Proteolysis of Isolated Mitochondria by Myocardial Lysosomal Enzymes

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1. Solubilized mitochondria and lysosomal fractions were obtained from guinea-pig heart by differential centrifugation and selective membrane disruption. 2. Mitochondria incubated at 37°C in the presence of lysosomal enzymes underwent proteolysis. The rate of protein degradation was inversely dependent on pH. 3. The use of proteinase inhibitors showed that at low pH the major enzyme involved in mitochondrial digestion was cathepsin D. 4. At neutral pH carboxyl proteinases were still active, but thiol proteinases accounted for most of the protein breakdown. 5. The role of lysosomal enzymes as mediators of mitochondrial damage in ischaemic myocardium is discussed.

The mechanism by which myocardial cells die during anoxia or ischaemia has been the subject of numerous studies. One of the theories that have been postulated is that digestive enzymes released from damaged lysosomes destroy key cellular components, resulting in irreversible damage (Wildenthal, 1978). The acidosis present in ischaemic myocardium would enhance the digestion of cellular components by lysosomal enzymes (Williamson *et al.*, 1976).

The lysosome theory of myocardial cell death is supported by the findings of Welman & Peters (1977) and Decker *et al.* (1977, 1979), which showed that lysosome-membrane disruption occurs between 20 and 30 min after the onset of ischaemia or anoxia and precedes morphological signs of cell death. However, opponents of this hypothesis have pointed out that the myocardium has relatively low lysosomal enzyme activity compared with tissues such as liver and kidney (Sobel, 1974). Furthermore, extensive protein catabolism in ischaemic myocardium has not been demonstrated (Kao *et al.*, 1976; Wildenthal & Griffen, 1977). This raises the question of the capacity of myocardial lysosomal enzymes to damage key cellular components. The purpose of the present study was to assess the capacity of lysosomal enzymes from cardiac muscle to digest the protein of an important cellular component, the mitochondrion. The enzymes responsible for proteolysis of mitochondria were characterized by the use of specific inhibitors, and the effect of pH on the rate of proteolysis was also determined.

Materials and Methods

Animals

Adult male Dunkin–Hartley guinea pigs (500–

600 g body wt.) were used in these experiments. Unstarved animals were anaesthetized with halothane and the hearts were rapidly excised and placed in KCl (0.25 M) at 4°C. Two hearts were used for each fractionation. The free wall of the left ventricle was removed from both hearts and weighed. The tissue was chopped with scissors and homogenized in 10 vol. of KCl in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.) by 25 strokes with a tight-fitting pestle.

Fractionation

The homogenate was fractionated as outlined in Scheme 1. All procedures were carried out at 0–4°C unless stated otherwise. The nuclear fraction was sedimented by centrifuging the homogenate at 600 g for 10 min. The post-nuclear supernatant was removed to a centrifuge tube and KCl was added to the nuclear pellet to adjust it to the original volume of the homogenate. The nuclear fraction was resuspended by six strokes in a Dounce homogenizer. Two portions (0.1 ml each) of post-nuclear supernatant were removed for enzyme assays, and the remainder was centrifuged at 20 000 g for 30 min to sediment the mitochondria and lysosomes. The postlysosomal supernatant was removed and stored on ice. The mitochondria and lysosome pellet was resuspended by six strokes in a Dounce homogenizer with a solution of KCl (0.25 M) containing 0.5 mg of digitonin (Sigma)/ml, equal to one-quarter of the postnuclear-supernatant volume. This mixture was approx. 0.1 mg of digitonin/mg of protein. After incubation at 37°C for 5 min to solubilize the lysosomal enzymes, an equal volume of sucrose (0.25 M) was added. The preparation was then cooled to 4°C and centrifuged at 20 000 g for 30 min to separate the mitochondria.

Scheme 1. *Fractionation of guinea-pig left-ventricle tissue*

Abbreviations; PNS, postnuclear supernatant; N, nuclear fraction; PLS, postlysosomal supernatant; ML, mitochondria and lysosomes; FLE, free lysosomal enzymes; M, mitochondrial fraction.

The supernatant, containing the free lysosomal enzymes, was removed and the mitochondrial pellet was resuspended in half the postnuclear-supernatant volume of sucrose (0.25M) to provide the mitochondrial fraction. The recovery of mitochondria and lysosomes in the various fractions was estimated by measurements of succinate dehydrogenase activity for mitochondria and *N*-acetyl- β -glucosaminidase activity for lysosomes. Succinate dehydrogenase was assayed by the method of Prospero (1974). Samples with high succinate dehydrogenase activity were diluted 1:14 with the buffer used in the reaction mixture. *N*-Acetyl- β -glucosaminidase activity was measured fluorimetrically as described by Welman *et al.* (1978).

Protein concentration of the fractions was measured by the technique of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as a standard. Free amino acid concentrations were determined by the ninhydrin method (Moore, 1968), with leucine (Sigma) as the standard.

Proteolysis of mitochondria and the effect of pH and dithiothreitol

The effects of the lysosomal preparation at various pH values on mitochondrial proteolysis were

determined by the following procedures. Samples of the mitochondrial fraction were incubated with an equal volume of the lysosomal enzyme preparation in stoppered tubes in a shaker bath at 37°C. The incubation media were buffered at pH 7, 6 or 5 by adding 1 vol. of potassium phosphate buffer (0.3M) to 9 vol. of a mixture of mitochondrial fraction and free lysosomal enzymes. In a further series of experiments, dithiothreitol (Sigma) in aqueous solution was added to a final concentration of 2.0mM. This addition was made to assess fully the effects of thiol proteinases (Huisman *et al.*, 1973). At the end of the incubation period of 2, 4 or 6 h, 10 vol. of trichloroacetic acid (5%, w/v) at 4°C was added. The precipitated protein was sedimented at 3000g for 10 min and the supernatant fractions containing free amino acids were separated. The pellets and supernatant fractions were stored overnight at 4°C.

Controls for the experiment were carried out by omitting either the mitochondrial fraction or the free-lysosomal-enzyme fraction and making up the volume with the appropriate solutions. The mitochondrial fraction was also incubated with the sucrose/KCl solution in the absence of digitonin. In addition, zero-time incubations were carried out as blank controls.

The acid-soluble supernatant fractions were assayed for free amino acids and the precipitated protein was measured after re-solubilization in NaOH (8M).

Effects of inhibitors of proteolysis

The incubation procedures described above were repeated in the presence of proteinase inhibitors. Pepstatin A (Sigma) was dissolved in methanol and added to the incubation media to give a final concentration of 4.8 μ M-inhibitor and 1% (v/v) methanol (Aoyagi *et al.*, 1972). Control incubations with 1% methanol were carried out simultaneously.

Leupeptin (Sigma) and moniodoacetate (Sigma) were added as aqueous solutions. The concentration of leupeptin tested was 33 μ M. Moniodoacetate was added to give 10mM in the incubation media. Dithiothreitol was omitted from incubations containing leupeptin or moniodoacetate.

Pepstatin A and moniodoacetate were also tested at 10 times the concentrations stated above, and incubations were carried out with combinations of the three inhibitors.

To simplify the expression of the results, the degree of enzymic proteolysis was calculated by using the increase in free amino acids at pH 5 for 6 h in the absence of dithiothreitol as 100%. The actual concentration of amino acid produced depended on the protein concentration of the mitochondrial fraction, which varied between 3 and 5mg/ml in different preparations. In order to pool the data, either the percentage change in amino acids, calculated as described above, or the change in amino acids per mg of mitochondrial protein can be used. Results were compared by paired Student's *t* tests.

Results

Fractionation

The recoveries of the marker enzymes for mitochondria and lysosomes in the fractions of guinea-

pig heart are shown in Table 1. The total activity of succinate dehydrogenase calculated from the nuclear + post-nuclear-supernatant fractions was 650 ± 39 munits/ml (mean $\pm$ s.d. for 20 expts.). This was taken as 100% for calculation of recoveries in the other fractions. The lysosomal marker, *N*-acetyl- β -glucosaminidase, had a mean total activity of 67.6 ± 1.6 munits/ml.

Table 1 shows that two-thirds of the mitochondria and lysosomes were recovered in the postnuclear-supernatant fraction. Most of the mitochondria were sedimented at 20000g, and the slight loss of succinate dehydrogenase activity is attributed to washing of the pellet. The lysosomal marker was not completely sedimented at 20000g, in agreement with previous findings of 70% sedimentability of this enzyme in postnuclear-supernatant fractions (Welman & Peters, 1976). Treatment with digitonin solubilized 76% of the *N*-acetyl- β -glucosaminidase activity in the mitochondria-and-lysosome pellet; consequently the mitochondrial fraction contained some lysosomal enzyme activity.

Incubation of mitochondria at various pH values

When the free-lysosomal-enzyme preparation was incubated in the absence of mitochondria, a small increase in free amino acid concentration was detected (Table 2). This may have resulted from the action of lysosomal proteinases on some contaminating proteins in this fraction, as the lysosomal enzymes themselves are usually stable at pH 5.

Incubation of the mitochondrial fraction, in the presence of the free-lysosomal-enzyme carrier solution alone, also gave rise to an increase in free amino acid concentration, as would be expected from the contamination of this fraction by lysosomal enzymes. The degree of proteolysis was about one-third of that seen in the presence of free lysosomal enzymes and correlated with the proportion of *N*-acetyl- β -glucosaminidase in the mitochondrial fraction (Table 2). The contribution that digitonin

Table 1. *Succinate dehydrogenase and N-acetyl- β -glucosaminidase activity in fractions of guinea-pig left ventricle* Results are means ($\pm$ s.d.) for 20 experiments; 1 unit $\equiv$ 1 μ mol/min. Recoveries were calculated as activity in fraction as percentage of total activity, after correction for volume differences.

Fraction	Succinate dehydrogenase activity		<i>N</i> -Acetyl- β -glucosaminidase activity	
	(munits/ml)	(% recovered)	(munits/ml)	(% recovered)
Total (N + PNS)	650 ± 39	(100)	67.6 ± 1.6	(100)
Nuclear (N)	224 ± 20	(34)	25.3 ± 2.8	(37)
Postnuclear supernatant (PNS)	426 ± 45	(66)	42.3 ± 3.2	(63)
Mitochondria + lysosomes (ML)	620 ± 43	(48)	53.7 ± 3.5	(40)
Postlysosomal supernatant (PLS)	13 ± 1	(2)	14.1 ± 1.3	(21)
Mitochondria (M)	579 ± 26	(45)	11.8 ± 1.0	(9)
Free lysosomal enzymes (FLE)	6 ± 1	(1)	38.7 ± 1.5	(29)

Table 2. *Amino acid production in controls at pH 5*

Results are means ($\pm$ S.E.M.) expressed in $\mu\text{mol/g}$ of protein and (in parentheses) as % of amino acid produced at 6 h in incubations containing combined fractions in the absence of dithiothreitol.

Time (h)	No. of expts. ...	Mitochondrial fraction with digitonin 10	Mitochondrial fraction without digitonin 5	Free lysosomal enzymes 5
2		17.4 ± 2.9 (9)	14.6 ± 1.3 (7)	10.5 ± 2.8 (5)
4		46.8 ± 1.8 (23)	39.3 ± 2.8 (20)	13.6 ± 1.6 (7)
6		75.2 ± 1.7 (37)	65.3 ± 2.6 (32)	26.5 ± 2.3 (13)

Table 3. *Amino acid produced by combined mitochondrial and lysosomal fractions*

Results are means ($\pm$ S.E.M.) for (a) 15 or (b) 5 experiments. For details see the text.

Time (h)	Amino acid produced ($\mu\text{mol/g}$ of protein)		
	pH 5	pH 6	pH 7
(a) Without dithiothreitol			
2	69.2 ± 5.1	30.3 ± 1.5	17.2 ± 2.4
4	125.5 ± 7.2	67.5 ± 2.1	36.3 ± 3.0
6	201.4 ± 8.1	98.1 ± 3.0	43.0 ± 4.1
(b) With dithiothreitol			
2	89.7 ± 6.3	33.1 ± 4.3	18.0 ± 4.0
4	156.4 ± 8.2	70.4 ± 4.6	35.4 ± 5.1
6	223.1 ± 5.1	111.3 ± 6.0	45.1 ± 6.0

Fig. 1. *Hydrolysis of mitochondrial protein at various pH values*

Results (expressed as percentage of hydrolysis at pH 5 after 6 h incubation without dithiothreitol) are means ($\pm$ S.E.M.) for ten experiments. For full details see the text.

made to the proteolysis of mitochondria is shown in Table 2 and varied from 19% at 2 h to 15% at 6 h at pH 5.

The combination of the mitochondrial and free-lysosomal-enzyme fractions at pH 5 and 37°C

produced changes in amino acid concentration as shown in Table 3. Fig. 1 illustrates the rates of amino acid production at various pH values, showing that proteolysis progressed at a constant rate over 6 h at each pH. The extent of protein degradation increased with decreasing pH, as expected, but it is noteworthy that even at pH 7 protein breakdown was occurring. The addition of dithiothreitol increased both the initial rate and final degree of proteolysis at pH 5. At 2 h incubation 30% more amino acids were freed, and at 4 and 6 h the increases were 24 and 10% respectively. At pH 6 the addition of the thiol group had less effect, and no significant effect was seen at pH 7.0.

Effects of inhibitors

The effects of inhibitors on mitochondrial proteolysis in the presence of free lysosomal enzymes at pH 5 is shown in Table 4. Leupeptin caused a small (15%) inhibition of proteolysis ($P < 0.005$). Moniodoacetate was more effective, decreasing proteolysis by 35% ($P < 0.001$). Pepstatin A had a marked effect, the production of amino acids being decreased by 80%. The combination of pepstatin A and moniodoacetate did not appear to be additive; proteolysis was inhibited by 85%, which was similar to the effect of pepstatin A alone. Pepstatin A and leupeptin also gave the same results as pepstatin A

Table 4. *Effect of lysosomal-enzyme inhibitors on hydrolysis at pH 5*Results are mean ($\pm$ S.E.M.) for ten experiments. For full details see the text. * $P < 0.001$ for difference from control

Time (h)	Hydrolysis (%)				
	Control	+Leupeptin	+Monoiodoacetate	+Pepstatin	+Pepstatin and monoiodoacetate
2	33 $\pm$ 5	28 $\pm$ 2	22 $\pm$ 4*	7 $\pm$ 4*	5 $\pm$ 4*
4	64 $\pm$ 6	61 $\pm$ 3	38 $\pm$ 11*	12 $\pm$ 3*	11 $\pm$ 3*
6	100	83 $\pm$ 5	63 $\pm$ 10*	17 $\pm$ 6*	13 $\pm$ 5*

Table 5. *Effect of lysosomal-enzyme inhibitors on hydrolysis at pH 6*Results are means ($\pm$ S.E.M.) for ten experiments. For full details see the text. * $P < 0.001$ for difference from control.

Time (h)	Hydrolysis (%)			
	Control	+Monoiodoacetate	+Pepstatin	+Pepstatin and monoiodoacetate
2	14 $\pm$ 2	8 $\pm$ 3*	6 $\pm$ 1*	2 $\pm$ 2*
4	32 $\pm$ 3	22 $\pm$ 6*	12 $\pm$ 1*	4 $\pm$ 1*
6	48 $\pm$ 5	37 $\pm$ 5*	16 $\pm$ 2*	6 $\pm$ 2*

Table 6. *Effect of lysosomal-enzyme inhibitors on hydrolysis at pH 7*Results are means ($\pm$ S.E.M.) for ten experiments. For full details see the text. * $P < 0.001$ for difference from control.

Time (h)	Hydrolysis (%)			
	Control	+Monoiodoacetate	+Pepstatin	+Pepstatin and monoiodoacetate
2	8 $\pm$ 2	5 $\pm$ 3*	7 $\pm$ 1*	4 $\pm$ 1*
4	15 $\pm$ 4	9 $\pm$ 4*	13 $\pm$ 2*	8 $\pm$ 1*
6	21 $\pm$ 3	11 $\pm$ 3*	20 $\pm$ 4*	10 $\pm$ 4*

alone. The inhibitory effects were independent of the time of incubation up to 6 h.

At pH 6 monoiodoacetate had a similar inhibitory effect as at pH 5, decreasing proteolysis by 31% (Table 5). On the other hand, pepstatin A was less effective at pH 6 than at pH 5, although there was still a marked inhibition (63%). At this pH the combination of pepstatin A and monoiodoacetate appeared to be partially additive, giving 88% inhibition of proteolysis.

Monoiodoacetate caused a similar inhibition of proteolysis at pH 7 as at lower pH (30%). However, pepstatin A had much less effect at pH 7, the mean decrease in proteolysis being only 21%. At this pH the combined effects of pepstatin A and monoiodoacetate were completely additive, giving 50% inhibition (Table 6). Methanol, the carrier for pepstatin, did not affect the rate of proteolysis in the controls. The experiments with higher concentrations of pepstatin A and monoiodoacetate gave the same results as those reported above with the lower doses. When incubations included both pepstatin A

and dithiothreitol, there was no significant difference in proteolysis from those incubations containing pepstatin A only.

Total protein concentrations measured in the pellets obtained after trichloroacetic acid precipitation showed no significant change, even after 6 h at pH 5, where the production of free amino acids was most marked.

Discussion

The results of this study show that mitochondrial protein can be degraded by myocardial lysosomal enzymes *in vitro*. The rate of proteolysis is inversely related to pH and is linear with time at each pH in the range tested.

Despite the evidence of proteolysis, there was no detectable change in total protein in the incubated mitochondria. However, cathepsin action is mainly to degrade large proteins into smaller polypeptides (Barrett, 1973; Huismann *et al.*, 1974). The findings with monoiodoacetate, which inhibits thiol enzymes,

show that cathepsin D is capable of generating free amino acids and that neither thiol cathepsins nor endopeptidases are essential to initiate protein degradation by cathepsin D (EC 3.4.23.5), as previously thought (Huismann *et al.*, 1973).

Leupeptin, which inhibits cathepsin B (EC 3.4.22.1) other thiol proteinases (Aoyagi *et al.*, 1969) and some serine proteinases, had a very limited effect on inhibiting proteolysis of mitochondria. This is in agreement with the previous finding of low cathepsin B activity in guinea-pig heart (Welman & Peters, 1976).

Pepstatin A, in the concentration used in most of these experiments, specifically inhibits carboxyl proteinases. Cathepsin D is a major lysosomal proteinase in myocardium and is a member of this group. Our results show that at low pH the carboxyl proteinases are the dominant lysosomal enzymes involved in mitochondrial proteolysis.

Previous studies using purified lysosomal enzymes from rat liver have shown proteolysis of denatured mitochondria, at neutral as well as acid pH (Rubio & Grisolia, 1977; Sawant *et al.*, 1964). We have tried to prevent damage to the mitochondria by avoiding freezing and thawing, hypo-osmoticity and storage of the fractions. Digitonin was chosen as a means of disrupting lysosomal membranes, as they are more sensitive to this detergent because of their relatively high cholesterol content. The concentration of digitonin employed (0.1 mg/mg of protein) was below that found to damage mitochondrial outer membranes (Kirschbaum, 1976). It was insufficient to solubilize the lysosomal enzymes completely, although latent activity was unmasked by much lower concentrations.

The work of Mellors *et al.* (1967) on mitochondria that had not been denatured showed that lysosomal enzymes could cause swelling and uncoupling of oxidative phosphorylation. Numerous studies on heart muscle, such as those of Jennings *et al.* (1969), have demonstrated structural and functional abnormalities of mitochondria resulting from anoxia and ischaemia. The release of cathepsin D and other lysosomal enzymes into the cytoplasm during anoxia and ischaemia has been demonstrated (Decker *et al.*, 1977, 1979). The proteolysis of mitochondria by cathepsin D in the acidic environment of the ischaemic cell may render it irreversibly damaged.

The capacity of myocardial lysosomes to degrade mitochondrial protein at both neutral and acidic pH lends support to the lysosome hypothesis of ischaemic cell death (Wildenthal, 1978). It also suggests a potential role of lysosomal enzymes in

contributing to myocardial damage in other pathological conditions where acidosis is not a feature.

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