Turnover of matrix proteins in mammalian mitochondria

Walter HUTH¹, Stefan ROLLE and Ilona WUNDERLICH

Georg-August-Universität Göttingen, Institut für Biochemie und Molekulare Zellbiologie, Humboldtallee 23, D-37073 Göttingen, Germany

In cultured hepatocytes the turnover of several mitochondrial matrix proteins (e.g. acetyl-CoA acetyltransferase) appears to be initiated by CoA-mediated, sequential transformation into CoA-modified forms. This modification favours the notion that intramitochondrial degradation by a matrix-resident ATP-dependent protease may be preceded by a specific modification by CoA. In a mitochondrial matrix fraction the MgATP-dependent decrease in anti-CoA immunoreactivity coincided with both a decrease in the anti-protein immunoreactivity of acetyl-CoA acetyltransferase and/or of 3-ketoacyl-CoA thiolase, and with the appearance of proteolytic fragments. A closer analysis of the degradation pattern revealed, however, a breakdown of the unmodified acetyl-CoA acetyltransferase and of its CoA-modified form, A1, whereas the form that is more highly modified by CoA, A2, proved to be inaccessible towards an ATP-dependent protease. In mammalian mitochondrial matrix, proteins can be degraded selectively by a matrix-resident ATP-dependent pro-

tease. The process of CoA modification results finally in the protection of matrix proteins from degradation. In cultured hepatocytes, leupeptin, an inhibitor of lysosomal proteases, did not affect the steady-state level of the mitochondrial matrix protein acetyl-CoA acetyltransferase. However, leupeptin mediated a specific accumulation of mitochondrial matrix proteins in the cytosolic fractions of hepatocytes cultured over a 24 h period. The levels of acetyl-CoA acetyltransferase, 3-ketoacyl-CoA thiolase and glutamate dehydrogenase proteins increased 1.9-, 2.0- and 2.2-fold respectively. Their status as mature, oligomeric, but enzymically inactive enzymes strongly suggests that they originate from a leakage of autophagosomes, a constituent of the non-selective autophagic/lysosomal pathway for degradation of whole mitochondria.

Key words: autophagy, CoA modification, hepatocytes, protein degradation.

INTRODUCTION

Covalent modification of proteins by CoA is a type of a post-translational modification process. This modification has been demonstrated for the mutant β -subunit of F_1 -ATPase of *Escherichia coli* [1], for a flavodoxin of *Klebsiella pneumoniae* [2], and for the rat liver mitochondrial matrix enzymes acetyl-CoA acetyltransferase (EC 2.3.1.9), 3-ketoacyl-CoA thiolase (EC 2.3.1.16) [3,4] and hydroxymethylglutaryl-CoA synthase (EC 4.1.3.5) [5]. Several CoA-modified mitochondrial matrix proteins have been detected by anti-CoA antibodies in the rat liver [6]. The modification results in the appearance of multiple enzyme forms [3,7,8]. For example, the acetyl-CoA acetyltransferase protein, exists in four additional forms: A1, A2, A3 and A4 [8]. Of these, two forms (A1 and A2) can be routinely visualized by a combined method of chromatography and immunoelectrophoresis from a small quantity of rat liver and/or from hepatocytes [3,9]. Forms A1 and A2 proved to be CoA-modified forms [3]. The unmodified acetyl-CoA acetyltransferase was transformed by CoA modification into form A1, and subsequently into form A2, with half-lives of transformation of 38.0 h and 24.5 h respectively. The more highly modified form, A2, was presumably transformed into form A3 with a half-life of 7.2 h [9]. This sequential CoA modification favours the notion that the CoA-modified forms may represent intermediates in an initiating turnover process of the acetyl-CoA acetyltransferase protein, i.e. the degradation of this matrix protein by a mitochondrial matrix protease and/or an extramitochondrial proteolytic system may be preceded by a specific modification with its substrate/product CoA. Alternatively, this reversible CoA-modification process of mitochondrial matrix proteins may protect these proteins from degradation by a matrix protease.

ATP-dependent proteases, matrix or membrane localized, exist in mitochondria of eukaryotic cells. A Lon-like ATP-dependent protease that is highly homologous with bacterial Lon protease has been demonstrated in the matrix of mitochondria of rat liver [10] and the bovine adrenal cortex [11], and in human mitochondria [12]. The mitochondrial matrixes additionally contain the ATP-dependent Clp XP protease [13]. The ATP-dependent AAA-proteases of the inner membrane of mitochondria [14,15] face either the intermembrane space (i-AAA proteases; Yme1p) or the matrix (m-AAA proteases; Yta10p and Yta12p). They mediate the ATP-dependent degradation of mitochondrial membrane proteins. A novel ATP-dependent protease has been demonstrated in the intermembrane space of mitochondria [16].

Mammalian mitochondrial matrix proteins exhibit different turnover rates [17]. Little is known about the physiological protein substrates of the ATP-dependent matrix proteases [18–21]. In fact, it is not known at present which proteolytic system, i.e. of the mitochondrial matrix ATP-dependent proteases, the lysosomes or the cytosolic proteasome, is responsible for the different turnover rates of the mammalian mitochondrial matrix proteins during the normal operation of the organelle. The process of a macroautophagy of whole mitochondria by autophagosomes/autolysosomes [22–24], activated during stages of nutrient deprivation [25], should result in the same degradation rates for all mitochondrial proteins. The existence of CoA-modified forms of mitochondrial matrix proteins and of ATP-dependent proteases in the matrix of mitochondria prompted us to study the effect of MgATP on the amount and the pattern of the endogenously occurring CoA-modified proteins in a mitochondrial matrix protein fraction. In addition, the influence of lysosomal and/or proteasomal inhibitors on the turnover of mitochondrial matrix proteins was analysed in cultured hepatocytes. The tools used in these investigations were immunoblots

Abbreviation used: p[CH₂]₃ppA, adenosine 5'-[β , γ -methylene]triphosphate.

¹ To whom correspondence should be addressed (e-mail whuth@gwdg.de).

using monospecific antibodies raised against CoA and/or several mitochondrial matrix proteins, and pulse-chase experiments using [35 S]methionine.

EXPERIMENTAL

The materials were obtained from the following sources: ATP, adenosine 5'-[β , γ -methylene]triphosphate (p[CH $_2$]ppA), phosphocreatine, leupeptin and creatine kinase were from Boehringer Mannheim (Mannheim, Germany); MG 132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal ('LLL') and lactacystin were from Calbiochem (Bad Soden, Germany); Percoll and SDS/PAGE calibration proteins were from Pharmacia Biotech (Freiburg, Germany); PMSF, metrizamide and tosylphenylalanylchloromethane-treated trypsin were from Sigma-Aldrich (Deisenhofen, Germany); sodium orthovanadate was from AppliChem (Darmstadt, Germany); acrylamide was from Serva (Heidelberg, Germany); Immobilon-P membranes were from Millipore Waters (Eschborn, Germany); Medium M199 was from Biochrom KG (Berlin, Germany); and L-[35 S]methionine, L-[3 H]methionine, enhanced chemiluminescence Western blotting reagents, biotinylated molecular-mass markers and Hyperfilm were from Amersham (Braunschweig, Germany). All other chemicals were of high purity, and were obtained from Merck (Darmstadt, Germany).

Animals

Male Wistar rats were obtained from Winkelmann (Borchen, Germany) and were kept on a 12 h day–12 h night cycle with free access to water and diet from Ssniff (Soest, Germany). The animals were killed under ether anaesthesia, and livers were immediately excised.

Preparation of the mitochondrial matrix fraction from rat livers

Mitochondria and a pure mitochondrial matrix protein fraction [yield of $95.1 \pm 5.2\%$, as calculated from glutamate dehydrogenase (EC 1.4.1.2) activity] were prepared as described previously [6], except that protease inhibitors were omitted. The matrix protein fraction was stabilized in 50 mM potassium phosphate, pH 7.2, and 20% (v/v) glycerol.

Incubation of mitochondrial matrix fractions and phosphocellulose chromatography

For immunoreactive studies, samples of 100 μ g of mitochondrial matrix proteins in 100 mM Tris/HCl buffer, pH 8.1, were either subjected directly to SDS/PAGE or were incubated (60 min,

37 °C) before SDS/PAGE in the absence (controls) or presence of the following compounds: 10 mM MgSO $_4$ and 5 mM of either ATP or p[CH $_2$]ppA, supplemented with an ATP-regenerating system (50 mM phosphocreatine and 16 units of creatine kinase). To examine effects of MgATP and/or trypsin on the degradation of the acetyl-CoA acetyltransferase protein, 25 mg of mitochondrial matrix proteins was incubated in a 50 mM potassium phosphate buffer, pH 7.8, for 60 min at 37 °C in the absence or presence of 5 mM MgATP supplemented with an ATP-regenerating system, and/or in the presence of a CoA-trapping system containing 15 mM acetyl phosphate and 100 units of phosphotransacetylase. Additionally, the matrix fractions were incubated (10 min, 37 °C) in presence of 500 μ g of trypsin. The reaction was stopped by addition of 1 mM PMSF. The mitochondrial matrix protein fraction was also used for a partial purification of acetyl-CoA acetyltransferase and its CoA-modified forms using a two-dimensional method of phosphocellulose chromatography followed by 'fused rocket' immunoelectrophoresis, as described previously [3,9].

Pulse-chase experiments

Cell culture

Hepatocyte cultures in medium M199 and pulse-chase experiments using L-[35 S]methionine [10 μ Ci/dish of specific radioactivity 70 mCi/mmol, and methionine (final concentration 50 μ M)] were performed as described by Schwerdt and Huth [9]. Following the pulse-chase procedure, the cells were directly harvested or incubated for 24 h in M199 medium in the absence or presence of inhibitors at the concentrations shown in the Figures and Tables. MG132 was dissolved in DMSO at a final concentration in the cell-culture medium of 0.3% (v/v).

Cell organelles

Cells (10 dishes) were disrupted by a ball-bearing Dounce homogenizer in 0.25 M sucrose medium containing leupeptin (5 μ g/ml), 1 mM EDTA and 1 mM PMSF to inhibit proteolysis. The cytosolic fraction was the supernatant of two successive centrifugations of the cell homogenate (first, at 2100 g for 10 min, and the supernatant was then spun at 105 000 g for 60 min). The pellet from the first centrifugation containing nuclei, mitochondria and significant portions of lysosomes, presumably due to trapping organelles in the cytoskeleton [26], was resuspended in the sucrose medium and homogenized using a tight-fitting homogenizer. This homogenate was washed three times, and then centrifuged at 300 g for 10 min to separate nuclei and unbroken cells to obtain a post-nuclear supernatant.

Table 1 Preferential degradation of the unmodified acetyl-CoA acetyltransferase and of the CoA-modified form A1 by a mitochondrial matrix ATP-dependent protease and/or by trypsin

Following an incubation of mitochondrial matrix fraction in the absence (control) or presence of MgATP and/or trypsin, the acetyl-CoA acetyltransferase protein was resolved into its unmodified enzyme and into its CoA-modified forms, as demonstrated in Figure 4. The immunoreactive areas (mm 2) were photographically enlarged and were calculated using a trapezium sum program. To compare the experiments from different preparations of the mitochondrial matrix fractions, the values (means $\pm$ S.D.) are expressed as percentages of the total immunoreactive area of the respective control experiments. The numbers of experiments are given in parentheses. Statistics were calculated as compared with the respective state of the control experiments. NS, not significant.

Additions	Immunoreactive area (%)		CoA-modified form	
	Total protein	Unmodified enzyme	A1	A2
None (17) (control)	100	49.0 $\pm$ 6.1	37.3 $\pm$ 5.5	13.7 $\pm$ 4.4
MgATP (7)	79.5 $\pm$ 11.1	37.4 $\pm$ 7.1 ($P < 0.01$)	27.2 $\pm$ 5.2 ($P < 0.001$)	14.9 $\pm$ 2.0 (NS)
MgATP (2) (CoA trapping)	63.4	21.6	26.7	15.1
Trypsin (8)	64.7 $\pm$ 14.6	20.1 $\pm$ 7.2 ($P < 0.001$)	27.0 $\pm$ 7.4 ($P < 0.001$)	17.6 $\pm$ 5.9 (NS)

Table 2 Accumulation of mitochondrial matrix proteins in the cytosol of hepatocytes: effects of lysosomal and/or proteasomal inhibitors

The cytosolic fractions of 24 and/or 72 h cultured hepatocytes in the absence or in the presence of 100 μ M MG 132, 18.2 μ M lactacystin, 5 μ g/ml leupeptin and/or 5 μ g/ml leupeptin in the presence of 10 mM 3-methyladenine were analysed four times by immunoblotting using antibodies against acetyl-CoA acetyltransferase, 3-oxoacetyl-CoA thiolase and glutamate dehydrogenase, as outlined in Figure 6. The immunoreactivity of the mitochondrial acetyl-CoA acetyltransferase could be quantified specifically as the antibodies against the mitochondrial enzyme do not cross react with the cytosolic acetyl-CoA acetyltransferase [40]. The immunoblots were quantified densitometrically. The values of immunoreactive intensity (means $\pm$ S.D.) are expressed as percentages of immunoreactive intensity present in the cytosolic fraction of 24 and/or 72 h culture of hepatocytes in the absence of inhibitors (control) which were set at 100%. Numbers of experiments are given in parentheses. However, 11 experiments were performed for detection of 3-oxoacetyl-CoA thiolase in the absence or presence of leupeptin (24 h culture). Statistics were calculated as compared with the respective state of control experiments. nd, not determined; NS, not significant.

Culture (h)	Additions	Immunoreactive intensity (%)		
		Acetyl-CoA acetyltransferase	3-Oxoacetyl-CoA thiolase	Glutamate dehydrogenase
24 (5)	None (control)	100	100	100
24 (5)	Leupeptin	187 $\pm$ 53 ($P < 0.01$)	199 $\pm$ 32 ($P < 0.001$)	216 $\pm$ 77 ($P < 0.05$)
72 (3)	None (control)	100	100	nd
72 (4)	Leupeptin	256 $\pm$ 107 ($P < 0.05$)	235 $\pm$ 94 ($P < 0.05$)	nd
24 (6)	Leupeptin and 3-methyladenine	nd	103 $\pm$ 7 (NS)	nd
24 (5)	MG 132	119 $\pm$ 22 (NS)	133 $\pm$ 31 ($P < 0.05$)	nd
24 (3)	Lactacystin	114 $\pm$ 37 (NS)	80 $\pm$ 27 (NS)	nd

Table 3 Contamination of the cytosolic fractions by mitochondrial matrix and lysosomal enzymes

The cytosolic fractions (numbers of experiments are given in parentheses) were analysed for enzymically active glutamate dehydrogenase, 3-oxoacetyl-CoA thiolase and β -N-acetylhexosaminidase. The contamination by mitochondrial matrix enzymes were calculated from the activities of glutamate dehydrogenase and of 3-oxoacetyl-CoA thiolase, whereas a contamination by lysosomal enzymes was based on the activity of β -N-acetylhexosaminidase. The values (means $\pm$ S.D.) are expressed as percentages of the total cellular activity of the respective enzyme in the cell homogenate. Statistics were calculated as compared with the respective state of the control experiment. NS, not significant.

Culture (h)	Additions	Contamination (% of total enzymic activity)	
		Mitochondrial matrix enzymes	Lysosomal enzyme
24 (15)	None	3.4 $\pm$ 2.4	14.6 $\pm$ 6.1
24 (13)	Leupeptin	4.1 $\pm$ 2.2 (NS)	29.3 $\pm$ 19.9 (NS)
72 (4)	None	2.4 $\pm$ 1.0	16.9 $\pm$ 8.0
72 (4)	Leupeptin	3.8 $\pm$ 2.0 (NS)	20.8 $\pm$ 11.2 (NS)
24 (5)	MG132	3.1 $\pm$ 2.0 (NS)	27.0 $\pm$ 6.3 ($P < 0.05$)
24 (4)	Lactacystin	3.3 $\pm$ 2.4 (NS)	16.7 $\pm$ 7.0 (NS)
24 and 72 (19)	None	3.2 $\pm$ 1.8	15.1 $\pm$ 6.4
24 and 72 (17)	Leupeptin	4.2 $\pm$ 2.0 (NS)	26.7 $\pm$ 17.1 ($P < 0.05$)

For preparation of lysosomes, the post-nuclear supernatant was successively centrifuged at 2100 g for 10 min and at 34700 g for 10 min. The sediment of the second centrifugation (mitochondrial and lysosomal fraction) was loaded on to the bottom of a discontinuous metrizamide gradient, and the lysosomes were separated by flotation with a centrifugation of 50500 g for 30 min using an SW 41-TI Beckman rotor, as described by Storrie and Madden [26]. The yield of pure lysosomes that were banded at the 5–17% metrizamide interphase was approx. 4%, as calculated from β -N-acetylhexosaminidase (EC 3.2.1.52) activity. A mitochondrial extract from hepatocytes was prepared as described by Schwerdt and Huth [9].

Total organelle-specific enzyme activities (mitochondria and lysosomes) in the cell homogenate were measured following the lysis of the organelles with 0.5% (w/v) Triton X-100 for 1 h at 4 $^{\circ}$ C with constant stirring, and a centrifugation at 105000 g for 60 min. The contamination of the cytosolic fractions by mitochondrial matrix and lysosomal enzymes was estimated as the

percentage of the total activities of 3-ketoacetyl-CoA thiolase, glutamate dehydrogenase and of β -N-acetylhexosaminidase present in the cell homogenate, and is indicated in the Results section. Glutamate dehydrogenase and β -N-acetylhexosaminidase were assayed as described previously [27,28]. 3-Ketoacetyl-CoA thiolase was measured as described previously [3,29].

SDS/PAGE, immunochemical methods and size exclusion chromatography

SDS/PAGE under non-reducing or reducing conditions was performed as described by Laemmli [30] with 12% (w/v) polyacrylamide. Biotinylated molecular-mass markers were phosphorylase b (97.4 kDa), BSA (68 kDa), ovalbumin (46 kDa) carbonic anhydrase (31 kDa) and lysozyme (14.4 kDa). Polyclonal antibodies against CoA, acetyl-CoA acetyltransferase, 3-ketoacetyl-CoA thiolase and glutamate dehydrogenase were raised in rabbits, and were isolated as described previously [6,31]. The discontinuous system was used for semi-dry immunoblotting on Immobilon-P membranes [32]. For detection of the CoA-modified proteins, the enhanced chemiluminescence amplification system was employed using antibodies raised against CoA, acetyl-CoA acetyltransferase, 3-ketoacetyl-CoA thiolase and glutamate dehydrogenase. Of each experiment, four or five immunoblots, if not stated otherwise, were quantified densitometrically using an HP Scan Jet 4c/T scanner and W/N CAM 2.2 gel documentation software (Cybertech, Berlin, Germany). A standard curve using antibodies against CoA and/or acetyl-CoA acetyltransferase revealed the reaction occurred quantitatively up to the level of 10 and 0.1 ng of antigen respectively. For immunoprecipitation at 4 $^{\circ}$ C, the cytosolic fractions were pre-cleared by incubation with Protein A (the same amount which is used to precipitate the primary antibody) for 60 min with continuous shaking, followed by centrifugation (10000 g , 5 min). To the resultant supernatant, phosphoenolpyruvate carboxykinase antiserum (6 μ l of antiserum/100 μ g of cytosolic protein) was added, and this mixture was incubated for 2 h at room temperature. To precipitate the protein-antibody complex, the sample was incubated with Protein A (0.75 μ l/ μ g IgG) for 3 h at 4 $^{\circ}$ C with continuous shaking, and then pelleted by centrifugation. The immunoprecipitate was washed, removed from Protein A, and measured for the radioactivity as described by Schwerdt and Huth [9]. The specific radioactivity (expressed as c.p.m./100 μ g of cytosolic protein) was analysed by immuno-

precipitations in triplicate, with 100% representing the given pulse of ^{35}S -label at zero time for the chase. The specific radioactivities at $t = 24$ h of the chase were expressed as percentages of that at $t = 0$ h, and are given as means $\pm$ S.D. Size-exclusion chromatography of the cytosolic fractions were performed with a Sephacryl S200HR column calibrated with the molecular-mass markers β -amylase (200 kDa), alcohol dehydrogenase (159 kDa), BSA (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa). The fractions were screened by measuring the immunoreactivity towards antibodies against acetyl-CoA acetyltransferase, 3-ketoacyl-CoA thiolase and glutamate dehydrogenase, and by enzymic activity.

Statistical analysis

The statistical significance of the effects was tested using Student's t test analysis in comparison with the respective states in the control experiments as for MgATP and/or trypsin (see Table 1) and for leupeptin, lactacystin and/or MG132 (see Tables 2 and 3) and were given in P values.

RESULTS

The partly purified acetyl-CoA acetyltransferase protein exhibited a tendency to dissociate into its subunits, which apparently depends on the CoA-modification status of the protein. Figure 1 demonstrates that 7% of the total unmodified enzyme, but 22% of the total CoA-modified form A2, appeared as monomers. Therefore the CoA modification of proteins resulting in the appearance of monomers might facilitate their proteolytic degradation. To determine whether CoA-modified mitochondrial matrix proteins have an increased susceptibility towards ATP-dependent mitochondrial matrix proteases, an *in vitro* assay using the anti-CoA and anti-protein immunoreactivity of these proteins was established with a pure mitochondrial matrix fraction.

MgATP-mediated effects on CoA- and on protein-immunoreactivity of mitochondrial matrix proteins

Immunoblots of mitochondrial matrix proteins with anti-CoA antibodies confirmed previous results [6] on endogenously occurring CoA-modified proteins with molecular masses of 54, 45, 43 and 41 kDa (Figure 2, lanes 1, 4, 6 and 9). The CoA-modified 41 kDa protein represents the acetyl-CoA acetyltransferase, as

Figure 1 The oligomerization status of acetyl-CoA acetyltransferase depends on CoA modification

Partly purified unmodified acetyl-CoA acetyltransferase and its CoA-modified forms A1 and A2 were prepared from a rat liver mitochondrial matrix fraction by cation-exchange chromatography [3]. Of each protein form, 60 μg was loaded on to a sucrose-density gradient of 5–20% sucrose, and were run on a SW40TI rotor at 10000 g for 17 h. The gradient fractions were acid-precipitated, washed and analysed by Western blotting (SDS/PAGE) using anti-(acetyl-CoA acetyltransferase) antibodies, before densitometric quantification. The sucrose gradient was calibrated using BSA (66 kDa), alcohol dehydrogenase (150 kDa), catalase (232 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa).

Figure 2 MgATP-mediated decrease in the anti-CoA immunoreactivity of endogenous CoA-modified mitochondrial matrix proteins

Mitochondrial matrix fractions (100 μg of protein) were subjected to SDS/PAGE either directly or after incubation for 60 min at 37 $^{\circ}\text{C}$ in the absence (controls) or presence of MgATP or p[CH₂]ppA supplemented with an ATP-regenerating system (regen. s.). Additionally present in the incubation medium were 5 $\mu\text{g}/\text{ml}$ leupeptin (lane 7) and 1 mM vanadate (lane 8). In contrast with experiments (A)–(C), (D) was performed in the presence of sodium acetate buffer (pH 4.5) instead of Tris/HCl buffer (pH 8.1). Immunoblots were performed with anti-CoA antibodies.

has been demonstrated previously [6]. MgATP, requiring an ATP-regenerating system for maximal activity, lowered distinctly the immunoreactive intensity of all CoA-immunoreactive proteins (lanes 3 and 7). The incubation of mitochondrial matrix proteins in the absence of added MgATP did not result in a significant decrease in the observed immunoreactivity (lane 2). MgATP could not be replaced by the non-hydrolysable ATP analogue, p[CH₂]ppA (lane 5). The lysosomal inhibitor leupeptin did not abolish the MgATP-mediated effect on the anti-CoA immunoreactivity (lane 7). In contrast, vanadate, an inhibitor of the ATPase activity of mammalian-mitochondrial-matrix ATP-dependent Lon protease [10], suppressed appreciably the MgATP-mediated decrease in anti-CoA immunoreactivity (lane 8). Decrease in the anti-CoA immunoreactivity by MgATP was, however, also suppressed at pH 4.5 (lane 9), which is the pH optimum for lysosomal proteases, i.e. the reduction in the CoA-immunoreactivity cannot be traced back to a concomitant degradation by lysosomal proteases. The MgATP-mediated effects on the anti-CoA immunoreactivity might have been caused by non-proteolytic exchange of protein-bound CoA and/or by proteolytic degradation of CoA-modified proteins. To test both possibilities, the immunoreactivity of two matrix proteins was analysed. Figure 3 demonstrates MgATP effects on the protein immunoreactivity of the CoA-modified enzymes 3-ketoacyl-CoA thiolase and acetyl-CoA acetyltransferase (lanes 2 and 3). The loss of protein immunoreactivity of these two enzymes coincided with the appearance of immunoreactive fragments. These protein fragments, which exhibit smaller molecular masses than the corresponding subunits of 43 and 41 kDa for thiolase and acetyltransferase respectively, are probably of proteolytic origin.

The MgATP-mediated decrease in the CoA and protein immunoreactivity of the acetyl-CoA acetyltransferase and 3-ketoacyl-CoA thiolase enzymes in a mitochondrial matrix fraction, resulting in the appearance of proteolytic fragments,

Figure 3 MgATP-mediated decrease in the immunoreactivity of acetyl-CoA acetyltransferase and 3-ketoacyl-CoA thiolase is accompanied by the appearance of immunoreactive fragments

Mitochondrial matrix fractions (30 μ g of protein) were incubated for 60 min at 37 °C in the absence (controls) or presence of MgATP with an ATP-regenerating system (regen. s.), and then subjected to SDS/PAGE. Immunoblots were performed in lanes 1 and 2 with antibodies raised against 3-ketoacyl-CoA thiolase (Thiolase) and, in lanes 3 and 4, with antibodies against acetyl-CoA acetyltransferase (AAT). The incubation of the matrix proteins in the absence of MgATP (controls) did not result in a significant decrease in the acetyltransferase and/or the thiolase immunoreactivity when compared with matrix proteins subjected directly to SDS/PAGE (results not shown).

indicates the susceptibility of these proteins towards an ATP-dependent mitochondrial matrix protease. It is, however, unknown whether the CoA-modified forms of the mitochondrial matrix proteins are only, and/or preferentially, degraded by an ATP-dependent protease of mitochondrial matrix origin.

Differential proteolytic breakdown of the mitochondrial matrix protein acetyl-CoA acetyltransferase

The incubation of the mitochondrial matrix fraction in the absence of added MgATP did not affect the immunoreactive area covered by the acetyl-CoA acetyltransferase protein, as demonstrated by fused-rocket immunoelectrophoresis (Table 1). The values of 49.0 ± 6.1 , 37.3 ± 5.5 and 13.7 ± 4.4 % for the immunoreactive area of the unmodified enzyme, forms A1 and A2 respectively, show no significant differences to the values published previously [3]. MgATP, however, when added to the matrix fraction, mediated a distinct decrease in the total immunoreactive area covered by acetyl-CoA acetyltransferase (Figure 4). This decrease can be traced back to a significant decrease in the immunoreactive area of both the unmodified enzyme and the CoA-modified form A1. The immunoreactive area of the more highly CoA-modified form A2, however, remained stable (Table 1). This apparent stability of A2 might result from its continual replacement by a putative CoA-mediated sequential transformation of the unmodified enzyme into the CoA-modified forms during the incubation procedure. However, in the presence of a CoA-trapping system, form A2 remained stable. Furthermore, A2 proved to be stable on incubation with trypsin in the

Figure 4 MgATP-mediated decrease in the immunoreactivity of acetyl-CoA acetyltransferase depends on the CoA-modification status

Mitochondrial matrix fractions (25 mg of protein) were incubated at 37 °C for 60 min in the absence (control) or the presence of the MgATP-regenerating system. For resolution and the demonstration of acetyl-CoA acetyltransferase protein and their CoA-modified forms, the mitochondrial matrix proteins were chromatographed on a phosphocellulose column, and the fractions were subsequently visualized by fused-rocket immunoelectrophoresis, as described by Huth et al. [3].

absence of MgATP. Hence, in the mitochondrial matrix fraction, an ATP-dependent protease and/or trypsin preferentially degraded the unmodified acetyl-CoA acetyltransferase enzyme and the CoA-modified form A1, and not form A2 [3]. In these experiments immunoreactive fragments of proteolytic origin could not be detected by the two-dimensional method of cation-exchange chromatography/fused-rocket immunoelectrophoresis, which was performed to demonstrate the acetyltransferase and its CoA-modified forms [3]. These peptides were probably separated by the cation-exchange chromatographic step. The characteristic degradation pattern of the acetyl-CoA acetyltransferase protein, as demonstrated in Figure 4 and Table 1, provides an explanation for the decrease in CoA and protein immunoreactivities, as demonstrated in Figures 2 and 3.

The unmodified acetyl-CoA acetyltransferase and the A1 form proved to be accessible, whereas form A2 proved to be inaccessible, to matrix ATP-dependent proteases. Moreover, upon partial purification, form A2 exhibits *in vitro* (Figure 1) a distinct tendency to dissociate into its monomers. Thus CoA modification is a process that confers an inaccessibility towards proteases, and might induce instability to the native oligomeric protein. Therefore the question arose as to whether the monomers of CoA-modified matrix proteins might be translocated into the cytosol and degraded either by the proteasomes or the lysosomes.

Effect of lysosomal and/or proteasomal inhibitors on the steady-state level of the mitochondrial matrix protein acetyl-CoA acetyltransferase in cultured hepatocytes

Leupeptin, an inhibitor of lysosomal cysteine and serine proteases, also mediates the inhibition of the autophagic/lysosomal pathway for degradation of whole mitochondria [23]. MG132

Figure 5 Lysosomal and/or proteasomal inhibitors do not affect the steady-state level of mitochondrial acetyl-CoA acetyltransferase

Mitochondrial extracts (30 µg of protein) from hepatocytes, cultured in pulse-chase experiments, were subjected to SDS/PAGE. Lane 1, 0 h chase; lane 2, 24 h chase; lanes 3 and 4, 24 h chase in the presence of 5 µg/ml leupeptin and 100 µM MG132 respectively. Immunoblots were performed with anti-(acetyl-CoA acetyltransferase) antibodies. Protein bands from three experiments were quantified densitometrically. The immunoreactive intensity is expressed as the percentage of the relevant immunoreactive protein present at 0 h chase.

Figure 6 Leupeptin causes an accumulation of mitochondrial matrix proteins in the cytosol, as demonstrated by immunoblotting

Cytosolic fractions were prepared from hepatocytes cultured for 24 and/or 72 h in the absence or in the presence of 100 µM MG132, and/or 5 µg/ml leupeptin. From the cytosol, 200 µg of protein was subjected to SDS/PAGE under reducing conditions. The molecular masses of immunoreactive proteins were determined using a calibration curve with appropriate molecular-mass markers. The immunoblots were performed with antibodies against acetyl-CoA acetyltransferase (AAT), 3-ketoacyl-CoA thiolase, thiolase and glutamate dehydrogenase (Glu-DH). The immunoblots with the cytosolic fractions from hepatocytes cultured in the presence of 5 µg/ml leupeptin and 10 mM 3-methyladenine are not shown. In the controls, i.e. in the absence of inhibitors, the immunoreactive 3-ketoacyl-CoA thiolase and Glu-DH amounted to 5.0 ± 2.2 and $2.3 \pm 1.6\%$ of enzymic activities (mean values for 24 and 72 cultured hepatocytes) of the respective total cellular enzymic activities. The activity of the mitochondrial AAT could not specifically be measured, since the cytosol of hepatocytes also contains a specific AAT.

reversibly inhibits cytosolic proteasomes [33]. In cultured hepatocytes, these lysosomal and/or proteasomal inhibitors did not result in an increase in the total amount of acetyl-CoA acetyltransferase; that is, the sum of the unmodified enzyme and its modified forms (Figure 5). Leupeptin is reported to be less permeable in muscle cells [34], but to exert inhibiting effects in

the isolated perfused liver [35]. For controlling the effect of inhibitors in cultured hepatocytes, the breakdown of cytosolic phosphoenolpyruvate carboxykinase was analysed in pulse-chase experiments. This carboxykinase, which showed, after 24 h of the chase, a specific radioactivity of $27.7 \pm 2.5\%$ of that at $t = 0$, was degraded in accordance with its half-life of 11 h [36]. This degradation rate was not altered in the presence of MG 132 (specific radioactivity $22.5 \pm 4.2\%$). In the presence of leupeptin, however, the specific radioactivity of 62.4 ± 0.8 after 24 h of the chase indicates a distinct suppression of the breakdown of the cytosolic phosphoenolpyruvate carboxykinase.

The failure of the lysosomal and/or proteasomal inhibitors to mediate an elevation of the intramitochondrial steady-state level of acetyl-CoA acetyltransferase does not definitively exclude an extramitochondrial breakdown of this matrix protein. If lysosomal and/or proteasomal inhibitors do not affect a putative retrograde translocation of matrix proteins into the cytosol, then the proteins cannot accumulate intramitochondrially; however, they should accumulate in the cytosol.

Effect of the lysosomal and/or proteasomal inhibitors on the occurrence of mitochondrial matrix proteins in cytosolic and lysosomal fractions of hepatocytes

Following 24 h of culture of hepatocytes in the absence or in the presence of leupeptin, MG 132 and/or lactacystin, the cytosolic fractions were prepared and screened for the presence of mitochondrial matrix proteins. From the immunoblots (Figure 6) and their densitometric analyses (Table 2), it became evident that mitochondrial matrix proteins, e.g. acetyl-CoA acetyltransferase, 3-ketoacyl-CoA thiolase and glutamate dehydrogenase, can be detected in the cytosolic fractions. Leupeptin caused a significant 1.9-, 2.0- and 2.2-fold accumulation of immunoreactive acetyl-CoA acetyltransferase, 3-ketoacyl-CoA thiolase and glutamate dehydrogenase respectively. The leupeptin-mediated accumulation of the acetyltransferase and thiolase was intensified when the hepatocytes were cultured for 72 h, i.e. their respective immunoreactive intensities were increased 2.6- and 2.3-fold. However, in the presence of 3-methyladenine, an inhibitor of the autophagic sequestration of mitochondria [37], the accumulation of mitochondrial matrix proteins, e.g. 3-ketoacyl-CoA thiolase, in the cytosolic fractions was suppressed (Table 2). In the presence of proteasomal inhibitors, the lower extent of an accumulation of 3-ketoacyl-CoA thiolase with MG 132 seems not to be caused by an inhibition of proteasomes, but rather by an additional inhibitory effect of MG132 on lysosomal cysteine proteases [33]. Thus lactacystin, a more specific irreversible inhibitor of proteasomes [38,39], caused no accumulation of this mitochondrial matrix protein (Table 2).

To analyse the source of immunoreactive mitochondrial matrix proteins, the cytosolic fractions were screened for contamination by enzymically active mitochondrial and/or lysosomal proteins (Table 3), which should originate from the lysis of mitochondria, autophagosomes/autolysosomes and/or lysosomes. The cytosolic fractions from hepatocytes cultured in the absence of inhibitors (controls) exhibited contamination by matrix proteins of $3.4 \pm 2.4\%$ (24 h of culture) and 2.4 ± 1.0 (72 h culture). These proteins contribute to the respective immunoreactivity detected. However, none of leupeptin, MG132 or lactacystin caused a significant increase in the cytosolic activities of mitochondrial matrix enzymes in the cytosolic fractions prepared from 24 h- and/or 72 h-cultured hepatocytes. Leupeptin, in addition, did not cause a significantly enhanced contamination of the cytosolic fractions by mitochondrial matrix enzymes when its effect was calculated on the basis of all the values of 24- and 72 h-cultured

Figure 7 Characterization of the mitochondrial matrix proteins accumulated in the cytosol

Cytosolic fractions were prepared from hepatocytes cultured for 17 h in the presence of 5 μ g/ml leupeptin. (A) Immunoblot of the cytosolic fractions (50 μ g of protein), of mitochondrial matrix proteins (20 μ g of protein) and of purified acetyl-CoA acetyltransferase (AAT; 0.5 μ g) with antibodies raised against AAT. (B) Immunoblot of cytosolic fractions (200 μ g of protein), SDS/PAGE under non-reducing conditions, using anti-CoA antibodies. The molecular mass of the immunoreactive proteins were determined using a calibration curve with appropriate molecular-mass markers. (C) Size-exclusion chromatography on Sephacryl S200 HR columns. The values of K_d , distribution coefficients and of the molecular masses were taken from a calibration curve with appropriate molecular-mass markers (see the Experimental section). The cytosolic fractions (approx. 8 mg of protein) were run on the Sephacryl S200 column. The eluted fractions (1 ml) were concentrated and analysed for immunoreactive glutamate dehydrogenase (Glu-DH), the 3-ketoacyl-CoA thiolase (Thiolase) and the acetyl-CoA acetyltransferase (AAT) proteins using the respective antibodies. The glutamate dehydrogenase fraction was almost within the exclusion volume of the Sephacryl S200 column.

hepatocytes (Table 3). Thus, in the presence of leupeptin, the acetyl-CoA acetyltransferase, 3-ketoacyl-CoA thiolase and glutamate dehydrogenase immunoreactive proteins did not accumulate in the cytosolic fractions due to an increased leakage of mitochondria. However, in the presence of leupeptin, the cytosolic fractions (24 h- and 72 h-cultured hepatocytes) exhibited a significantly increased contamination by the lysosomal enzyme β -N-acetylhexosaminidase (Table 3), indicating an enhanced leakage of lysosomes.

To characterize the enzymes further, the size, CoA modification and oligomerization status of the accumulated mitochondrial matrix proteins in the cytosolic fractions were analysed. Acetyl-CoA acetyltransferase, but not 3-ketoacyl-CoA thiolase, is known to be synthesized as a precursor with a cleavable N-terminal mitochondrial targeting sequence [41,42]. The cleavage of this targeting sequence proceeds in the matrix compartment of mitochondria. Figure 7(A) demonstrates that the accumulated acetyl-CoA acetyltransferase is present as the mature form of the

Figure 8 Leupeptin-mediated accumulation of mitochondrial matrix proteins in a lysosomal fraction

Lysosomal fractions were prepared from hepatocytes cultured for 17 h in the absence or presence of 5 μ g/ml leupeptin. Immunoblots of lysosomal fractions (270 μ g protein) with antibodies against 3-ketoacyl-CoA thiolase (Thiolase) and glutamate dehydrogenase (Glu-DH) are shown. Immunoreactive acetyl-CoA acetyltransferase could also be demonstrated in the presence of leupeptin. The immunoblot, however, was not demonstrated as the antibodies resulted additionally in a so-called 'immunoreactive ladder' and the specific protein could not be clearly shown.

protein. In addition, acetyl-CoA acetyltransferase is known to be CoA-modified within mitochondria [3]. Figure 7(B) shows that leupeptin, in contrast with its effect on the protein immunoreactivity, does not mediate an accumulation of CoA immunoreactivity of acetyl-CoA acetyltransferase, i.e. CoA has been released during the process of accumulation. Size-exclusion chromatography revealed that the mitochondrial matrix proteins do not accumulate as monomers, but as single oligomeric peaks (Figure 7C): glutamate dehydrogenase and 3-ketoacyl-CoA thiolase appeared with molecular masses of approx. 250 [43] and 169 [42] kDa respectively. The acetyl-CoA acetyltransferase protein appeared, in addition to its tetrameric mass of 164 kDa [41], in trimeric, dimeric and monomeric forms, which apparently are due to the tendency of the tetrameric acetyl-CoA acetyltransferase to dissociate into monomers, as has been demonstrated already (see Figure 1).

The lysosomal fractions prepared from hepatocytes, cultured in the absence and/or the presence of leupeptin, proved to be free both of enzymically active glutamate dehydrogenase and of an enzymic activity with acetoacetyl-CoA as a measure for 3-ketoacyl-CoA thiolase and/or acetyl-CoA acetyltransferase. However, these mitochondrial matrix proteins could be detected only and/or in higher amounts in the lysosomal fractions from hepatocytes that had been cultured in the presence of leupeptin (Figure 8). Moreover, we found that these lysosomal fractions banding in the 5–17% metrizamide interphase also contain autophagic vacuoles, i.e. autolysosomes and autophagosomes could be revealed by electron microscopy (results not shown), as had been shown previously [24].

DISCUSSION

The degradation of mitochondrial matrix proteins can take place selectively, by intramitochondrial ATP-dependent proteases, or in lysosomes or proteasomes following a retrograde translocation of matrix proteins into the cytosol, and non-selectively via the macroautophagy of whole mitochondria (autophagic/lysosomal pathway).

Degradation by mitochondrial matrix ATP-dependent proteases

In a culture of hepatocytes, the turnover of several mitochondrial matrix proteins appears to be associated with post-translational

modification by CoA [3,4]. *In vitro*, CoA binding proved to be a reversible process that is thiol-dependent and thiol-labile, suggesting a covalent attachment of CoA to the proteins by forming mixed disulphides [8]. The active site of human acetyl-CoA acetyltransferase is composed of Cys¹²⁶, His³⁸⁵ and Cys⁴¹³ [44]. The tetrameric acetyl-CoA acetyltransferase (100 % activity, on the basis of its specific activity in units/mg of protein) is modified by CoA, and is thereby transformed into the partially active tetrameric form A1 (72.5 % activity; apparently one subunit is modified) with a half-life of 38.0 h, and subsequently into the tetrameric form A2 (53.1 % activity, apparently two subunits are modified) with a half-life of 24.5 h, and then additionally into forms A3 and A4, exhibiting 24.0 and 0.5 % of the original specific enzymic activity respectively [3,8,9]. The CoA-modified forms might be the targets for intramitochondrial degradation by a matrix-resident ATP-dependent protease, or might be protected from proteolytic breakdown.

In a mitochondrial matrix fraction, MgATP mediated a preferential degradation of the unmodified acetyl-CoA acetyltransferase and of the CoA-modified form A1, whereas the more highly CoA-modified form A2 remained stable. This matrix ATP-dependent proteolytic activity, inhibited by vanadate but not by leupeptin, resembles the ATP-dependent Lon-like protease of eukaryotic mitochondria [10]. Little is known about the physiological role of the ATP-dependent Lon protease in mammalian mitochondria [19–21]. However, its expression seems to be associated with the rate of biogenesis of mitochondria [45]. In yeast, the Lon protease mediates the selective turnover of mitochondrial proteins, e.g. of the β -subunits of the general matrix peptidase and of F_1 -ATPase, but not of the matrix protein α -ketoglutarate dehydrogenase [46]. In addition, the Lon protease is required for the expression and assembly of certain mitochondrially encoded proteins [47,48], and is involved in the degradation of misfolded proteins [49]. The protein substrates of the matrix ATP-dependent ClpXP protease are unknown in mammalian mitochondria. To date, it cannot be definitely established whether the Lon-like protease or the ClpXP protease is responsible for the degradation of, e.g. the unmodified acetyl-CoA acetyltransferase in the mitochondrial matrix fraction from rat liver. The matrix-processing peptidase strictly specific for mitochondrial precursor proteins and the intermediate peptidase both cleave proteins in an ATP-independent manner [50–52].

As mentioned above, the process of CoA modification of mitochondrial matrix proteins appears to lead to the establishment of an intrinsic reversible equilibrium among the unmodified acetyl-CoA acetyltransferase and its CoA-modified forms. Both the matrix ATP-dependent proteases and trypsin failed to effect degradation of the more highly CoA-modified form A2 of acetyl-CoA acetyltransferase. These results suggest that the CoA modification, by forming mixed disulphides with cysteine residues or an exchange reaction with protein disulphide groups, causes this inaccessibility of modified proteins. However, the inaccessibility towards proteases apparently depends on the CoA-modification status, and becomes detectable only when an oligomeric protein is modified to a greater extent.

Turnover of mitochondrial matrix proteins in cultured hepatocytes

Micrographs showing mitochondria within autophagic vacuoles (autophagosomes/autolysosomes) provide evidence that under certain conditions whole mitochondria are completely degraded by lysosomes [22–24]. This process of macroautophagy is activated in the liver and other tissues during the first stages of nutrient deprivation [25,53], and during apoptosis [54].

In the cytosolic fractions of cultured hepatocytes, the lysosomal inhibitor leupeptin caused an accumulation of mitochondrial matrix proteins, such as acetyl-CoA acetyltransferase, 3-ketoacyl-CoA thiolase and glutamate dehydrogenase, and a significantly enhanced contamination by lysosomal enzymes. The mitochondrial matrix proteins accumulate in mature, oligomeric but enzymically inactive states, but not as monomers. Thus there is no indication that matrix proteins exit mitochondria as enzymically inactive monomers. The mature matrix proteins in the cytosolic fractions clearly originate from functional forms organized in the mitochondrial matrix compartment, but not via a simple leakage from mitochondria as enzymically active oligomers. Rather, with respect to the contamination of the cytosolic fractions by lysosomal enzymes and to the oligomeric but enzymically inactive status of the matrix proteins, they derive from a leakage from mitochondria-containing autophagosomes. Autophagosomes accumulate in hepatocytes in the presence of leupeptin [23], and consequently mitochondrial matrix proteins could be detected in higher amounts in the lysosomal and the cytosolic fractions. Their enzymically inactive status clearly relates to the acidification of the late autophagosomes during the autophagic/lysosomal pathway [25]. The origin of the mitochondrial matrix proteins in the cytosolic fractions from autophagosomes is confirmed further by the effect of 3-methyladenine, an inhibitor of the autophagic sequestration of mitochondria [37], which suppressed this leupeptin-mediated accumulation of mitochondrial matrix proteins. Leupeptin did not mediate an increase in the intramitochondrial steady-state level of the matrix protein acetyl-CoA acetyltransferase, suggesting that the sequestration of mitochondria by the early autophagosomes is not inhibited during the process of macroautophagy of whole mitochondria. Thus, also in the presence of leupeptin, the turnover of acetyl-CoA acetyltransferase, i.e. the rates of its synthesis and of its degradation, proved to be in balance.

In mitochondria, experiments with proteins, particularly the matrix proteins exhibiting heterogeneous turnover rates, have shown that only a part of their breakdown can occur via the autophagy of whole mitochondria [17,55,56]. Several matrix proteins are targets for CoA-mediated transformation [3,7,9] and/or degradation by a mitochondrial matrix protease. These two selective processes apparently compete for matrix proteins, e.g. for the unmodified acetyl-CoA acetyltransferase protein. Thus, its apparent half-life of 38 h, as measured in cultured hepatocytes [9], should be the result of the rates of both its transformation into modified forms, as well as of its proteolytic degradation. The CoA modification of mitochondrial matrix proteins was found to be a reversible process [4,8]. Both ATP-dependent degradation and CoA-dependent modification are thought to provide a link between the metabolic state and turnover of proteins [57] during the normal operation of mitochondria, i.e. within the framework of the energy charge of the cell, the CoA-modification status might control the selective degradation of several mitochondrial matrix proteins. This post-translational modification does not ensure an enhanced susceptibility of the modified proteins towards proteolytic breakdown, but results in inaccessibility towards matrix ATP-dependent proteases. The process of CoA modification clearly protects proteins from degradation.

We are grateful to Irmelin Probst and Kirsten Unthan-Fechner for supplying hepatocytes, to Ulrike Möller for performing parts of the immunoblots and to Peter Schwartz (Zentrum Anatomie) for performing the electron microscopy. The antibody

serum against the cytosolic phosphoenolpyruvate carboxykinase was generously given by Bruno Christ. The work contains parts of the Doctoral thesis of Stefan Rolle at the Faculty of Medicine, University of Göttingen, Germany.

REFERENCES

- Odaka, M., Kiribuchi, K., Allison, W. S. and Yoshida, M. (1993) *In vivo* affinity label of a protein expressed in *Escherichia coli*. FEBS Lett. **336**, 231–235
- Thorneley, R. N. F., Gillian, C. A., Drummond, M. H., Eady, R. R., Huff, S., MacDonald, C. J. and Shneier, A. (1992) Posttranslational modification of Klebsiella pneumoniae flavodoxin by covalent attachment of coenzyme A, shown by ³¹P NMR and electrospray mass spectrometry, prevents electron transfer from the *nifJ* protein to nitrogenase. Biochemistry **31**, 1216–1224
- Huth, W., Arvand, M. and Möller, U. (1988) Identification of [1-¹⁴C]pantothenic acid mediated modified mitochondrial proteins. Eur. J. Biochem. **172**, 607–614
- Huth, W., Worm-Breitgoff, C., Möller, U. and Wunderlich, I. (1991) Evidence for an *in vivo* modification of mitochondrial proteins by coenzyme A. Biochim. Biophys. Acta **1077**, 1–10
- Charlier, Jr., H. A., Narasimhan, C. and Mizioro, H. M. (1997) Inactivation of 3-hydroxy-3-methylglutaryl-CoA synthase and other acyl-CoA-utilizing enzymes by 3-oxobutylsulfoxyl-CoA. Biochemistry **36**, 1551–1558
- Huth, W., Pauli, C. and Möller, U. (1996) Immunochemical detection of CoA-modified mitochondrial matrix proteins. Biochem. J. **320**, 451–457
- Middelton, B. (1973) The oxoacyl-coenzyme A thiolases of animal tissues. Biochem. J. **132**, 717–730
- Quandt, L. and Huth, W. (1984) Modulation of rat liver mitochondrial acetyl-CoA acetyltransferase activity by a reversible chemical modification with coenzyme A. Biochim. Biophys. Acta **784**, 168–176
- Schwerdt, G. and Huth, W. (1993) Turnover and transformation of mitochondrial acetyl-CoA acetyltransferase into CoA-modified forms. Biochem. J. **292**, 915–919
- Desautels, M. and Goldberg, A. L. (1982) Demonstration of an ATP-dependent, vanadate-sensitive endoproteases in the matrix of rat liver mitochondria. J. Biol. Chem. **257**, 11 673–11 679
- Watabe, S. and Kimura, T. (1985) Adrenal cortex mitochondrial enzyme with ATP-dependent protease and protein-dependent ATPase activities. J. Biol. Chem. **260**, 14 498–14 504
- Wang, N., Gottesman, S., Willingham, M. C., Gottesman, M. M. and Maurizi, M. R. (1993) A human mitochondrial ATP-dependent protease that is highly homologous to bacterial Lon protease. Proc. Natl. Acad. Sci. U.S.A. **90**, 11 247–11 251
- Bross, P., Andresen, B. S., Knudsen, I., Kruse, T. A. and Gregersen, N. (1995) Human ClpP protease: cDNA sequence, tissue expression and chromosomal assignment of the gene. FEBS Lett. **377**, 249–252
- Rep, M., van Dijk, J. M., Suda, K., Schatz, G., Grivell, L. A. and Suzuki, C. K. (1996) Promotion of mitochondrial membrane complex assembly by a proteolytically inactive yeast Lon. Science (Washington, D.C.) **274**, 103–106
- Leonhard, K., Guiard, B., Pellechia, G., Tzagoloff, A., Neupert, W. and Langer, T. (2000) Membrane protein degradation by AAA proteases in mitochondria: extraction of substrates from either membrane surface. Mol. Cell **5**, 629–638
- Sitte, N., Dubiel, W. and Kloetzel, P. M. (1998) Evidence for a novel ATP-dependent protease from the rat liver mitochondrial intermembrane space: purification and characterisation. J. Biochem. (Tokyo) **123**, 408–415
- Hare, J. F. and Hodges, R. (1982) Turnover of mitochondrial matrix polypeptides in hepatoma monolayer cultures. J. Biol. Chem. **257**, 12 950–12 953
- Suzuki, C. K., Rep, M., van Dijk, J. M., Suda, K., Grivell, L. A. and Schatz, G. (1997) ATP-dependent proteases that also chaperone protein biogenesis. Trends Biochem. Sci. **22**, 118–123
- Desautels, M. and Goldberg, A. L. (1982) Liver mitochondria contain an ATP-dependent, vanadate-sensitive pathway for the degradation of proteins. Proc. Nat. Acad. Sci. U.S.A. **79**, 1869–1873
- Watabe, S., Hara, T., Kohne, H., Hiroi, T., Yago, N. and Nakazawa, T. (1993) *In vitro* degradation of mitochondrial proteins by ATP-dependent protease in bovine adrenal cortex. J. Biochem. (Tokyo) **113**, 672–676
- Watabe, S., Kohn, H., Kouyama, H., Hiroi, T., Yago, N. and Nakazawa, T. (1994) Purification and characterization of a substrate protein for mitochondrial ATP-dependent protease in bovine adrenal cortex. J. Biochem. (Tokyo) **115**, 648–654
- Marzella, L., Sandberg, P. O. and Glaumann, H. (1980) Autophagic degradation in rat liver after vinblastine treatment. Exp. Cell Res. **128**, 291–301
- Kovács, A. L., Reith, A. and Seglen, P. O. (1982) Accumulation of autophagosomes after inhibition of hepatocytic protein degradation by vinblastine, leupeptin or a lysosomotropic amine. Exp. Cell Res. **137**, 191–201
- Marzella, L., Ahlberg, J. and Glaumann, H. (1982) Isolation of autophagic vacuoles from rat liver: morphological and biochemical characterization. J. Cell Biol. **90**, 144–154
- Dunn, Jr., W. A. (1994) Autophagy and related mechanisms of lysosome-mediated protein degradation. Trends Cell Biol. **4**, 139–143
- Storrie, B. and Madden, E. A. (1990) Isolation of subcellular organelles. Methods Enzymol. **182**, 203–225
- Schmidt, E. (1974) Glutamate dehydrogenase. In Methoden der enzymatischen Analyse (Bergmeyer, H. U., ed.), pp. 689–696, Verlag Chemie Weinheim
- von Figura, K. (1977) Human β -N-Acetylglucosaminidase. Eur. J. Biochem. **80**, 525–533
- Seubert, W., Lamberts, J., Kramer, R. and Ohly, B. (1968) On the mechanism of malonyl-CoA independent fatty acid synthesis. The mechanism of elongation of long chain fatty acids by acetyl-CoA. Biochim. Biophys. Acta **164**, 498–517
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature (London) **227**, 680–685
- Huth, W. and Alves, F. (1985) Mitochondrial acetyl-CoA acetyltransferase from various organs from rat: forms pattern and CoASH modification. Biochim. Biophys. Acta **830**, 274–281
- Kyhse-Anderson, I. (1984) Electrophoretic blotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods **10**, 203–209
- Goldberg, A. L., Akopian, T. N., Kisselev, A. F. and Lee, D. H. (1997) Protein degradation by the proteasome and dissection of its *in vivo* importance with synthetic inhibitors. Mol. Biol. Rep. **24**, 69–75
- Ariyoshi, H., Okahara, K., Sakon, M., Kambayashi, J., Kawashima, S., Kawasaki, T. and Monden, M. (1998) Possible involvement of m-calpain in vascular smooth muscle cell proliferation. Arterioscler. Thromb. Vasc. Biol. **18**, 493–498
- Renfrew, C. A. and Hubbard, A. L. (1991) Degradation of epidermal growth factor receptor in rat liver. J. Biol. Chem. **266**, 21 265–21 273
- Christ, B., Nath, A., Bastian, H. and Jungermann, K. (1988) Regulation of the expression of the phosphoenolpyruvate carboxykinase gene in cultured rat hepatocytes by glucagon and insulin. Eur. J. Biochem. **178**, 373–379
- Gordon, P. B., Høyvik, H. and Seglen, R. O. (1992) Prelysosomal and lysosomal connections between autophagy and endocytosis. Biochem. J. **283**, 361–369
- Dick, L. R., Cruikshank, A. A., Grenier, L., Melandri, F. D., Nunes, S. L. and Stein, R. L. (1996) Mechanistic studies on the inactivation of the proteasome by lactacystin. J. Biol. Chem. **271**, 7273–7276
- Roberts, B. (1997) Evidence of proteasome-mediated cytochrome P-450 degradation. J. Biol. Chem. **272**, 9771–9778
- Schwabe, D. and Huth, W. (1979) Immunochemical aspects, molecular and kinetic properties of multiple forms of acetyl-CoA acetyltransferase from rat liver mitochondria. Biochim. Biophys. Acta **575**, 112–120
- Fukao, T., Kamijo, K., Osumi, T., Fujiki, Y., Yamaguchi, S., Orii, T. and Hashimoto, T. (1989) Molecular cloning and nucleotide sequence of cDNA encoding the entire precursor of rat mitochondrial acetoacetyl-CoA thiolase. J. Biochem. (Tokyo) **106**, 197–204
- Amaya, Y., Arakawa, H., Takiguchi, M., Ebina, Y., Yokota, S. and Mori, M. (1988) A noncleavable signal for mitochondrial import of 3-oxoacyl-CoA thiolase. J. Biol. Chem. **263**, 14 463–14 470
- King, K. S. and Frieden, C. (1970) The purification and physical properties of glutamate dehydrogenase from rat liver. J. Biol. Chem. **245**, 4391–4396
- Fukao, T., Nakamura, H., Song, X.-Q., Nakamura, K., Orii, K. E., Kohno, Y., Kano, M., Yamaguchi, S., Hashimoto, T., Orii, T. and Kondo, N. (1998) Characterization of N93S, I312T, and A333P missense mutations in two Japanese families with mitochondrial acetoacetyl-CoA thiolase deficiency. Hum. Mutat. **12**, 245–254
- Luciakova, K., Sokolikova, B., Chloupkova, M. and Nelson, B. D. (1999) Enhanced mitochondrial biogenesis is associated with increased expression of mitochondrial ATP-dependent Lon protease. FEBS Lett. **444**, 186–188
- Suzuki, C. K., Suda, K., Wang, N. and Schatz, G. (1994) Requirement for the yeast gene Lon in intramitochondrial proteolysis and maintenance of respiration. Science (Washington, D.C.) **264**, 273–276
- Van Dyck, L., Neupert, W. and Langer, T. (1998) The ATP-dependent PIM1 protease is required for the expression of intron-containing genes in mitochondria. Genes Dev. **12**, 1515–1524
- Van Dijk, J. M., Kutejova, E., Suda, K., Perecko, D., Schatz, G. and Suzuki, C. K. (1998) The ATPase and protease domain of yeast mitochondrial Lon: roles in proteolysis and respiration-dependent growth. Proc. Natl. Acad. Sci. U.S.A. **95**, 10 584–10 589
- Wagner, I., Arit, H., Van Dyck, L., Langer, T. and Neupert, W. (1994) Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria. EMBO J. **13**, 5135–5145

-
- 50 Pollock, R. A., Hartl, F. U., Cheng, M. Y., Ostermann, J., Horwich, A. and Neupert, W. (1988) The processing peptidase of yeast mitochondria: the two co-operating components MPP and PEP are structurally related. *EMBO J.* **7**, 3493–3500
- 51 Saavedra-Alanis, V. M., Rysavy, P., Rosenberg, C. E. and Kalousek, F. (1994) Rat liver mitochondrial processing peptidase. Both α - and β -subunits are required for activity. *J. Biol. Chem.* **269**, 9284–9289
- 52 Kalousek, F., Isaya, G. and Rosenberg, L. E. (1992) Rat liver mitochondrial intermediate peptidase (MIP): purification and initial characterization. *EMBO J.* **11**, 2803–2809
- 53 Mortimore, G. E., Pösö, A. R. and Lardeux, B. R. (1989) Mechanism and regulation of protein degradation in liver. *Diabetes Metab. Rev.* **5**, 49–70
- 54 Lemasters, J. J., Nieminen, A. L., Qian, T., Trost, L. C., Elmore, S. P., Nishimura, Y., Crowe, R. A., Cascio, W. E., Bradham, C. A., Brenner, D. A. and Herman, B. (1998) The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim. Biophys. Acta* **1366**, 177–196
- 55 Chandler, C. S. and Ballard, F. J. (1985) Distribution and degradation of biotin-containing carboxylases in human cell lines. *Biochem. J.* **232**, 385–393
- 56 Grisolia, S., Hernandez-Yago, J. and Knecht, E. (1985) Regulation of mitochondrial protein concentration: a plausible model which permit assessing protein turnover. *Curr. Top. Cell. Regul.* **27**, 387–396
- 57 Gottesman, S. and Maurizi, M. R. (1992) Regulation by proteolysis: energy-dependent proteases and their targets. *Microbiol. Rev.* **56**, 592–621
-

Received 1 October 2001/21 January 2002; accepted 1 March 2002