Effects of protein-degradation inhibitors on the inactivation of tyrosine aminotransferase, tryptophan oxygenase and benzopyrene hydroxylase in isolated rat hepatocytes

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The following three potent inhibitors of hepatocytic proteolysis were investigated to see if they would inhibit the intracellular inactivation of enzymes: chymostatin and leupeptin (proteinase inhibitors) and methylamine (a lysosomotropic weak base). Chymostatin inhibited the inactivation of two of the three enzymes tested: tyrosine aminotransferase (EC 2.6.1.5) and tryptophan oxygenase (tryptophan 2,3-dioxygenase, EC 1.13.11.11). Leupeptin had no effect on any of the enzymes, whereas methylamine had only a weak inhibitory effect on tyrosine aminotransferase inactivation. Apparently proteolytic cleavage (probably by a non-lysosomal proteinase, since only chymostatin is effective) is involved in the inactivation of tyrosine aminotransferase and tryptophan oxygenase. The third enzyme, benzopyrene hydroxylase (flavoprotein-linked mono-oxygenase, EC 1.14.14.1), is probably inactivated by a non-proteolytic mechanism.

At least four possibilities exist for the first step in the inactivation/degradation of enzymes: proteolytic cleavage; (irreversible) loss of prosthetic groups; removal of enzyme from its normal position; or specific inactivation reactions as, e.g., breaking or making of disulphide bonds. All four possibilities are probably represented in the cell. In the present paper we report data on the importance of proteolytic cleavage in the inactivation of three enzymes.

Isolated hepatocytes are in a state of highly activated protein degradation when incubated in an amino-acid-free medium like the one used in our previous studies (Seglen, 1977; Seglen *et al.*, 1979). The degradation can be inhibited by several types of agents, the most powerful ones being leupeptin, chymostatin and lysosomotropic amines such as methylamine (Seglen *et al.*, 1979; Grinde & Seglen, 1980).

Leupeptin and chymostatin are proteinase inhibitors, in the form of modified oligopeptides, isolated from bacteria (Umezawa & Aoyagi, 1977). Leupeptin is known to inhibit the lysosomal cathepsins A, B, H and L, whereas chymostatin inhibits cathepsin A, B, D and H as well as a non-lysosomal serine proteinase from liver (Umezawa & Aoyagi, 1977; Barrett, 1977).

Methylamine is a lysosomotropic weak base and seems to inhibit lysosomal protein degradation fairly selectively and completely (Seglen *et al.*, 1979; Seglen *et al.*, 1980; Grinde & Seglen, 1980; Kovács

et al., 1981). Accepting this view of methylamine action, leupeptin inhibits approx. 85% of the lysosomal proteolysis, as well as some 10–20% of non-lysosomal proteolysis; similar data for chymostatin indicates about 45% inhibition of lysosomal and more than 50% inhibition of non-lysosomal proteolysis (Grinde & Seglen, 1980).

The three enzymes chosen for the present study all exhibit a fairly rapid turnover in the cell. Proteins with short half-lives are supposedly preferably degraded outside the lysosomes (Ballard, 1977; Seglen *et al.*, 1979).

Two of the enzymes, tyrosine aminotransferase and tryptophan oxygenase, contain prosthetic groups, whereas benzopyrene hydroxylase probably is dependent on membrane integration in order to be active.

Experimental

Cells

Isolated hepatocytes were prepared from the liver of 16 h-starved male Wistar rats (240–280 g body wt.) by the method of collagenase perfusion (Seglen, 1976), and incubated as suspensions in 50 ml conical flasks containing 4–6 ml of cell suspension (30–60 mg wet wt./ml). The flasks were gyrorotated (90 rev./min) in a water bath (37°C). The cells were incubated in a modified version of a medium especially designed for hepatocytes (Schwarze *et al.*,

1981). This medium is an organic buffered-salt solution fortified with glucose, pyruvate, vitamins, amino acids and the antibiotic gentamicin. The modification consisted in excluding the amino acids, including 1% albumin and lowering the NaCl concentration to an osmolality of 290 mosm. Cell integrity, as tested with the Trypan Blue-exclusion method, was generally above 85% after the incubations. The incubations were stopped by cooling 0.5–1.0 ml samples quickly to 0°C by placing them in ice/water.

Measuring protein degradation

Protein degradation was measured as the release of [^{14}C]valine from protein prelabelled *in vivo*. For this purpose, 50 μCi of [^{14}C]valine was intravenously injected 24 h before the cell preparation. Degradation rates were calculated as percentage of the total protein radioactivity at the start of the experiment. Sample preparation and measurement were performed as described previously (Seglen, 1978; Seglen *et al.*, 1979).

Tyrosine aminotransferase assay

The assay for tyrosine aminotransferase was modified from that described by Diamondstone (1966) and is based on the deamination of tyrosine to *p*-hydroxyphenylpyruvate and the further conversion of this substance into *p*-hydroxybenzaldehyde. Cell samples (0.5 ml) were frozen and thawed three times in liquid N_2 to release the enzymes. A portion (3 ml) of reaction buffer (21.5 mM- α -oxoglutarate and 0.075 mM-pyridoxal phosphate in 0.4 M-potassium phosphate buffer, pH 7.35) was added and cell debris removed by centrifugation (2000 *g*, 60 min). The supernatant (enzyme preparation) was left for 2 days at 4°C. Three parallel samples of the enzyme preparation (0.1 ml) were then added to 1.6 ml of reaction buffer and preincubated for 45 min in a shaking water bath (37°C). The reaction was started by adding 1.5 ml of freshly made tyrosine solution (13 mM in the same potassium phosphate buffer, 50°C) and stopped 10 min later by adding 0.2 ml of 10 M-NaOH. The samples were mixed thoroughly and left for 30 min at room temperature. The amount of *p*-hydroxybenzaldehyde was measured spectrophotometrically at 331 nm. One unit of enzyme catalyses the formation of 1 μmol of product/min.

Tryptophan oxygenase assay

Tryptophan oxygenase was assayed by a method measuring the conversion of tryptophan into kynurenine (Seglen & Jervell, 1969a). The cell samples were frozen and thawed as described above and 17 ml of reaction buffer (2.5 mM-tryptophan and 2 μM -haemoglobin in 0.02 M-sodium phosphate buffer, pH 7.0) was added. Portions (4 ml) were incubated in a

shaking water bath at 37°C and reactions in duplicate samples were stopped after 20 min and after 40 min incubation, by adding 1 ml of 15% (v/v) metaphosphoric acid. The rate of kynurenine formation is at a maximum at this period. The protein precipitate was removed by centrifugation (2000 *g* in 10 min), and the supernatants were filtered and neutralized with 1 M-NaOH. The amount of kynurenine formed during the 20 min period was measured spectrophotometrically at 365 nm. A unit of enzyme activity catalyses the formation of 1 μmol of kynurenine/h.

Benzopyrene hydroxylase assay (EC 1.14.14.1)

The assay for benzopyrene hydroxylase was based on the fluorimetric measurement of benzopyrene metabolites. Our method is modified from that described by Dehnen *et al.* (1973). The cell samples were transferred directly from incubation to liquid N_2 , since the enzyme did not appear to be stable when kept at 0°C. The samples were diluted to 10 mg wet wt. of cells/ml with 0.05 M-Trizma (Tris) buffer, pH 7.4, containing 3 mM-MgCl₂. Then 0.4 ml of this enzyme solution (three parallel samples) was added to 0.5 ml of Trizma buffer containing 3 mM-MgCl₂, 10 mM-glucose 6-phosphate, 1 mM-NADP and 0.5 unit of glucose 6-phosphate dehydrogenase/ml. This mixture was preincubated for 1 min at 25°C in a shaking water bath, and the reaction was started by adding 50 μl of 2 mM-benzo[*a*]pyrene dissolved in acetone. After 8 min incubation at 25°C the reaction was stopped by adding 100 μl of 10% (w/v) Triton X-100 in triethylamine and 100 μl of 0.5% EDTA. The samples were shaken and transferred to ice. After 30 min the amount of product was measured as the difference in fluorescence intensity between 520 nm and 475 nm when the samples were excited at 435 nm.

Biochemical reagents

[^{14}C]Valine (Amersham CFB75; 285 Ci/mol, 50 mCi/litre, 0.18 mM) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.; leupeptin and chymostatin from The Peptide Institute, 476 Ina, Mino-shi, Osaka 562, Japan; methylamine from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; dexamethasone from Merck, Sharp and Dohme B. V., Haarlem, The Netherlands; other biochemicals were from Sigma.

Results and discussion

Effects of the inhibitors on total protein degradation

When the protein has been prelabelled by an intravenous injection of [^{14}C]valine 24 h before cell preparation, the measured rate of radioactivity

release seems to come close to the real rate of protein degradation (Seglen, 1977; Seglen *et al.*, 1979). Employing such a labelling procedure in our system, leupeptin, chymostatin and methylamine individually inhibit protein degradation by approx. 70%, leupeptin being slightly less active than the two others (Fig. 1a).

To investigate enzyme inactivation, the protein-synthesis inhibitor cycloheximide was used at 0.1 mM, a concentration high enough to stop 90% of hepatocytic protein synthesis (Woodside, 1976; Seglen, 1977). Cycloheximide does, however, also inhibit protein degradation in the cells (Poli *et al.*, 1981), in our system by 20–25% (Fig. 1b). The inhibitor did not influence cell integrity in our system. The protein-degradation rates in the presence of the degradation inhibitors are about the same whether cycloheximide is included or not. In the presence of cycloheximide the degradation inhibitors cause a 50–65% inhibition of protein degradation, their effects being fairly unchanged throughout 10 h of incubation (Fig. 1b). Leupeptin, chymostatin and methylamine should therefore be suitable for studying the importance of proteolysis in enzyme inactivation.

Fig. 1. Effects of leupeptin, chymostatin and methylamine on protein degradation in the presence and absence of cycloheximide

Hepatocytes were isolated from rats prelabelled for 24 h *in vivo* with [^{14}C]valine. The cells were incubated for up to 10 h in the absence (○) of any inhibitor or in the presence of 0.5 mM-leupeptin (△, ▲), 0.2 mM-chymostatin (▽, ▼) or 10 mM-methylamine (□, ■). Closed symbols indicate the additional presence of 0.1 mM-cycloheximide. The net release of [^{14}C]valine during the incubation was measured and expressed as percentage of the initial protein radioactivity. The Figure shows results from experiments with cells from two different rats (a and b). Each point represents the mean result for two to three cell samples, with standard errors in the range 1–3%.

Inactivation of tyrosine aminotransferase

The administration *in vivo* of dexamethasone plus glucagon increases the tyrosine aminotransferase activity to at least 8–9 times the normal value (results not shown). In cells where the enzyme has been induced, the activity falls significantly during the incubation. With cycloheximide added to the medium, the activity decreases with a half-life of approx. 2 h (Fig. 2b). A similar turnover rate for the enzyme has also been found by other methods (Kenney, 1962; Jervell & Seglen, 1969; Ernest & Feigelson, 1979), indicating that the inactivation of the induced enzyme is occurring normally in the cells.

The effects of leupeptin, chymostatin and methylamine on tyrosine aminotransferase inactivation were examined, in the presence and in the absence of cycloheximide (Fig. 2). In the absence of cycloheximide the degradation inhibitors could possibly decrease the amount of enzyme by inhibiting its synthesis. In the presence of cycloheximide no synthesis of the enzyme would be expected to occur, but cycloheximide was shown to inhibit protein degradation by itself (Fig. 1), and it might also

Fig. 2. Effects of leupeptin, chymostatin and methylamine on the inactivation of tyrosine aminotransferase. Hepatocytes were isolated from rats given 1 mg of dexamethasone and 0.1 mg of glucagon intraperitoneally 6 h before the cell preparation. The cells were incubated for up to 5 h without any addition (○) or with 0.5 mM-leupeptin (△, ▲), 0.2 mM-chymostatin (▽, ▼) or 10 mM-methylamine (□, ■). Closed symbols indicate the additional presence of 0.1 mM-cycloheximide. (In a the inhibitors were given after 2 h of incubation.) Tyrosine aminotransferase was assayed and the results are given as percentage of enzyme activity related to the enzyme activity before the incubations. In (a) 100% is equivalent to 21 units/g wet wt. of cells, and in (b) to 19 units/g of cells. Each point represents the mean result for three cell samples; standard errors were in the range 1–4%.

inhibit other relevant cell processes. However, the same results were obtained both with and without cycloheximide added to the medium. Chymostatin inhibited the enzyme inactivation very strongly, whereas methylamine had a slight inhibitory effect and leupeptin had no effect.

There exist a number of reports referring to the inactivation of tyrosine aminotransferase (see Millward, 1980; Katunuma, 1977). Pyridoxine deficiency has been found to result in a 40% accumulation of the apoform of the enzyme, without changing the degradation rate (Kenney *et al.*, 1978), indicating that loss of coenzyme is not an important first step in the enzyme degradation. Carbamoylation (Johnson *et al.*, 1973) or phosphorylation (Kenney *et al.*, 1973) has been proposed as a reversible, but necessary, step before the hydrolysis of tyrosine aminotransferase. Our results do, however, indicate that proteolysis is the most important step in the inactivation of the enzyme. The proteinase involved would be expected to be non-lysosomal, since chymostatin, but not leupeptin (and methylamine only slightly) inhibits the inactivation. Chymostatin was previously found to inhibit a larger portion of the non-lysosomal (amine-resistant) than the lysosomal protein degradation in isolated hepatocytes, whereas leupeptin acts fairly selectively on lysosomal degradation (Grinde & Seglen, 1980).

The non-lysosomal serine proteinase from liver, which is inhibited by chymostatin (Katunuma, 1977), might be responsible for the inactivation. This proteinase preferably attacks enzymes which use pyridoxal phosphate as coenzyme, and tyrosine aminotransferase contains four pyridoxal phosphate groups in its active form. Katunuma *et al.* (1976) did, however, report that, in a cell-free system, tyrosine aminotransferase is a poor substrate for this proteinase. More recent evidence, furthermore, indicates that the proteinase may reside in mast cells rather than in hepatocytes (Woodbury *et al.*, 1978a,b; Libby & Goldberg, 1980), in which case it would be irrelevant.

The lack of effect of leupeptin and the small effect of methylamine do not necessarily imply that none of the tyrosine aminotransferase molecules are inactivated in the lysosomes. Enzymes internalized in the lysosomes could be inactivated by the conditions there, without going through proteolysis. The small effect of methylamine might be a result of methylamine being inhibitory to the internalization of the enzyme into lysosomes.

Inactivation of tryptophan oxygenase

Tryptophan oxygenase is known to have a turnover rate fairly similar to that of tyrosine aminotransferase (Feigelson *et al.*, 1959; Schimke *et al.*, 1965; Seglen & Jervell, 1969b), and like tyrosine aminotransferase it carries a prosthetic group, which

is haem. Tryptophan oxygenase can be hormonally induced to as much as 10 times its normal value (results not shown). Cells incubated with cycloheximide then gave the expected 2 h half-life for the enzyme (Fig. 3b).

As with tyrosine aminotransferase, chymostatin decreased the rate of tryptophan oxygenase inactivation, as shown in the presence of cycloheximide (Fig. 3b); neither leupeptin nor methylamine had any effect. In the absence of cycloheximide (Fig. 3a) the results were somewhat more difficult to interpret. Chymostatin, leupeptin and methylamine have all been shown to cause some inhibition of protein synthesis in isolated hepatocytes, chymostatin and methylamine being the most inhibitory (Grinde & Seglen, 1980). Apparently the inhibitors do disturb the synthesis of tryptophan oxygenase, but not the synthesis of the two other enzymes tested in the present study. This would explain why, in the absence of cycloheximide, tryptophan oxygenase activity falls more rapidly with, than without, leupeptin or methylamine in the medium (Fig. 3a). The synthesis of tryptophan oxygenase has been shown to be considerably more sensitive to cycloheximide than the synthesis of tyrosine aminotransferase in the perfused liver (Seglen, 1971).

Chymostatin probably has the same effect as

Fig. 3. Effects of leupeptin, chymostatin and methylamine on the inactivation of tryptophan oxygenase. Hepatocytes were isolated from rats given 1 mg of dexamethasone and 0.1 mg of glucagon intraperitoneally 6 h before cell preparation. The cells were incubated for up to 3 h without any addition (○) or with 0.5 mM-leupeptin (△, ▲), 0.2 mM-chymostatin (▽, ▼) or 10 mM-methylamine (□, ■). Closed symbols indicate the additional presence of 0.1 mM-cycloheximide. Tryptophan oxygenase activity was assayed and the results given as percentages of the enzyme activity before the incubations. In (a) 100% is equivalent to 20 units of enzyme activity/g wet wt. of cells and in (b) to 5 units/g of cells. Each point represents the mean result for duplicate cell samples, with standard errors in the range 1–5%.

methylamine and leupeptin on tryptophan oxygenase synthesis; its inhibitory effect on the inactivation of the enzyme, however, keeps the amount of enzyme at about the same value as in control cells.

Apparently some non-lysosomal proteinase is important in the inactivation of tryptophan oxygenase as well. Rudek *et al.* (1978) have found that 3% of the tryptophan oxygenase enzymic activity and 5% of its antigenic activity is associated with the lysosomes when the cells are treated with the lysosomal protein-degradation inhibitor chloroquine. Our data do not rule out that a substantial fraction of the enzyme molecules may be internalized into lysosomes at some stage during their inactivation/degradation.

Inactivation of benzopyrene hydroxylase

Benzopyrene hydroxylase is not a well-characterized enzyme, but a benzopyrene-metabolizing activity found associated with the microsomal membrane, and most likely dependent on membrane integration in order to be active (Conney *et al.*, 1957).

The enzyme activity was induced to 3–4 times its normal value (results not shown) with 20-methylcholanthrene (1,2-dihydro-3-methylbenz[*j*]aceanthrylene). In the absence of cycloheximide, no significant fall in the activity was observed, whereas in the presence of cycloheximide a drastic fall was

noted (Fig. 4). In both cases, however, none of the three protein-degradation inhibitors had any effect on the amount of enzyme activity. If benzopyrene hydroxylase activity is dependent on an integrated position in the endoplasmic reticulum, its release from this membrane, and thereby its inactivation, might precede its degradation.

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Fig. 4. Effects of leupeptin, chymostatin and methylamine on the inactivation of benzopyrene hydroxylase. Hepatocytes were isolated from rats given 8 mg of 20-methylcholanthrene (dissolved in 1 ml of corn oil) 40 h before the cell preparation. The cells were incubated for up to 4 h without any addition (○) or with 0.5 mM-leupeptin (△, ▲), 0.2 mM-chymostatin (▽, ▼) or 10 mM-methylamine (□, ■). Closed symbols indicate the additional presence of 1 mM-cycloheximide. Benzopyrene hydroxylase was assayed and the results given as percentages of enzyme activity before the incubations. Each point represents the mean result for duplicate cell samples, with standard errors in the range 1–5%.

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