proteinases have been found in liver and muscle, but none have been proved to play a role in this soluble ATP-dependent proteolytic pathway. Mammalian tissues, including muscle, liver and erythrocytes, do contain a high-M_r (approx. 650000–700000) multifunctional proteinase (DeMartino & Goldberg, 1979; Rose *et al.*, 1979; Boches *et al.*, 1980; Wilk & Orlowski, 1980; Hardy *et al.*, 1981; DeMartino, 1983; Dahlmann *et al.*, 1983, 1985; L. Waxman, K. Tanaka & A. L. Goldberg, unpublished work), which after purification can be stimulated 2-fold by ATP (DeMartino & Goldberg, 1979; Boches *et al.*, 1980; DeMartino, 1983) without the involvement of Ub. However, unlike the bacterial and mitochondrial enzymes, it does not require Mg^{2+} or ATP hydrolysis for activity.

Soluble ATP-dependent proteolytic systems have not been obtained from other mammalian cells, other than reticulocytes and murine erythroleukaemia cells, a precursor of reticulocytes (Waxman *et al.*, 1985; Rieder

Abbreviations used: Ub, ubiquitin; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; Cbz, benzyloxycarbonyl; DCIC, 3,4-dichloroisocoumarin; DTT, dithiothreitol; MNA, methoxynaphthylamine; CF, Conjugating Fraction.

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et al., 1985). In such cells, these proteolytic systems may be particularly active in order to degrade various soluble and organellar proteins during cell maturation into erythrocytes. However, studies with mutants of fibroblasts indicate an important role for the ATP+Ub-dependent proteolytic pathway in other nucleated cells (Ciechanover *et al.*, 1984; Finley *et al.*, 1984). It is therefore unclear why extracts with similar properties have not been obtained from other tissues, such as liver, where the degradation of various enzymes is known to require ATP (Hershko & Tomkins, 1971; Milman *et al.*, 1975; Goldberg & St. John, 1976). Possibly, such tissues contain regulatory factors, inhibitors or additional proteinases that mask or inactivate the ATP-dependent proteolytic system. The demonstration of this pathway in liver and muscle extracts would be of particular biochemical importance since most work on the turnover of specific enzymes has been carried out in liver cells and the physiological regulation of protein breakdown has been extensively studied in skeletal muscle.

EXPERIMENTAL

Reagents

N-Ethylmaleimide (NEM), ATP, yeast hexokinase, adenosine 5′-[$\beta\gamma$-imido]triphosphate, ADP, haemin, heparin, poly(Glu-Tyr) (in a 1:1 molar ratio), α-casein, yeast cytochrome *c* and soya-bean trypsin inhibitor were purchased from Sigma. RNAase was obtained from Worthington, and bovine serum albumin (BSA) and egg-white lysozyme were purchased from Miles. [^{3}H]Formaldehyde was obtained from New England Nuclear and Na^{125}I from Amersham. Cbz-Gly-Leu-Phe-CH_2Cl and 3,4-dichloroisocoumarin (DCIC) were gifts from Dr. J. Powers (Georgia Institute of Technology). Phe-Ala-Arg-CH_2Cl was provided by Dr. E. Shaw (Brookhaven National Laboratory) and egg-white cystatin by Dr. V. Turk (J. Stefan Institute, Ljubljana, Yugoslavia). The proteinase inhibitors chymostatin and leupeptin were generously given by Dr. H. Umezawa (Microbial Chemistry Research Foundation, Tokyo, Japan), and Ep-475 (E-64-C) [L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(3-methyl)butane] was kindly provided by Dr. K. Hanada (Taisho Pharmaceutical Co., Saitama, Japan). Ubiquitin was purified from human erythrocytes (Ciechanover *et al.*, 1982; Wilkinson *et al.*, 1980) and globin was prepared from haemoglobin (Guidotti, 1967). Decavanadate was synthesized from V_2O_5 (Alfa Products, Danvers, MA, U.S.A.) (Vijaya *et al.*, 1984). ^{125}I-BSA, ^{125}I-lysozyme ^{125}I-RNAase and ^{125}I-α-casein were prepared by using chloramine-T (Moore *et al.*, 1977). [^{3}H]Globin, [^{3}H]soya-bean trypsin inhibitor and [^{3}H]cytochrome *c* were prepared by reductive methylation (Rice & Means, 1971).

Preparation of extracts

Male New Zealand White rabbits (3–4 kg) (Millbrook Farms, MA, U.S.A.) were killed by asphyxiation with CO_2, and the liver and psoas muscles were rapidly excised. The liver was perfused with 0.9% NaCl and minced; the muscles were trimmed of fat and connective tissue, and then ground on a prechilled meat grinder. Approx. 200 g of muscle or 100 g of liver (wet wt.) were suspended in 3 ml (per g of tissue) of ice-cold buffer containing 5 mM-Tris (pH 8), 1 mM-2-mercaptoethanol, 1% glycerol, 1 mM-EDTA and 1 mM-EGTA, and homogenized in a 1-litre Waring blender for 1 min at top speed. Chymostatin and Ep-475 (final concns. 50 μM) were added during extraction, to prevent the possible proteolytic inactivation of Ub (Haas *et al.*, 1985). After the pH was adjusted to 7.0, homogenates were centrifuged at 30000 *g* for 30 min. Crude extracts were prepared by centrifugation of the supernatants at 100000 *g* for 45 min, and glycerol was added with stirring to 20% (final concn.). Subsequent steps were carried out at 4 °C.

Muscle and liver extracts were fractionated by DEAE-cellulose (Whatman DE-52) chromatography as described previously for reticulocyte extracts (Hershko *et al.*, 1983; Tanaka *et al.*, 1983). The bound material ('Fraction II'), which contains most of the proteolytic activity and the enzymes required for attachment of Ub to proteins, was eluted with 0.5 M-NaCl, and dialysed overnight against 20 mM-Tris/HCl (pH 7.6)/0.5 mM-dithiothreitol (DTT)/1 mM-magnesium acetate/0.1 mM-EDTA/20 mM-KCl/20% glycerol. We recovered in Fraction II 6–8% of the protein in the high-speed extract from muscle and 20–25% of the protein in the high-speed supernatant from liver of that applied to a 30 ml DEAE-cellulose column. The material from muscle and liver which did not bind to DEAE-cellulose ('Fraction I') was boiled and concentrated on CM-cellulose (Ciechanover *et al.*, 1982). Both muscle and liver Fraction I were shown to contain Ub, on the basis of their ability to stimulate ATP-dependent proteolysis in reticulocyte Fraction II (results not shown).

Assays

Protein content was measured by the procedure of Lowry *et al.* (1951), with BSA as the standard. Proteolytic activity with ^{125}I-lysozyme or other radiolabelled substrates was measured as described previously for reticulocyte extracts (Tanaka *et al.*, 1983, 1984). Generally, 200–400 μg of soluble protein was incubated in a volume of 0.2–0.6 ml in buffer containing 50 mM-Tris (pH 8), 10 mM-magnesium acetate, 1 mM-DTT and radiolabelled substrate, in the presence or absence of 5 mM-ATP and 7.5–12.5 μg of Ub. After 1–2 h at 37 °C, the reaction was stopped by the addition of trichloroacetic acid (7% final concn.), and the acid-soluble radioactivity was determined by γ-radiation counting or liquid-scintillation counting. The breakdown of endogenous cell proteins was estimated by the net appearance of free tyrosine. Since this amino acid can be neither synthesized nor degraded in skeletal muscle (Fulks *et al.*, 1975), its appearance in dialysed preparations of 100000 *g*-extract supernatants and Fraction II from muscle must reflect protein degradation. Tyrosine appearance is probably also a valid measure of protein breakdown in dialysed liver extracts and Fraction II, since, after dialysis, these preparations should show little or no capacity for tyrosine degradation or synthesis. Hydrolysis of the fluorimetric substrate Cbz-Ala-Arg-Arg-methoxynaphthylamine (MNA) (Enzyme Systems Products, Livermore, CA, U.S.A.) was determined as described previously (Waxman & Goldberg, 1985). ATP hydrolysis in tissue extracts was estimated by measuring the release of P_i by a colorimetric assay (Waxman & Goldberg, 1982).

All assays were linear with time. The data shown in the Figures and Tables were obtained within each experiment and are the averages of duplicate or triplicate deter-

minations which agreed within 5%. Experiments were repeated two or more times, and good agreement between experiments was found for the chromatographic and enzymic properties of the proteolytic and fluorimetric activities under investigation. However, the specific activity of the degradative system has been highly variable from preparation to preparation, probably owing to the instability of the various components of the ATP-dependent pathway.

RESULTS

ATP-stimulated proteolysis in liver and muscle extracts

In dialysed reticulocyte extracts, ATP stimulates the degradation of ^{125}I-lysozyme 5–10-fold (Fagan *et al.*, 1986). After DEAE-cellulose chromatography of these extracts to remove Ub and haemoglobin (i.e. in 'Fraction II' as designated by Ciechanover *et al.*, 1978), ATP stimulates ^{125}I-lysozyme degradation about 2-fold, but when Ub is added with the ATP a further 2–5-fold increase in proteolysis is seen (Tanaka *et al.*, 1983).

In analogous experiments with crude extracts (100000 *g* supernatant) of muscle or liver, the degradation of exogenous radiolabelled substrates was stimulated at most 2-fold by ATP (Table 1; Etlinger *et al.*, 1981; Haas *et al.*, 1985). However, when the breakdown of endogenous soluble proteins was measured in these muscle extracts, ATP stimulated their degradation 12-fold (Table 1). Subsequent DEAE-cellulose chromatography of muscle to remove Ub allowed a large purification of this proteolytic system. The bound fraction (i.e. Fraction II) contained only 5–10% of the applied protein, but showed a 9-fold increase in specific activity against endogenous proteins and a 6-fold increase against ^{125}I-lysozyme. After this chromatographic step, ^{125}I-lysozyme degradation in the eluted fraction was stimulated approx. 6-fold and tyrosine release from endogenous proteins was stimulated more than 13-fold when ATP and Ub were added together (Table 1).

In liver extracts (100000 *g* supernatant), no release of tyrosine from endogenous proteins was seen with or without ATP. When liver extracts were fractionated similarly on DEAE-cellulose to remove free Ub, approx. 75–85% of the protein in the 100000 *g* supernatant was also removed. In these preparations, unlike those from reticulocytes or muscle, ^{125}I-lysozyme degradation and tyrosine release from endogenous protein were still stimulated less than 2-fold by the addition of ATP and Ub (Table 1). Several possible explanations for the low amount of ATP-stimulated proteolysis in the liver extracts were tested. It is unlikely that these results are due to the artifactual inactivation of Ub by cathepsin B-like proteinases, as was proposed by Haas *et al.* (1985), or to the oxidation of essential thiol groups by heavy metals, since both muscle and liver were homogenized in the presence of the proteinase inhibitors chymostatin and Ep-475 and the metal chelators EDTA and EGTA. The possibility also existed that liver extracts rapidly hydrolysed the added ATP, since these assays did not utilize an ATP-regenerating system. We therefore measured the ATPase activity of the muscle and liver extracts under standard incubation conditions (as in Table 1). These extracts (400 μg) hydrolysed ATP more slowly than did 50 μl of reticulocyte extract (results not shown), where ATP-stimulated proteolysis is linear for several hours (Tanaka *et al.*, 1983). Thus the lack of an ATP stimulation of proteolysis in liver extracts is not due to rapid depletion of ATP.

Table 1. Effect of ATP on protein breakdown in crude extracts and Fraction II from skeletal muscle and liver

Portions (400 μg) of crude extract or of Fraction II from skeletal muscle or liver were incubated with 5 μg of ^{125}I-lysozyme (30000 c.p.m.) for 1 h at 37 °C in buffer containing 50 mM-Tris/HCl (pH 8), 10 mM-magnesium acetate and 1 mM-DTT, in the presence or absence of 5 mM-ATP and 10 μg of Ub. To measure tyrosine release from endogenous proteins, 5 mg of crude extract or 5 mg of Fraction II from skeletal muscle or liver was incubated for 1.5 h at 37 °C in the above buffer. When proteolysis in Fraction II from muscle was measured, ATP alone stimulated the breakdown of ^{125}I-lysozyme approx. 3–4-fold (results not shown). However, free Ub may be present, owing to the release from endogenous proteins by isopeptidase activity (Hershko, 1983).

	^{125}I-lysozyme degradation (ng/h)		Tyrosine release from endogenous proteins (nmol/1.5 h)	
	−ATP	+ATP	−ATP	+ATP
Crude extract				
Muscle	92	203	0.1	1.2
Liver	72	138	0	0
	−ATP	+ATP +Ub	−ATP	+ATP +Ub
Fraction II				
Muscle	130	835	0.8	10.8
Liver	42	77	1.5	2.3

Partial purification of an ATP+Ub-dependent proteolytic system

In reticulocyte extracts, we have identified two high-M_r proteinases which are co-eluted on DEAE-cellulose (Waxman *et al.*, 1986, 1987). One of these has properties characteristic of the M_r-670000 multifunctional proteinase previously described in erythrocytes and a variety of mammalian tissues (DeMartino & Goldberg, 1979; Boches *et al.*, 1980; Wilk & Orlowski, 1980, 1983; Hardy *et al.*, 1981; DeMartino, 1983; Dahlmann *et al.*, 1985). The other enzymic activity, which was termed 'ubiquitin-conjugate-degrading enzyme' or 'UCDEN' (Waxman *et al.*, 1987), was much larger (Waxman *et al.*, 1987), required ATP, and only degraded proteins that were conjugated to Ub (Waxman *et al.*, 1986, 1987; Hough *et al.*, 1986). To determine whether muscle and liver contained a similar ATP+Ub-dependent proteinase, Fraction II prepared from these tissues was subjected to gel filtration on Sephacryl S-300. As seen previously with analogous preparations from reticulocytes (Waxman *et al.*, 1986, 1987), individual column fractions had little or no proteolytic activity against ^{125}I-lysozyme (Figs. 1*a* and 1*b*). In the fractions containing liver proteins with M_r values greater than 450000, ATP stimulated ^{125}I-lysozyme degradation 2-fold (Fig. 1*b*), but the addition

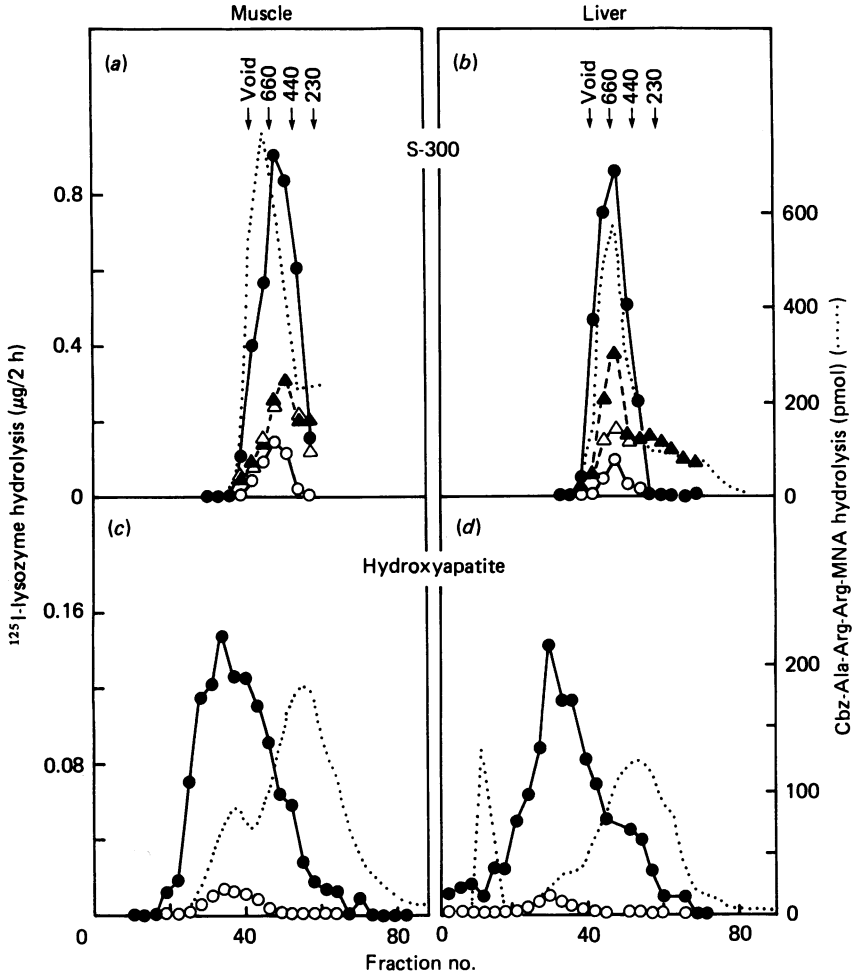

Fig. 1. Gel filtration and hydroxyapatite chromatography of an ATP+Ub-dependent proteinase from muscle and liver

Samples of muscle Fraction II (125 mg; *a*) and of liver Fraction II (150 mg; *b*) were applied directly to a column of Sephacryl S-300 (1.5 cm × 62 cm) equilibrated in 20 mM-Tris/HCl (pH 7.6)/20 mM-NaCl/1 mM-magnesium acetate/0.1 mM-EDTA/0.5 mM-DTT/20% glycerol. Fractions (2 ml) were collected, and 25 μl portions were assayed for the presence of the latent high-M_r proteinase (Waxman *et al.*, 1987) with Cbz-Ala-Arg-Arg-MNA for 15 min (*a*) or 30 min (*b*) at 37 °C. Portions (*a*, 75 μl; *b*, 200 μl) were assayed for the degradation of ^{125}I-lysozyme either with or without 5 mM-ATP present, or with 5 mM-ATP and 7.5 μg of Ub in the presence or absence of 300 μg of muscle CF. After 2 h at 37 °C, the reaction was stopped by the addition of trichloroacetic acid (7% final concn.) and the acid-soluble radioactivity was determined. Thyroglobulin (M_r 660000), ferritin (440000), catalase (230000), aldolase (158000), albumin (67000), ovalbumin (43000) and chymotrypsinogen (25000) were used to calibrate the column [indicated by arrows, with values ($\times 10^{-3}$)]. The muscle and liver fractions (42–54) which contained ATP+Ub-dependent proteolytic activity from the Sephacryl S-300 were pooled and applied to a column of hydroxyapatite (1.5 cm × 9 cm) equilibrated in the same buffer. Bound proteins were eluted with a linear gradient (total 200 ml) of 0–0.25 M-KH_2PO_4 (pH 7.6); 2 ml fractions were collected, and 25 μl portions were assayed for the hydrolysis of Cbz-Ala-Arg-Arg-MNA for 30 min at 37 °C. Portions of muscle (*c*; 120 μl) and of liver (*d*; 250 μl) column fractions were assayed for proteolytic activity as above with ^{125}I-lysozyme as substrate without any addition or in the presence of 5 mM-ATP, 7.5 μg of Ub and 300 μg of muscle CF for 2 h at 37 °C. The release of acid-soluble radioactivity was linear for 2 h. Key: ●, +CF+ATP+Ub; ○, +CF, no ATP; ▲; +ATP; △, no ATP. 'Void' indicates void volume, determined with Blue Dextran.

of Ub with the ATP had no further stimulatory effect (results not shown). The low proteolytic activity that was stimulated 2-fold by ATP was probably due to the presence of the M_r-670000 multifunctional proteinase (DeMartino & Goldberg, 1979), which we identified (Fig. 1*b*) by its ability to hydrolyse the fluorimetric peptide Cbz-Ala-Arg-Arg-MNA (Tanaka *et al.*, 1986; L. Waxman, K. Tanaka & A. L. Goldberg, unpublished work).

These high-M_r fractions should lack the enzymes that conjugate Ub to proteins. To test if muscle and liver contain these activities, the lower-M_r proteins (30000–300000) from the Sephacryl S-300 column were pooled and concentrated 5-fold by ultrafiltration in an Amicon pressure cell. The pooled material from muscle and liver as well as the crude extracts and Fraction II from these tissues all conjugated Ub to ^{125}I-lysozyme (Fig. 2) in a similar fashion to analogous preparations from reticulocytes (Waxman *et al.*, 1986, 1987). Therefore this low-M_r fraction is referred to as the Conjugating Fraction (CF). When ATP was present, liver and muscle CF linked multiple Ub moieties with ^{125}I-lysozyme to produce a number of higher-M_r bands, as shown by SDS/polyacrylamide-gel electrophoresis analysis and autoradiography (Fig. 2). Moreover, in accord with

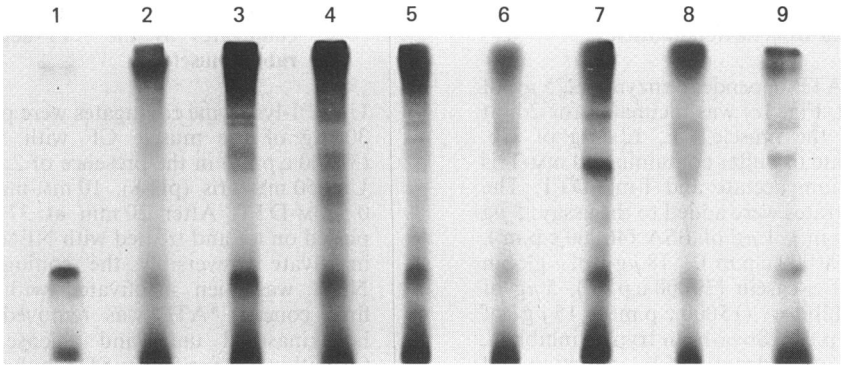

Fig. 2. Demonstration of Ub conjugation to ^{125}I-lysozyme by extracts of liver, muscle and reticulocytes

The ability of various fractions from liver, muscle and reticulocytes to conjugate Ub to ^{125}I-lysozyme was determined by incubating (in a total volume of 80 μl) 300 μg of liver or muscle crude extract, Fraction II or CF, or 150 μg of reticulocyte Fraction II or CF, with 50 mM-Tris/HCl (pH 8), 10 mM-magnesium acetate, 2 mM-DTT, 75 μM-haemin (to block degradation of the conjugates), 5 mM-ATP, 10 μg of Ub and 3 μg of ^{125}I-lysoyzme (120000 c.p.m.). After 1 h at 37 °C, the mixtures were prepared for SDS/polyacrylamide-gel electrophoresis on 10%-polyacrylamide gels (Laemmli, 1970). After electrophoresis, the gels were fixed and dried and autoradiographed for 2 days at room temperature with Kodak X-Omat AR film and an intensifying screen. Lanes: 1, ^{125}I-lysozyme without incubation; 2, liver crude extract; 3, liver Fraction II; 4, liver CF, 5, reticulocyte Fraction II; 6, reticulocyte CF; 7, muscle crude extract; 8, muscle Fraction II; 9, muscle CF. The direction of electrophoresis in the Figure was from top to bottom.

observations by Hough & Rechsteiner (1986) with reticulocyte extracts, a significant fraction of the conjugates formed by liver and muscle extracts was of very high M_r and had low mobility on SDS/polyacrylamide-gel electrophoresis. In reticulocytes, such aggregates have been shown to be the preferred substrates for an ATP-requiring proteinase (Hershko *et al.*, 1984; Hough *et al.*, 1986).

The CF showed little or no proteolytic activity when assayed with or without ATP or Ub. Addition of the CF to the muscle and liver fractions containing proteins larger than M_r 450000 increased the degradation of ^{125}I-lysozyme more than 6-fold, provided that ATP and Ub were present (Fig. 1). Thus, like reticulocytes (Waxman *et al.*, 1986, 1987), muscle and liver contain the ATP+Ub-dependent pathway, although in liver this system appears latent and the components must be isolated and reconstituted for ATP-dependent proteolysis to be demonstrated.

These tissues seem to contain two distinct high-M_r proteinases, an ATP+Ub-dependent activity as well as the multifunctional proteinase that degrades proteins and peptides. Both proteolytic activities were eluted from Sephacryl S-300 at approx. M_r 650000–700000, based on calibration of the column with thyroglobulin and ferritin (Figs. 1*a* and 1*b*). However, this matrix cannot resolve polypeptides of M_r greater than 600000. These two proteinases from muscle could be resolved from each other when the proteins that were eluted at M_r 450000 or greater on Sephacryl S-300 were pooled and chromatographed on Sepharose 6B. The multifunctional proteinase was eluted with thyroglobulin at approx. M_r 670000, and the Ub-conjugate-degrading activity was eluted ahead of phosphorylase kinase (M_r 1280000), and had an approximate M_r of 1500000. Similar values were found for the sizes of these two enzymes in reticulocytes (Waxman *et al.*, 1987).

The muscle and liver multifunctional proteinase could also be separated from the Ub-conjugate-degrading activity by chromatography on hydroxyapatite (Figs. 1*c* and 1*d*). For both the muscle and liver enzymes, the peak of ATP+Ub-dependent activity was eluted at approx. 50 mM-phosphate, whereas the major peak of Cbz-Ala-Arg-Arg-MNA-hydrolysing activity (i.e. the multifunctional proteinase) was eluted at approx. 110 mM-phosphate (Figs. 1*c* and 1*d*). Thus the ATP+Ub-dependent pathway is clearly present in both muscle and liver, and does not require the high-M_r multifunctional proteinase.

Breakdown of proteins by the ATP+Ub-dependent system from muscle

In addition to the degradation of endogenous muscle proteins (Table 1) and ^{125}I-lysozyme (Table 1, Fig. 1), the reconstituted mixtures of the CF and the ATP+Ub-dependent proteolytic fraction pooled from the hydroxyapatite column degraded a variety of proteins in an ATP+Ub-dependent fashion. The magnitude of the stimulation of protein breakdown by ATP depended on the substrate (Table 2). For several proteins there was very little or no degradation in the absence of ATP. In the presence of ATP, lysozyme and denatured soya-bean trypsin inhibitor are very rapidly hydrolysed to acid-soluble fragments by the partially purified system from muscle, whereas denatured BSA, a much larger protein, is hydrolysed much more slowly (Table 2). α-Casein, globin and cytochrome *c* were also rapidly hydrolysed in the presence of ATP, but these substrates also were hydrolysed at significant rates in the absence of ATP. The low amount of ATP-independent proteolytic activity may be due to an unidentified enzyme, since the multifunctional proteinase had been removed by chromatography on hydroxyapatite. In these preparations, breakdown of denatured RNAase was primarily ATP-independent. ^{125}I-lysozyme appears to be the most useful substrate for routine studies of the proteolytic system from muscle, since its degradation was particularly rapid and completely dependent on ATP.

Table 2. Degradation of various proteins by an ATP-dependent proteolytic system from skeletal muscle

The partially purified ATP-dependent enzyme (225 μg of pooled fractions 23–39, Fig. 1*c*) was incubated for 2 h at 37 °C with 300 μg of the muscle CF, 12.5 μg of Ub, 5 mM-ATP, and substrate in buffer containing 50 mM-Tris (pH 8), 10 mM-magnesium acetate and 1 mM-DTT. The following labelled substrates were added to the assay: 5 μg of lysozyme (30000 c.p.m.), 1 μg of BSA (46000 c.p.m.), 1 μg of RNAase (93000 c.p.m.), 18 μg of globin (44000 c.p.m.), 5 μg of α-casein (30000 c.p.m.), 5 μg of soya-bean trypsin inhibitor (15000 c.p.m.), 15 μg of cytochrome *c* (60000 c.p.m.). Soya-bean trypsin inhibitor, cytochrome *c* and BSA were denatured by reduction and alkylation, and RNAase was oxidized with performic acid.

Substrate	Proteolysis (ng/h) −ATP	Proteolysis (ng/h) +ATP +Ub
^{125}I-lysozyme	0	590
[^{3}H]Soya-bean trypsin inhibitor	3	337
^{125}I-BSA	1	20
^{125}I-α-casein	25	393
[^{3}H]Globin	34	377
[^{3}H]Cytochrome *c*	49	163
^{125}I-RNAase	60	76

Table 3. Effect of ATP on degradation of Ub–lysozyme conjugates by the ATP-dependent proteinase from rabbit muscle

Ub–^{125}I-lysozyme conjugates were prepared by incubating 300 μg of the muscle CF with 5 μg of ^{125}I-lysozyme (30000 c.p.m.) in the presence of 2.5 mM-ATP, 12.5 μg of Ub, 50 mM-Tris (pH 8), 10 mM-magnesium acetate and 0.5 mM-DTT. After 30 min at 37 °C, the mixture was placed on ice and treated with NEM (2 mM) for 15 min to inactivate irreversibly the conjugating enzymes. Free NEM was then inactivated with excess DTT (4 mM final concn). *ATP was removed by the addition of hexokinase (1 unit) and glucose (10 mM). Conjugate formation was verified by SDS/polyacrylamide-gel electrophoresis of the incubation mixtures as described in Fig. 2. The gels were then autoradiographed to check for formation of high-M_r complexes. Samples (225 μg) of the partially purified ATP-dependent enzyme from muscle (fractions 23–39, Fig. 1*b*) were then incubated as shown for 2 h at 37 °C. ^{125}I-lysozyme alone or in the presence of the CF without ATP+Ub was not degraded.

Additions	^{125}I-lysozyme degradation (c.p.m./2 h)	^{125}I-lysozyme degradation (% of control)
ATP (5 mM)	2202	100
No ATP*	163	7
No ATP* + adenosine 5′-[βγ-imido]triphosphate (5 mM)	0	0

Degradation of Ub–protein conjugates

Additional experiments demonstrated directly that the muscle enzyme which degraded ^{125}I-lysozyme in the presence of CF, Ub and ATP was in fact an ATP-requiring proteinase capable of degrading Ub–protein conjugates. The active fractions eluted from the hydroxyapatite column were pooled and incubated with Ub–protein conjugates. The conjugates were formed by incubating ^{125}I-lysozyme, ATP and Ub for 30 min at 37 °C. In the control samples (in the absence of ATP), this reaction was stopped by the addition of NEM, and then hexokinase and glucose were added to metabolize the ATP. As shown in Table 3, subsequent addition of ATP greatly stimulated the degradation of ^{125}I-lysozyme–Ub conjugates to acid-soluble products. The removal of added ATP by addition of hexokinase and glucose prevented the breakdown of the lysozyme–Ub conjugates. The non-hydrolysable ATP analogue adenosine 5′-[βγ-imido]triphosphate was ineffective in stimulating the degradation of ^{125}I-lysozyme–Ub conjugates. Thus we have isolated from mammalian tissues, by chromatography on DEAE-cellulose, Sephacryl and hydroxyapatite, a proteinase which requires ATP hydrolysis to degrade Ub–protein conjugates.

Sensitivity of the ATP+Ub-dependent proteolytic activity to inhibitors

To characterize further the ATP-dependent proteolytic activity from muscle, the effects of potential inhibitors were tested on the pooled fractions from the hydroxyapatite column. NEM, a thiol-blocking reagent, and DCIC, an irreversible inhibitor of serine proteinases (Harper *et al.*, 1985), inhibited nearly completely the ATP+Ub-dependent proteolytic activity. Both these reagents were also found to inhibit the ATP-dependent pathway in reticulocyte lysates (Fagan *et al.*, 1986) and the ATP+Ub-dependent proteinase isolated from reticulocytes (Waxman *et al.*, 1986). In these experiments, the muscle Ub-conjugate-degrading enzyme was incubated with the inhibitor, and then the excess NEM and DCIC were inactivated by the addition of DTT (Harper *et al.*, 1985) before mixing with the CF. Therefore, under these conditions, these reagents blocked degradation by inhibiting the ATP-dependent proteinase rather than by affecting the conjugation of Ub to ^{125}I-lysozyme.

Decavanadate, which inhibits several ATP-dependent enzymes (DeMaster & Mitchell, 1973; Choate & Mansour, 1979), and certain peptide chloromethyl ketones, which inactivate chymotrypsin (Cbz-Gly-Leu-Phe-CH_2Cl) (Kurachi *et al.*, 1973) or trypsin (Phe-Ala-Arg-CH_2Cl) (Kettner & Shaw, 1981), can block both the ATP-dependent degradative pathway and the Ub-conjugate-degrading activity in reticulocyte lysates (Waxman *et al.*, 1986, 1987). The ATP+Ub-dependent proteinase from muscle was inhibited by decavanadate and by these peptide chloromethyl ketones (Table 4).

Haemin blocks completely ATP-dependent proteolysis in reticulocyte lysates (Etlinger & Goldberg, 1980), but does not affect the conjugation of Ub to proteins (Haas & Rose, 1981; Tanaka *et al.*, 1984). Similarly, haemin inhibits completely the ATP+Ub-dependent proteolytic activity isolated from muscle (Table 4), like that from reticulocytes (Waxman *et al.*, 1986, 1987). The polyanions poly(Glu-Tyr) (Table 4) and poly(Glu-Ala-Tyr) were also found to be potent inhibitors of this process in muscle (results not shown) and in reticulocytes (Waxman *et al.*, 1986) and also of the ATP-independent degradation of oxidant-treated haemoglobin in reticulocyte

Table 4. Effect of inhibitors on ATP+Ub-dependent proteolysis and Ub conjugation in rabbit muscle

Partially purified ATP-dependent enzyme (225 μg) was preincubated at 18 °C in the presence of inhibitor, 50 mM-Tris (pH 8) and 10 mM-magnesium acetate. After 30 min, DTT was added (final concn. 2 mM). Then 300 μg of the CF and ^{125}I-lysozyme (5 μg; 30000 c.p.m.) were added, with or without ATP (5 mM) and Ub (10 μg). The mixtures were then incubated for 90 min at 37 °C. To determine whether each of the inhibitors tested blocked Ub conjugation to ^{125}I-lysozyme, each inhibitor was pre-treated with 2 mM-DTT in 50 mM-Tris/HCl (pH 8)/10 mM-magnesium acetate in a volume of 60 μl at room temperature for 30 min. CF (150 μg), ^{125}I-lysozyme, Ub and ATP were then added to each sample in a volume of 20 μl, and the mixtures incubated for 1 h at 37 °C. Samples were prepared for SDS/polyacrylamide-gel electrophoresis and electrophoresed on 10%-polyacrylamide slab gels (1.5 mm thick) (Laemmli, 1970). After electrophoresis, the gels were stained with Coomassie Blue to locate the lanes containing protein and then dried. The lanes were then cut out and counted directly for radioactivity in a gamma spectrometer. Each inhibitor was tested in duplicate.

	^{125}I-lysozyme degradation		^{125}I-lysozyme conjugation	
Inhibitor	(μg/h)	(% inhibition)	(c.p.m. × 10^{-3})	(% inhibition)†
None	1.24	0	8.9	0
NEM (1 mM)	0	100	11.7	0
DCIC (100 μM)	0.16	87	9.6	0
Decavanadate (100 μM)	0.69	44	8.1	9
Phe-Ala-Arg-CH_2Cl (100 μM)	0.54	56	8.7	2
Cbz-Gly-Leu-Phe-CH_2Cl (100 μM)	0.58	53	7.7	13
Haemin (100 μM)	0	100	*	
Poly(Glu-Tyr) (0.1 mg/ml)	0	100	0.2	98
Heparin (0.1 mg/ml)	0.48	61	2.4	73
Cystatin (0.1 mg/ml)	0	100	8.2	8
Leupeptin (100 μM)	1.22	2	10.9	0
Ep-475 (100 μM)	1.24	0	8.5	4

* Haemin has been shown previously not to inhibit conjugation of Ub to proteins (Haas & Rose, 1981; Tanaka *et al.*, 1984).

† Since the conjugation of Ub to ^{125}I-lysozyme has not been shown to be linear with time in these preparations, the values given for percentage inhibition of conjugation by these substances are merely to help discriminate between strong and weak inhibitors of this process.

lysates (Fagan *et al.*, 1986). Another polyanion, heparin, also inhibited by 61% the ATP+Ub-dependent degradation of ^{125}I-lysozyme (Table 4).

Interestingly, egg-white cystatin, a potent polypeptide inhibitor of many thiol proteinases, inhibits completely at 0.1 mg/ml the ATP+Ub-dependent proteolytic activity isolated from muscle (Table 4) and the ATP-dependent process in reticulocyte Fraction II (L. Waxman & J. M. Fagan, unpublished work). This effect is noteworthy, since a homologous protein inhibitor is present in mammalian tissues (Kominami *et al.*, 1981; Green *et al.*, 1984). The inhibition by cystatin is surprising, since neither the ATP-Ub-dependent proteolytic activity from muscle (Table 4) nor the analogous ATP-dependent activity from intact reticulocytes (Boches & Goldberg, 1982) was diminished by the addition of leupeptin or Ep-475. The last two agents are low-M_r inhibitors of lysosomal thiol proteinases (Aoyagi & Umezawa, 1975; Hanada *et al.*, 1978) and the Ca^{2+}-activated proteinase (Waxman, 1981). (The Ca^{2+}-activated proteinase cannot be involved in this degradative process, since addition of Ca^{2+} or the presence in these buffers of 0.1 mM-EGTA had no effect on the ATP+Ub-dependent proteolytic activity from muscle or from reticulocytes.) Therefore, the effect of cystatin probably involves a distinct action unrelated to its ability to inhibit thiol proteinases.

To confirm that these effects are on the proteolytic step, we examined the ability of cystatin as well as the other inhibitors used in this study to block conjugation of Ub to ^{125}I-lysozyme. In the presence of 2 mM-DTT, cystatin, NEM, DCIC, decavanadate, Cbz-Gly-Leu-Phe-CH_2Cl, leupeptin and Ep-475 do not affect the conjugation of Ub to lysozyme. When NEM was not inactivated with DTT, it completely blocked conjugation of ^{125}I-lysozyme to Ub, as expected from the known sensitivity of this process to thiol-blocking reagents (Hershko *et al.*, 1983). Therefore these compounds probably act by inhibiting the proteinase. On the other hand, in the presence of DTT, poly(Glu-Tyr) and heparin inhibit the ATP+Ub-dependent process, at least in part by inhibiting the conjugation of Ub to lysozyme (Table 4).

DISCUSSION

Previously, only in cell-free preparations from reticulocytes (Etlinger & Goldberg, 1977) and murine erythroleukaemia cells (Rieder *et al.*, 1985; Waxman *et al.*, 1985) was it possible to demonstrate a clear ATP requirement for proteolysis. In dialysed extracts of muscle and liver, at most only a 2-fold stimulation of the degradation of radiolabelled substrates could be measured on addition of ATP (Table 1; Etlinger *et al.*, 1981; Haas *et al.*, 1985). In contrast, in the present studies we found that muscle extracts degrade endogenous proteins in an ATP-dependent fashion. Thus these preparations carry out ATP-dependent proteolysis, but the radiolabelled proteins generally used as substrates (^{125}I-lysozyme and [^{14}C]casein), unlike endogenous proteins, also may be degraded by other enzymes that are independent of ATP. After further purification by ion-exchange chromatography, these preparations were more active and showed a 6-fold stimulation of ^{125}I-lysozyme degradation on re-addition of Ub and ATP. Although liver extracts and

Fraction II did not show such ATP-dependence with endogenous or exogenous substrates, we have isolated from both rabbit liver and skeletal muscle a fraction capable of conjugating Ub to proteins, and a high-M_r proteinase which requires ATP hydrolysis to degrade Ub–protein conjugates. When combined, these isolated components gave ATP-dependent proteolysis. The conjugate-degrading enzyme appears identical in size, chromatographic behaviour and inhibitor-sensitivity with the ATP-dependent enzyme isolated from reticulocytes (Waxman *et al.*, 1986, 1987). Furthermore, this Ub-conjugate-degrading enzyme in muscle, liver or reticulocytes (Waxman *et al.*, 1986, 1987) could be resolved chromatographically from the multifunctional high-M_r proteinase (Figs. 1*c* and 1*d*), which appears not to play an essential role in this ATP-dependent process.

It is possible that, by partial purification, we have removed inhibitors or other enzymes which mask or inactivate the ATP+Ub-dependent proteolytic system in the crude extracts. It has been reported that lysosomal proteinases can inactivate Ub and thereby inhibit ATP-dependent proteolysis (Haas *et al.*, 1985). However, even in the presence of inhibitors of lysosomal thiol proteinases, both crude extracts of muscle and liver and liver Fraction II still showed little activation of ^{125}I-lysozyme degradation by ATP and Ub (Table 1). The lack of an ATP stimulation was not due to a failure in Ub-conjugate formation. On the contrary, these preparations all were very active in conjugating Ub to ^{125}I-lysozyme, to form high-M_r aggregates similar to those shown to be good substrates in reticulocyte extracts (Hershko *et al.*, 1984; Hough *et al.*, 1986).

An attractive explanation for the lack of ATP stimulation of proteolysis in cell-free preparations of liver and other tissues is that they contain specific inhibitors of the conjugate-degrading enzyme. No evidence for such an inhibitor was found in the DEAE-cellulose flow-through fraction from muscle or liver, since this material did not inhibit ATP-dependent proteolysis when mixed with reticulocyte Fraction II (L. Waxman & J. M. Fagan, unpublished work). One possible candidate for an endogenous inhibitor in Fraction II is a proteinase inhibitor related to the cystatin or stefin family (Turk *et al.*, 1985). Purified egg-white cystatin was found to inhibit completely the partially purified ATP+Ub-dependent proteolytic activity from muscle without affecting Ub conjugation (Table 4). Since the ATP+Ub-dependent proteolytic process is not affected by leupeptin and Ep-475, it is unlikely that cystatin blocks this process by inhibiting thiol proteinases. Therefore, this polypeptide probably has additional biochemical actions. Inhibitors of the cystatin or stefin family are present in a variety of tissues, and in particularly high concentration in liver (Kominami *et al.*, 1981; Green *et al.*, 1984). Changes in the amount or activity of such inhibitors *in vivo* could be a mechanism which influences the rate at which intracellular proteins are degraded.

The non-lysosomal ATP-dependent proteolytic pathway initially described in reticulocytes is thought to be involved in the degradation of abnormal proteins (Etlinger & Goldberg, 1977) as well as proteins that are lost during cell maturation (Muller *et al.*, 1980; Boches & Goldberg, 1982; Speiser & Etlinger, 1982; Magnani *et al.*, 1986). Our findings provide the first clear biochemical evidence for such a degradative system in other mammalian tissues. It should now be possible to examine directly whether this ATP-dependent degradative pathway is responsible for the inactivation of short-lived liver enzymes or for the breakdown of various muscle proteins under physiological and pathological conditions known to affect protein turnover.

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