α_2 -Macroglobulin used to isolate intracellular endopeptidases from mammalian cells in culture

Leif A. SLOT and Klavs B. HENDIL*

August Krogh Institute, 13 Universitetsparken, DK 2100 Copenhagen O, Denmark

Extracts of cell cultures labelled with [³H]leucine were incubated with human α_2 -macroglobulin (α_2 M), a plasma proteinase inhibitor. The proteinase- α_2 M complexes were then precipitated with immobilized monoclonal antibodies to α_2 M and analysed by SDS/polyacrylamide-gel electrophoresis. Parallel experiments were done with methylamine-inactivated α_2 M to check for unspecific binding of cell proteins to α_2 M. Several ³H-labelled cell proteins bound to active, but not to inactivated, α_2 M. Such proteins are likely to be proteinases. Putative endopeptidases of subunit M_r 112 000, 78 000, 53 000, and in some experiments 88 000 and 16 000, were trapped by α_2 M in supernatant fractions from IMR90 human fibroblasts, EBTr bovine fibroblasts and HeLa human carcinoma cells. No additional proteins were trapped in the presence of ATP. The M_r -78 000 endopeptidase was identified as calpain II by immunoblotting. At pH 5.3 putative endopeptidases of subunit M_r 80 000, 53 000 and 28 000–32 000 were trapped from IMR90-fibroblast extracts. Immunoblotting showed that both cathepsin B and cathepsin D were present in the M_r -28 000–32 000 electrophoretic bands. The use of α_2 M and immobilized antibody to α_2 M thus allows a rapid enrichment of endopeptidases from cell extracts. Some potentials and limitations of the method are discussed.

INTRODUCTION

Proteins in living cells are continuously broken down and replaced by newly synthesized protein (Goldberg & St. John, 1976). Much of the degradation takes place in the cytoplasm rather than in lysosomes (Bigelow *et al.*, 1981; Hershko & Ciechanover, 1982), but the proteinases responsible for the turnover of intracellular proteins are not yet identified (Mayer & Doherty, 1986; Rechsteiner, 1987). Unknown intracellular proteinases are also involved in the post-translational processing of proteins by limited proteolysis. To gain insight into these important processes, it will be necessary to isolate and characterize cellular proteinases.

Endopeptidases seem to be present in all tissues, though generally in low amounts. Their purification is often hampered by the presence in the same tissues of inhibitors that mask the proteinase activity (Lenney, 1980). Furthermore, once purified some of the proteinases have proved to be confined to mast cells or other specific cell types, so that they are unlikely to be involved in the protein turnover or processing that takes place in all cells.

We have therefore investigated the approach of using α_2 -macroglobulin (α_2 M) to isolate endopeptidases from homogeneous cell populations grown in culture. α_2 M from human blood plasma inhibits an unusually broad spectrum of endopeptidases of all four catalytic classes (metallo-, serine, aspartic and cysteine proteinases). α_2 M works as a molecular trap for enzymically active endopeptidases: once the proteinase has cleaved a peptide bond within the 'bait' region of α_2 M, the inhibitor undergoes an irreversible conformational change that sterically entraps the proteinase (Barrett, 1981; Travis & Salvesen, 1983). In the experiments reported here, we

have incubated cell extracts with α_2 M to allow reaction between proteinases and α_2 M. The α_2 M-proteinase complexes were then precipitated with immobilized monoclonal antibodies against α_2 M, and denatured and analysed by SDS/polyacrylamide-gel electrophoresis.

EXPERIMENTAL

Materials

Materials were purchased from the following sources: Sephacryl S200 and S300, Procion Red-Sepharose, Sepharose CL6B and Protein A-Sepharose (Pharmacia, Uppsala, Sweden); DEAE-cellulose type DE52 (Whatman, Maidstone, Kent, U.K.); fetal-calf serum and newborn-calf serum (Gibco, Paisley, Scotland, U.K.); benzyloxycarbonylarginine methylcoumarylamide (Cambridge Research Chemicals, Harston, Cambridgeshire, U.K.). Unless otherwise stated, other biochemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Zn²⁺-chelate-Sepharose CL4B was prepared as described by Porath *et al.* (1975). Eagle's minimal essential medium with non-essential amino acids (Eagle, 1959) was prepared in the laboratory from ingredient salts, amino acids (BDH, Poole, Dorset, U.K.) and vitamins (Merck, Darmstadt, Germany). L-[4,5-³H]Leucine and N-succinimidyl[2,3-³H]propionate were obtained from Amersham International (Amersham, Bucks., U.K.).

The following buffers were used: PBS (phosphate-buffered saline: 137 mM-NaCl, 2.7 mM-KCl, 4.1 mM-Na₂HPO₄, 0.73 mM-KH₂PO₄, pH 7.4); buffer A (0.5 mM-Na₂EDTA, 137 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, pH 7.4); buffer B (300 mM-Tris/HCl, pH 7.5, 600 mM-KCl, 6 mM-MgCl₂, 3 mM-CaCl₂, 15 mM-leucine, 9 mM-cysteine, 60 mM-

Abbreviation used: α_2 M, α_2 -macroglobulin.

* To whom correspondence should be addressed.

lysine, 200 units of penicillin G/ml and 0.2 mg of streptomycin sulphate/ml); buffer C (100 mM-sodium acetate, pH 5.3, 200 mM-KCl, 20 mM-lysine, 3 mM-cysteine and 0.5 % of Triton X-100); buffer D (500 mM-NaCl, 10 mM-Tris/HCl, pH 7.5, 0.1 % bovine serum albumin).

α_2 M was purified from heparinized human plasma essentially as described by Sottrup-Jensen *et al.* (1983) but omitting the adsorption to lysine-Sepharose. Proteinase-inhibitor activity of α_2 M was determined with trypsin as described by Ganrot (1966). The trypsin (bovine trypsin, diphenylcarbamoyl chloride-treated; Sigma type XI), in turn, was determined to be 41 % active by active-site titration with *p*-nitrophenyl guanidinobenzoate (Chase & Shaw, 1970). The α_2 M preparations bound 0.8–2.0 mol of active trypsin/mol of α_2 M.

α_2 M was radioactively labelled to 38 Ci/mmol with *N*-succinimidyl[2,3-³H]propionate (Amersham) as suggested by the manufacturer. Methylamine-inactivated α_2 M was prepared as described by Gonias & Pizzo (1983) and dialysed against 1000 vol. of 10 mM-Tris/HCl, pH 7.4. Trypsin- α_2 M complexes were made by incubating α_2 M (5 mg/ml) with bovine trypsin (Sigma, 1 mg/ml; 41 % active) in 100 mM-Tris/HCl (pH 8.1)/20 mM-CaCl₂ for 30 min at 37 °C. Soya-bean trypsin inhibitor was then added to 2 mg/ml, and the preparation was separated on a column (1.2 cm × 100 cm) of Sephacryl S200 eluted with 50 mM-Tris/HCl (pH 7.4)/5 mM-EDTA/500 mM-NaCl. The front peak containing α_2 M was concentrated to 10 mg/ml by ultrafiltration and stored at –20 °C.

Calpain II was purified from bovine lung by (NH₄)₂SO₄ precipitation, DEAE-cellulose chromatography and gel filtration on Sephacryl S300, essentially as described by Waxman (1981). These steps were followed by affinity chromatography on Procion Red-Sepharose (Hathaway *et al.*, 1982). Calpain was labelled to 1.5 Ci/mmol with *N*-succinimidyl[2,3-³H]propionate as described for α_2 M.

Antibodies

Monoclonal antibody to human α_2 M (Slot & Hendil, 1987) was coupled to CNBr-activated Sepharose CL6B (2.4 mg of IgG/ml of gel; Axén *et al.*, 1967). Antibody against bovine calpain II was purified from egg yolk (Jensenius *et al.*, 1981) from hens immunized by four injections at 2-week intervals, each with 200 µg of calpain II in Freund's adjuvant. Complete Freund's adjuvant was used in the first immunization, and incomplete adjuvant in the following immunizations.

Antibodies were generously given by the following: sheep antisera to human cathepsins B and D (Dr. Alan Barrett, Cambridge, U.K.); rabbit antibody to human urokinase (Dr. Jan Grøndahl-Hansen, Copenhagen, Denmark); rabbit antibody to human non-pepsin aspartic proteinase (Dr. Pal Szecezi and Dr. Bent Foltman, Copenhagen, Denmark). Peroxidase-labelled rabbit anti-(chick IgG) antibody was purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands). All other antibodies were from Dakopatts (Copenhagen, Denmark).

Cells

IMR90 human fibroblasts, EBTr bovine fibroblasts and HeLa human carcinoma cells were initially obtained from Gibco (Paisley, Scotland, U.K.), the American

Type Culture Collection (Rockville, MD, U.S.A.) and the State Serum Institute (Copenhagen, Denmark) respectively. The cells were grown in 90 % (v/v) Eagle's minimal essential medium with non-essential amino acids, 8 % (v/v) newborn-calf serum and 2 % (v/v) fetal-calf serum (Gibco).

Cell extracts

Cells were grown to high densities in disposable glass roller bottles (650 cm²; Flow, Irvine, Scotland, U.K.) in medium with streptomycin sulphate (100 µg/ml) and penicillin G (100 units/ml) and labelled by incubation for 42–48 h in medium with 0.05 mM-[³H]leucine (20 Ci/mol), 100 ml per roller bottle. For harvest, each roller bottle was washed in 2 × 50 ml of buffer A and the cells were detached in 20 ml of buffer A. The cell suspension was washed with 3 × 10 ml of ice-cold PBS by centrifugations (500 *g*, 5 min) and homogenized in ice-cold degassed 250 mM-sucrose, pH 7.0 (3 ml/g of cells), by 20–30 strokes in a Dounce homogenizer with a tight-fitting pestle. More than 90 % of the cells were ruptured, as determined by phase-contrast microscopy.

In experiments with cytoplasmic proteinases the homogenate was mixed with 0.2 vol. of freshly prepared and degassed buffer B, and in experiments with acid proteinases with 1 vol. of degassed buffer C. For both types of preparation, the homogenate was centrifuged at 80000 *g* for 30 min in an MSE 10 × 10 rotor. The supernatants are referred to as 'high-speed supernatant'.

In some experiments an ATP-generator mixture was added to the supernatants, to give final concentrations of 10 mM-phosphocreatine, 0.5 mM-ATP and 0.05 mg of creatine kinase/ml.

Precipitation of cellular proteinase- α_2 M complexes

The buffered high-speed supernatants (usually 1 ml) were mixed with active or methylamine-inactivated α_2 M to a final concentration of 300 µg/ml. Preparations in pH 7.5 buffer were incubated for 18 h at room temperature, whereas preparations in pH 5.3 buffer were incubated for 2 h at 37 °C. The preparations were then centrifuged at 12000 *g* for 5 min, and the supernatant was tumbled for 1 h at room temperature with monoclonal antibodies to α_2 M, coupled to Sepharose [usually 0.25 ml of 50 % (v/v) gel]. The Sepharose beads were then transferred to a Pasteur pipette closed with a 2 mm glass bead that allowed buffer to pass slowly out of the pipette. This mini-column was washed in succession with 2 ml of buffer A, 3 ml of buffer D, 2 ml of 0.5 % Nonidet P40/1 % SDS and 2 ml of PBS. α_2 M was then eluted with boiling 2 % SDS. The eluate was analysed for protein, and the volume (about 400 µl) determined by weighing. The eluate was freeze-dried, and redissolved and boiled for 1 min in sample buffer (Laemmli, 1970) to give final concentrations of 10 % SDS and 500 µg of protein/ml.

Electrophoresis

Samples of α_2 M and cellular proteinases were electrophoresed in slab gels (Laemmli, 1970) with linear gradients of 7.5–15 % polyacrylamide. *M_r* markers (Kem-En-Tec, Copenhagen, Denmark) and their *M_r* values were: myosin (200000); β -galactosidase (116000); phosphorylase *b* (97400); bovine serum albumin (66000); ovalbumin (45000); carbonic anhydrase (29000); soya-bean trypsin inhibitor (20100); α -lactalbumin (14200).

After electrophoresis some lanes (with 5 μg of total protein per lane) were stained with AgNO_3 (Görg *et al.*, 1985). To detect radioactive proteins, other lanes (with 20 μg of protein per lane) were fluorographed at -70°C (Chamberlain, 1979) with preflashed Kodak XAR5 film. Fluorograms were scanned in a LKB Ultrosan XL densitometer (LKB, Bromma, Sweden). Protein from other lanes was blotted on to nitrocellulose sheets (BA85; Schleicher and Schuell, Dassel, Germany) and incubated with primary antibodies, followed by peroxidase-labelled secondary antibodies, and stained with 3-amino-9-ethylcarbazole (Heegaard & Bjerrum, 1988).

Enzyme assays

Cathepsin B was assayed as described by Barrett (1980). Trypsin was assayed with benzoylarginyl *p*-nitroanilide or with benzyloxycarbonylarginine methylcoumarylamide (Zimmerman *et al.*, 1977).

Protein analysis

The concentrations of pure proteins were determined from the A_{280} , with the following $A_{280}^{1\%}$ values; $\alpha_2\text{M}$ 8.93 (Hall & Roberts, 1978); calpain II 14.2 (Tsuiji & Imahori, 1981); IgG 14.3 (Hudson & Hay, 1980). The concentrations of $\alpha_2\text{M}$ -proteinase complexes were determined with *o*-phthalaldehyde (Peterson, 1983), with bovine serum albumin in 2% SDS as a standard.

Radioactivity

Radioactivity was determined by liquid-scintillation counting in Pico-fluor 30 (Packard, Downers Grove, IL, U.S.A.). Protein was dissolved in 0.1 M-NaOH before radioactivity counting. Differences in counting efficiencies between samples were determined by internal standardization.

RESULTS

Reaction of $\alpha_2\text{M}$ with calpain

Calpain II, a Ca^{2+} -activated cytoplasmic endopeptidase of M_r 110000, is found in small amounts in most mammalian tissues (Murachi, 1983). In initial experiments we used calpain as a model proteinase: if it proved possible to isolate calpain from cell homogenates, then other unknown proteinases could probably be isolated, too.

Like most other cysteine proteinases, calpain needs thiol compounds to stay enzymically active. However, preliminary experiments showed that $\alpha_2\text{M}$ was inactivated by dithiothreitol or by moderate concentrations of mercaptoethanol or cysteine. As a compromise, all incubation buffers were de-aerated before use, and mercaptoethanol or cysteine was added to 1–1.5 mM final concentration. Under these conditions $\alpha_2\text{M}$ lost about 8% of its activity/h (results not shown).

$[^3\text{H}]$ Calpain was incubated with $\alpha_2\text{M}$ in a molar ratio of calpain to active $\alpha_2\text{M}$ of 1:3, i.e. well below the reaction stoichiometry of 1–2 mol of proteinase/mol of $\alpha_2\text{M}$ (Barrett, 1981). Gel filtration showed that 52% of the $[^3\text{H}]$ calpain was eluted with $\alpha_2\text{M}$ (Fig. 1, trace A). The remaining radioactivity was distributed between free calpain and its autolytic degradation products. In a parallel experiment where $[^3\text{H}]$ calpain was incubated with $\alpha_2\text{M}$ but in a high-speed supernatant from EBTr-cell homogenates, only 25% of the calpain became bound by $\alpha_2\text{M}$ (Fig. 1, trace B).

Fig. 1. Binding of $[^3\text{H}]$ calpain to α_2 -macroglobulin in buffer and in cell extract

$[^3\text{H}]$ Calpain (4.2 μg) was incubated in 1280 μl of 50 mM-Tris/HCl (pH 7.4)/5 mM- CaCl_2 /1 mM-mercaptoethanol. The buffer was made up either in water (A, C) or in a supernatant fraction (B) from an EBTr-cell homogenate prepared with 3 ml of 250 mM-sucrose per g wet wt. of cells. In A and B, 240 μg of $\alpha_2\text{M}$ was also added. After incubation for 2 h at room temperature, the samples were centrifuged at 12000 g for 5 min and the supernatant was applied to a column (2.5 cm $\times$ 90 cm) of Sephacryl S200 and eluted with 50 mM-Tris/HCl (pH 7.4)/5 mM-EDTA/500 mM-NaCl, at 5.6 ml/h. V_0 and V_t denote void and total volumes respectively.

Precipitation of $\alpha_2\text{M}$ -proteinase complexes

After incubation of $\alpha_2\text{M}$ and cell homogenate, the next step in the purification of cellular proteinases is the recovery of $\alpha_2\text{M}$, now with the trapped proteinase. We have therefore produced a monoclonal antibody against $\alpha_2\text{M}$ and coupled it to Sepharose. Fig. 2 shows that the immunoaffinity Sepharose had a binding capacity of about 5 mg of $\alpha_2\text{M}$ /g wet wt. of Sepharose.

Effects of incubation time and $\alpha_2\text{M}$ concentration

Experiments not shown here revealed that the reaction between $[^3\text{H}]$ calpain (1 $\mu\text{g}/\text{ml}$) and $\alpha_2\text{M}$ (50 $\mu\text{g}/\text{ml}$) runs to completion within 1 hr of incubation at room temperature. The precipitation of $[^3\text{H}]$ calpain from buffered cell extract was lower than from buffer alone, even when large amounts of $\alpha_2\text{M}$ were used, as shown in Fig. 3. Cell extracts contain calpastatin, an inhibitor of calpain (Murachi, 1983). The binding of calpain to calpastatin is reversible, whereas the reaction between proteinases and $\alpha_2\text{M}$ essentially is not (Barrett, 1981). One might therefore expect that, with time, all calpain should eventually end up as complexes with $\alpha_2\text{M}$.

Fig. 2. Binding of trypsin- α_2 M to Sepharose-coupled monoclonal antibody to α_2 M

Antibody coupled to Sepharose (20 μ l of a 50 % suspension) was tumbled for 1 h at room temperature in 500 μ l of 50 mM-Tes (pH 8.0)/10 mM- CaCl_2 containing 0.1 % gelatine and trypsin- α_2 M as indicated. After centrifugation (12000 g, 2 min), trypsin- α_2 M in the supernatant was assayed (Zimmerman *et al.*, 1977). The abscissa intercept shows that 20 μ l of 50 % Sepharose suspension binds about 50 μ g of α_2 M-proteinase complex.

Fig. 3. Precipitation of [³H]calpain from buffer and from cell extract with α_2 M and anti- α_2 M coupled to Sepharose

[³H]Calpain (0.26 μ g) was incubated for 1 h at room temperature in 200 μ l of 50 mM-Tris/HCl (pH 7.4)/1 mM-mercaptoethanol made up in either 0.1 % bovine serum albumin (○) or in a supernatant fraction from EBTr fibroblasts homogenized in 3 ml of 250 mM-sucrose per g wet wt. of cells. (●). CaCl_2 was then added (final concn. 10 mM) and α_2 M was added as indicated. The incubation was continued for another 2.5 h before addition of anti- α_2 M antibody coupled to Sepharose (100 μ l of a 50 % suspension). After another 1 h of incubation with tumbling, the samples were centrifuged (12000 g, 5 min) and the radioactivity in the supernatant was determined. The precipitation of [³H]calpain is calculated as the difference in radioactivity in the supernatant before and after incubation with immobilized antibody. Results shown are averages from two determinations.

However, extension of the incubation time did not improve the recovery of [³H]calpain from cell extracts (results not shown).

Isolation of proteinases from cell extracts at pH 7.5

Extracts of bovine EBTr fibroblasts were incubated either with active α_2 M or with α_2 M that had been inactivated with methylamine. Methylamine-inactivated α_2 M has a compact conformation like α_2 M-proteinase complexes and does not bind proteinases (Barrett, 1981). It was therefore used as a control for non-specific binding of proteins to α_2 M. After incubation, the α_2 M-proteinase complexes were precipitated with anti- α_2 M-Sepharose and analysed by electrophoresis. Silver-stained gels (Fig. 4, parts A and B) showed a multitude of protein bands in lanes both with active and with inactive α_2 M. Immunoblotting showed that bands at M_r 25000 and around M_r 50000 were subunits of mouse IgG from the immunoaffinity Sepharose (results not shown). Many of the other bands are fragments of α_2 M, since they reacted with polyclonal rabbit antibody to human α_2 M (Fig. 4, part C). The plethora of α_2 M degradation products makes it difficult to find putative cell proteinases that would show up as bands in lanes with active α_2 M, but not in lanes with inactive α_2 M. Also, even under ideal conditions the ratio of proteinase to α_2 M is expected to be low: at low concentrations of proteinase, α_2 M reacts with proteinases with a 1:1 stoichiometry (Travis & Salvesen, 1983). Thus, in a complex with 1 mol of α_2 M (M_r 716000) and 1 mol of proteinase of M_r , say, 50000, only 6 % of the mass of the complex would be proteinase. However, after labelling with ³H-amino acids, cell proteins in the gels can be observed by fluorography without interference by the unlabelled α_2 M.

Fluorograms showed that several cell protein bands were present in eluates from α_2 M-proteinase complexes (Fig. 4, part D). However, eluates from α_2 M that had been inactivated before the incubation with cell extracts also contained cell proteins (Fig. 4, part D). This unspecific background constitutes 40–50 % of the radioactive protein recovered in experiments with active α_2 M, as determined both from the areas under the curves in Fig. 4 (part D) and from direct scintillation counting of the α_2 M complexes. The immunoaffinity gel beds were usually washed with 70 bed vol. of buffer, and further washing did not decrease the background. Other vain attempts to decrease the background included purification of the α_2 M-proteinase complexes by gel filtration on Sepharose CL4B or preadsorption to mouse IgG immobilized on Protein A-Sepharose before incubation with immunoaffinity Sepharose, elution of the affinity columns with glycine buffer, pH 2, instead of SDS, and shorter incubation time of α_2 M with cell extracts. In ten experiments the difference in radioactivity recovered with active and inactive α_2 M respectively, i.e. the proteins specifically bound to active α_2 M, varied between 0.14 % and 0.6 % of the radioactivity in the high-speed supernatant from cell homogenates. The experiment shown in Fig. 4 is representative, but the number of protein bands on fluorograms of proteins from active and inactive α_2 M varied somewhat, making it difficult to decide for the minor bands whether they fulfilled the criteria for putative proteinases, namely that they bound to active, but not to inactivated, α_2 M. However, proteins that fulfilled these criteria, and are therefore probably cellular endopeptidases, were present at positions corresponding to an M_r of

Fig. 4. Recovery of proteinases from cytosolic extracts of IMR90 human fibroblasts incubated with α_2 M at pH 7.5

IMR90 cells were labelled with [3 H]leucine, homogenized and centrifuged (80 000 *g* for 30 min). The supernatant was incubated at pH 7.5 with either active or methylamine-inactivated α_2 M and tumbled with anti- α_2 M antibody coupled to Sepharose. The Sepharose beads were washed, and α_2 M-proteinase complexes were eluted and electrophoresed in acrylamide gels with SDS. A, B, Silver-stained total protein from experiments with active (A) or inactivated (B) α_2 M. C, Immunoblot of protein from an experiment with active α_2 M probed with rabbit anti- α_2 M antibody. Many of the protein bands in (A) prove to be degradation products of α_2 M. D, Cellular proteinases trapped by α_2 M: densitometer tracings of fluorograms of lanes with 3 H-labelled cell proteins bound to active (—) or inactivated (---) α_2 M. Putative proteinases appear as peaks in experiments with active, but not with inactivated, α_2 M. The apparent M_r values of such putative proteinases are indicated. E, F, Immunoblots of proteins from experiments with active (E) or inactivated (F) α_2 M, probed with anti-calpain antibody. Calpain binds only to active α_2 M and coincides with the M_r -79 000 band in (D). High- M_r covalent complexes of calpain and α_2 M are also seen.

about 112 000, 78 000 and 53 000 in all experiments with IMR90 human fibroblasts ($n = 2$), EBTr bovine fibroblasts ($n = 4$) and HeLa human carcinoma cells ($n = 3$). Bands at positions corresponding to M_r of 88 000 and 16 000 were present in EBTr and IMR90 cells in four out of these seven experiments. A series of bands of $M_r > 170$ 000 was also invariably more prominent in gels from incubations with active α_2 M. Some of these bands are likely to contain proteinases that have been covalently linked to α_2 M. The covalent binding of proteinases to α_2 M can be out-competed by small nucleophiles (Salvesen *et al.*, 1981; Sottrup-Jensen *et al.*, 1981), so our incubation

buffers contained 10 mM-lysine. Indeed, omission of lysine from the incubation buffer caused an approx. 2-fold increase in the amount of radioactive cell proteins present in high- M_r complexes (results not shown).

Part of the intracellular degradation of cellular proteins depends on ATP (Mayer & Doherty, 1986; Rechsteiner, 1987). No additional bands emerged, however, in experiments where an ATP-generator system was included during incubation of cell extracts with α_2 M (results not shown). Blots of proteinases recovered from IMR90 cells did not react with antibodies against human cathepsin B or D, human non-pepsin aspartic proteinase (Tarasova *et al.*, 1986), or tissue plasminogen activator, though blots from control lanes with the appropriate proteinases showed a strong positive reaction (results not shown). However, blots of proteinases isolated by incubation of α_2 M with human (IMR90 or HeLa) or bovine (EBTr) cells reacted with antibodies against the M_r -78 000 chain of bovine calpain, whereas a lane with protein from a parallel experiment with inactive α_2 M did not react (Fig. 4, parts E and F). In blots from electrophoresis of total cell protein, the antibody against calpain reacted only with the M_r -78 000 subunit of calpain (results not shown). The immunoreactivity in Fig. 4 (part E) of peptides of $M_r > 170$ 000 therefore confirms that some of the high- M_r peptides in the gels are covalent complexes of calpain with α_2 M. The putative proteinases of subunit M_r 112 000, 88 000 and 53 000 were not further characterized.

Isolation of proteinases at low pH

α_2 M remains active over a wide pH range (pH 5.0–8.5; Barrett, 1981), and we have also investigated the usefulness of α_2 M to trap proteinases active at low pH. Extracts of IMR90 human fibroblasts were incubated with active or methylamine-inactivated α_2 M. Proteinase- α_2 M complexes were then recovered from the supernatant and electrophoresed as described for neutral proteinases. Again, the silver-stained gels showed several protein bands that could be identified as degradation products of α_2 M by immuno-blotting (results not shown). However, the silver-stained gels also showed that active α_2 M had trapped proteins with M_r 28 000–32 000. These bands, and additional bands at M_r 53 000, 80 000 and above 130 000, are also evident in fluorograms of gels from experiments with [3 H]leucine-labelled cells, but not in experiments with inactive α_2 M (Fig. 5, part C). The bands of $M_r > 130$ 000 are likely to be covalent complexes of α_2 M (or its fragments) with proteinases. This suggestion is supported by immunoblotting, since Fig. 5 (parts F and G) shows that cathepsin B is present both in a M_r -28 000–32 000 form corresponding to free cathepsin, and in bands of $M_r > 130$ 000, corresponding to covalent complexes with α_2 M. The covalent binding of proteinases to α_2 M is much more prominent at low pH (Fig. 5) than at high pH (Fig. 4), although both buffers contained 10 mM-lysine to inhibit covalent binding of proteinases to α_2 M. Putrescine also suppresses this covalent binding (Sottrup-Jensen *et al.*, 1981), but the addition of 0.6 mM-putrescine to our incubations almost completely inhibited both covalent and non-covalent binding of the acid proteinases (results not shown). Cathepsin D, an aspartic proteinase, also bound to active but not to inactive α_2 M, and apparently mainly in a non-covalent fashion (Fig. 5, parts D and E). The protein in the bands of M_r 53 000 and 80 000 was not identified. Immunoblots of electro-

Fig. 5. Recovery of proteinases from extracts of IMR90 human fibroblasts, incubated with α_2 M at pH 5.3

IMR90 cells were labelled with [3 H]leucine, homogenized in the presence of Triton X-100, and centrifuged. The supernatant was incubated at pH 5.3 with either active or inactivated α_2 M and then tumbled with anti- α_2 M antibody immobilized on Sepharose. The Sepharose beads were washed and α_2 M-proteinase complexes eluted and electrophoresed in polyacrylamide gels with SDS. A, B, Silver-stained total protein from experiments with active (A) or inactivated (B) α_2 M. C, Cellular proteinases trapped by α_2 M: densitometer scans of fluorograms of lanes with 3 H-labelled cell proteins bound to active (—) or inactivated (----) α_2 M. Proteinases bind only to active α_2 M. D, E, Immunoblots of proteins from experiments with active (D) or inactive (E) α_2 M probed with anti-(cathepsin D) antibody. Cathepsin D is bound only to active α_2 M, and coincides with the M_r -32000 band in panel (C). F, G, Immunoblot as panels D and E, but probed with anti-cathepsin B antibody. Cathepsin B is present both covalently linked to α_2 M (high- M_r bands to the left) and as free chains of M_r 28000 and 32000.

phoretic lanes with α_2 M-proteinase complexes did not react with antibodies against calpain or non-pepsin aspartic proteinase (M_r 58000; Tarasova *et al.*, 1986), though control lanes with the appropriate proteinases reacted avidly (results not shown).

Similar results were obtained in a total of three experiments with IMR90 cells. The unspecific binding of 3 H-labelled cell proteins to inactive α_2 M constituted 20–30 % of the proteins bound to active α_2 M. The radioactive proteins that bound to active, but not to inactivated, α_2 M constituted a fraction of about 0.2 % of the radioactivity in the cell supernatant fraction.

DISCUSSION

We used methylamine-inactivated α_2 M to check the extent of unspecific binding of cell proteins to α_2 M. This background is rather high, since it constitutes 20–50 % of the cell proteins bound to active α_2 M, and it proved difficult to decrease. The binding to α_2 M of some proteins that are not proteolytic enzymes has been described previously (Barrett, 1981; O'Connor-McCourt & Wakefield, 1987).

3 H-labelled cell protein bands that are found in electrophoretic lanes from experiments with active α_2 M and not in lanes with methylamine-inactivated α_2 M are likely to be subunits of endopeptidases, truncated subunits of endopeptidases, or such intact or truncated endopeptidases or other proteins (Salvesen *et al.*, 1981) that have been covalently linked to α_2 M. Since a proteinase trapped in α_2 M is probably not accessible to be cleaved by other endopeptidases, truncated subunits are expected mainly from proteolytic enzymes that are enzymically active also in their cleaved form. Formation of covalent complexes of intracellular proteinases and α_2 M were seen in several experiments both with [3 H]-calpain and in immuno-blot. One would expect such covalent complexes to have an M_r value of at least that of the proteinase plus either 179000 or about 95000, since these are the M_r values of the subunits or proteolytic fragments of α_2 M carrying the cross-linking residue. Complexes with higher M_r are also expected (Wang *et al.*, 1983). For proteins of $M_r > 95000$, as those shown in Figs. 4 and 5, it is therefore difficult to decide whether they are subunits of high- M_r proteinases or covalent complexes of proteinases with α_2 M. On the other hand, peptides of $M_r < 95000$ are likely to be free subunits of peptidases. Incubation of α_2 M with cell extracts at pH 7.5 resulted in the entrapment of peptides of M_r 112000, 78000, 53000 and perhaps 88000 and 16000. The M_r -78000 peptide was identified as calpain but the M_r -112000, -88000, -53000 and -16000 chains were not identified. The M_r -112000 and -53000 chains are probably not subunits of the same enzyme, since they are present in variable proportions in different experiments (results not shown). The M_r -112000 chain may be a covalent complex of a proteinase (M_r about 16000) and the M_r -95000 fragment of α_2 M, or it may be a proteinase, perhaps the insulinase previously detected in several cell types (subunit M_r 110000; Shii *et al.*, 1985). However, a similar enzyme isolated from red blood cells was not inhibited by α_2 M (Kirschner & Goldberg, 1983). As far as the M_r -53000 chain is concerned, endopeptidases with similar subunit M_r (50000 and 57000) have been isolated from red blood cells by Golovtchenko-Matsumoto *et al.* (1982) and Witheiler & Wilson (1972) respectively.

After incubation of α_2 M with cell extracts at low pH, a series of proteins with M_r 28000–32000 were trapped. Several cathepsins have M_r values in this range (Barrett & McDonald, 1980), and cathepsins B and D were identified by immuno-blotting. Proteins of M_r 53000 and 80000 were also trapped by active α_2 M, and the former protein may be identical with a proteinase described by Obled *et al.* (1984) in rat muscle lysosomes. The M_r -80000 chain did not react with antibodies to calpain, and it may belong to a new acid proteinase. Potentially the catalytic class of such unknown proteinases can be determined with the use of class-specific inhibitors, some

of which should decrease the binding of proteinase to $\alpha_2\text{M}$.

Most, but not all, endopeptidases react with $\alpha_2\text{M}$. Some of those that do not are probably sterically hindered owing to their high M_r (Travis & Salvesen, 1983). Indeed, in preliminary experiments we could detect high enzymic activity of a cytoplasmic high- M_r proteinase (M_r 700 000; Wilk & Orlowski, 1980) in extracts from HeLa cells, but none was precipitated by $\alpha_2\text{M}$. The high- M_r proteinase is composed of subunits of M_r 32 000–25 000 (Wilk & Orlowski, 1980; Dahlmann *et al.*, 1985), and no proteins in this range were found by gel electrophoresis of proteinases trapped from HeLa extracts at pH 7.5 (Fig. 5), so this abundant proteinase is not bound by $\alpha_2\text{M}$.

Despite these limitations, the use of $\alpha_2\text{M}$ to trap intracellular endopeptidases deserves further refinements and applications. With the present method, the peptidases are recovered as complexes with $\alpha_2\text{M}$. The method involves only incubation of raw cell extract with $\alpha_2\text{M}$ and an immunoprecipitation, but the enrichment during these few steps is considerable: in many cell types cathepsin B constitutes a fraction of less than 10^{-3} of bulk cell protein (Kirschke *et al.*, 1980), and calpain is present in tissues at a concentration of about 10^{-4} of bulk protein (Elce *et al.*, 1984). Nonetheless these proteinases and other putative proteinases constitute the majority of cell proteins trapped by $\alpha_2\text{M}$. This enrichment opens several opportunities: since proteinases bound to $\alpha_2\text{M}$ retain their enzymic activity toward low- M_r substrates (Barrett, 1980; Harpel & Hayes, 1980), it is possible to analyse the proteinase- $\alpha_2\text{M}$ complexes with chromogenic or fluorogenic substrates, even for enzymes that are inhibited by endogenous inhibitors or are present in trace amounts in the cell homogenate, so that they need partial purification to allow detection.

The $\alpha_2\text{M}$ -proteinase complexes may also be used to produce monoclonal antibodies against the unknown proteinases.

The experiments were initiated by K.B.H. during a sabbatical year at the University of Utah. K.B.H. thanks the Fulbright Commission and Det Thottske Rejselegat for support, and Dr. Martin Rechsteiner for his hospitality, stimulating discussions and comments on the manuscript. Financial support from the Danish Natural Science Foundation and Novo's Fond is also gratefully acknowledged. Mrs. Anne-Marie Lauridsen provided excellent technical assistance.

REFERENCES

- Axn, R., Porath, J. & Ernback, S. (1967) *Nature (London)* **214**, 1302–1304
- Barrett, A. J. (1980) *Biochem. J.* **187**, 909–912
- Barrett, A. J. (1981) *Methods Enzymol.* **80**, 737–754
- Barrett, A. J. & McDonald, J. K. (1980) *Mammalian Proteases: A Glossary and Bibliography*, vol. 1, Academic Press, London
- Bigelow, S., Hough, R. & Rechsteiner, M. C. (1981) *Cell* **25**, 83–93
- Chamberlain, J. P. (1979) *Anal. Biochem.* **98**, 132–135
- Chase, T. & Shaw, E. (1970) *Methods Enzymol.* **19**, 20–27
- Dahlmann, B., Kuehn, L., Rutschmann, M. & Reinauer, H. (1985) *Biochem. J.* **228**, 161–170
- Eagle, H. (1959) *Science* **130**, 432–437
- Elce, J. S., Baenziger, J. E. & Young, D. R. C. (1984) *Biochem. J.* **220**, 507–512
- Ganrot, P. O. (1966) *Clin. Chim. Acta* **14**, 493–501
- Goldberg, A. L. & St. John, A. C. (1976) *Annu. Rev. Biochem.* **45**, 747–803
- Golovtchenko-Matsumoto, A. M., Matsumoto, I. & Osawa, T. (1982) *Eur. J. Biochem.* **121**, 463–467
- Gonias, S. L. & Pizzo, S. V. (1983) *Biochemistry* **22**, 536–546
- Grg, A., Postel, W., Weger, J., Sciavava, H. W. & Boesken, W. H. (1985) *Sci. Tools* **32**, 5–9
- Hall, P. K. & Roberts, R. C. (1978) *Biochem. J.* **173**, 27–38
- Harpel, P. C. & Hayes, M. B. (1980) *Anal. Biochem.* **108**, 166–175
- Hathaway, D. R., Werth, D. K. & Haeberle, J. R. (1982) *J. Biol. Chem.* **257**, 9072–9077
- Heegaard, N. H. H. & Bjerrum, O. (1988) in *Handbook of Immunoblotting of Proteins* (Bjerrum, O. J. & Heegaard, N. H. H. eds.), pp. 1–25, CRC Press, Boca Raton, FL
- Hershko, A. & Ciechanover, A. (1982) *Annu. Rev. Biochem.* **51**, 335–364
- Hudson, L. & Hay, F. C. (1980) *Practical Immunology*, 2nd edn., Blackwell, Oxford
- Jensenius, J. C., Andersen, I., Hau, J., Crone, M. & Koch, C. (1981) *J. Immunol. Methods* **46**, 63–68
- Kirschke, H., Langner, J., Riemann, S., Wiederanders, B., Ansoerge, S. & Bohley, P. (1980) *Protein Degradation in Health and Disease: Ciba Found. Symp.* **75**, 15–31
- Kirschner, R. J. & Goldberg, A. L. (1983) *J. Biol. Chem.* **258**, 967–976
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lenney, J. F. (1980) *Curr. Top. Cell. Regul.* **17**, 25–57
- Mayer, R. J. & Doherty, F. (1986) *FEBS Lett.* **198**, 181–193
- Murachi, T. (1983) *Trends Biochem. Sci.* **8**, 167–169
- Obled, A., Ouali, A. & Valin, C. (1984) *Biochimie* **66**, 609–616
- O'Connor-McCourt, M. D. & Wakefield, L. M. (1987) *J. Biol. Chem.* **262**, 14090–14099
- Peterson, G. L. (1983) *Methods Enzymol.* **91**, 95–119
- Porath, J., Carlsson, J., Olsson, I. & Belfrage, G. (1975) *Nature (London)* **258**, 598–599
- Rechsteiner, M. (1987) *Annu. Rev. Cell Biol.* **3**, 1–30
- Salvesen, G. S., Sayers, C. A. & Barrett, A. J. (1981) *Biochem. J.* **195**, 453–461
- Shii, K., Baba, S., Yokono, K. & Roth, R. A. (1985) *J. Biol. Chem.* **260**, 6503–6506
- Slot, L. A. & Hendil, K. B. (1987) *Scand. J. Clin. Lab. Invest.* **47**, 393–397
- Sottrup-Jensen, L., Petersen, T. E. & Magnusson, S. (1981) *FEBS Lett.* **128**, 123–126
- Sottrup-Jensen, L., Stepanik, T. M., Wierzbicki, D. M., Jones, C. M., Lnblad, P. B., Kristensen, T., Mortensen, S. B., Petersen, T. E. & Magnusson, S. (1983) *Ann. N.Y. Acad. Sci.* **421**, 41–60
- Tarasova, N. I., Szecsi, P. & Foltmann, B. (1986) *Biochim. Biophys. Acta* **880**, 96–100
- Travis, J. & Salvesen, G. S. (1983) *Annu. Rev. Biochem.* **52**, 655–709
- Tsuji, S. & Imahori, K. (1981) *J. Biochem. (Tokyo)* **90**, 233–240
- Wang, D., Yuan, A. I. & Feinman, R. D. (1983) *Ann. N.Y. Acad. Sci.* **421**, 90–97
- Waxman, L. (1981) *Methods Enzymol.* **80**, 664–680
- Wilk, S. & Orlowski, M. (1980) *J. Neurochem.* **35**, 1172–1182
- Wittheler, J. & Wilson, D. B. (1972) *J. Biol. Chem.* **247**, 2217–2221
- Zimmerman, M., Ashe, B., Yurewicz, E. C. & Patel, G. (1977) *Anal. Biochem.* **78**, 47–51