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Mitochondria contain a proteolytic system which can recognize and degrade oxidatively-denatured proteins

Olivier MARCILLAT, Yin ZHANG, Sharon W. LIN and Kelvin J. A. DAVIES*
Institute for Toxicology and Department of Biochemistry, University of Southern California, 1985 Zonal Avenue, HSC-PSC 614-616, Los Angeles, CA 90033, U.S.A.

When incubated with mitochondria in an air atmosphere, menadione and doxorubicin (which redox cycle with the respiratory chain to produce oxygen radicals), as well as xanthine oxidase plus xanthine (which generate superoxide and H_2O_2), stimulated the degradation of newly-synthesized ([^{3}H]leucine-labelled) mitochondrial polypeptides. No stimulation was observed in an N_2 atmosphere, ATP was not required, and xanthine oxidase was not effective without xanthine. Various forms of oxidative stress induced varying degrees of protein cross-linking, protein fragmentation and proteolysis, as judged by gel electrophoresis and amino acid analysis. To learn more about the proteolytic enzymes involved in degradation, we undertook studies with purified protein substrates which had been exposed to oxidative stress ($^{\cdot}OH$ or H_2O_2) *in vitro*. Despite mitochondrial contamination with acid proteases of lysosomal (and other) origin, pH profiles revealed distinct proteolytic activities at both pH 4 and pH 8. The pH 8 activity preferentially degraded the oxidatively-denatured forms of haemoglobin, albumin and superoxide dismutase; was unaffected by digitonin; and exhibited a several-fold increase in activity upon mitochondrial disruption (highest activity being found in the matrix). In contrast, the pH 4 activity was dramatically decreased by digitonin treatment (to reduce lysosomal contamination); was unaffected by mitochondrial disruption; and showed no preference for oxidatively-denatured proteins. The pH 8 activity was not stimulated by ATP, but was inhibited by EDTA, haemin and phenylmethylsulphonyl fluoride. In contrast, the contaminating pH 4 activity was only inhibited by pepstatin and leupeptin. Thus, our experiments reveal a distinct mitochondrial (matrix) proteolytic pathway which can preferentially degrade oxidatively-denatured proteins.

INTRODUCTION

The existence of intracellular proteolytic systems which can specifically recognize and degrade oxidatively-damaged or denatured proteins is now quite well established [1–27]. Such proteolytic systems or enzymes have been identified in the cytosol of both mammalian [1–9,17,27] and bacterial [2,3,6,10,11–20] cells. It has been suggested that these proteolytic systems may actually protect cells against oxidatively-damaged polypeptides and protein fragments [3,19,23], and this laboratory has proposed that they should be considered to form part of a cell's 'secondary antioxidant defenses' [3,6–11].

Mammalian cells contain a number of organelles which might be inaccessible to the cytosolic proteolytic systems discussed above. Thus it is pertinent to ask whether mammalian organelles contain their own 'secondary antioxidant' proteolytic systems. Our decision to begin our studies in this area with mitochondria was based on several considerations. First, mitochondria possess both an inner and an outer membrane which should make them quite impermeable to cytosolic proteases and peptidases. Secondly, the mitochondrial electron transport chain is one of the major intracellular sources of free-radical generation, both during normal ATP synthesis [28,29] and in the presence of quinonoid compounds which can redox cycle to generate large oxygen radical fluxes [30–32]. Thus it is reasonable to predict that mitochondrial proteins might be subject to oxidative stress. Thirdly, several laboratories have reported that mitochondria possess proteolytic enzymes [e.g. 2,3,21,23,33–41], at least one of which can preferentially degrade incomplete (puromycyl) peptides [37], and which appears to be an ATP-stimulated endoprotease [38].

Preliminary studies [2,3,21,22] suggested that certain oxygen radicals may damage mitochondrial proteins, causing both direct fragmentation (the term 'protein fragmentation' is used to describe the direct breakdown of proteins by oxygen radicals. Such processes have been found to involve both main-chain and side-chain scission by mechanisms which differ from peptide hydrolysis. In contrast, the terms 'protein degradation' and 'proteolysis' refer to peptide-bond hydrolysis by proteolytic enzymes) and increased proteolysis. The apparent success of these preliminary studies encouraged us to undertake more detailed investigations. In the present report we attempted to determine if activated oxygen species, generated exogenously or by the interaction of various agents with the mitochondrial electron transport chain, can cause both protein modification and protein degradation. Because it is virtually impossible to isolate completely pure mitochondria (see [37] for a discussion of this point) we made a major effort to discriminate between recognition and degradation by true intra-

Abbreviations used: PMSF, phenylmethylsulphonyl fluoride; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, dicyclohexylcarbodi-imide; Hb, haemoglobin.

* To whom all correspondence and reprint requests should be addressed.

mitochondrial proteases, and spurious results which might be introduced by contaminating lysosomal, mast cell and cytoplasmic proteases. We also tested the ability of mitochondrial proteases to recognize and degrade purified proteins which had been exposed to specific oxygen radicals. Finally, we examined the effects of potential protease inhibitors and activators on the degradation of oxidatively-denatured proteins by mitochondrial proteases.

EXPERIMENTAL PROCEDURES

Mitochondrial isolation

Rat liver mitochondria, beef liver mitochondria and beef heart mitochondria were used for various aspects of these experiments. Rat liver mitochondria were prepared according to Pedersen *et al.* [42] in a medium consisting of 0.07 M-sucrose, 0.22 M-D-mannitol, 0.002 M-Hepes, and 0.05% bovine serum albumin (using the high yield procedure). Beef liver mitochondria were prepared as described by Johnson & Lardy [43].

Beef heart mitochondria were isolated by the following modification to the method of Smith [44]. Beef hearts were sliced, minced and then homogenized in a buffer consisting of 0.15 M-sucrose, 0.001 M-EDTA and 0.01 M-Hepes (pH 7.6). The 20% (w/v) homogenates were centrifuged at 1600 *g* and the supernatants were carefully decanted and filtered through a double layer of gauze. The 1600 *g* pellets were resuspended, centrifuged again and the supernatants were decanted and filtered through gauze. The two 1600 *g*-filtered supernatants were combined and then centrifuged at 6000 *g* to produce a mitochondrial pellet. Mitochondrial pellets were washed three times (by centrifugation) and incubated (20 mg of protein/ml) with digitonin (50 μg/mg of protein) for 2 min at 0 °C to disrupt contaminating lysosomes. The digitonin-treated mitochondria were diluted with 10 volumes of ice-cold buffer, centrifuged at 7500 *g* and washed (by centrifugation) twice more before use.

Degradation of newly-synthesized mitochondrial proteins

Rat liver mitochondria were prepared as above. Mitochondrial polypeptides were labelled (in the presence of 0.36 mM-cycloheximide) by incorporation of [^{3}H]-leucine, as described by Desautels & Goldberg [37]. Degradation of these polypeptides was assessed by formation of acid-soluble counts, after addition of 10% (w/v) trichloroacetic acid [37]. Proteolysis (see Tables 1 and 2) was linear for at least 60 min (10, 15, 30, 45 and 60 min were tested).

Degradation of oxidatively-denatured proteins

Haemoglobin (Hb) (H 2500), bovine serum albumin (A 0281) and α-casein (C 7891) were obtained from Sigma. Superoxide dismutase (83-500-1) was obtained from Miles Scientific (Napperville, IL, U.S.A.). These proteins were radiolabelled by reductive methylation with [^{3}H]formaldehyde and $NaBH_4$ [45] and extensively dialysed. Oxidatively-denatured [^{3}H]Hb and [^{3}H]-albumin were obtained by exposure to the hydroxy radical ($^{\cdot}OH$), using ^{60}Co radiation under 100% N_2O, as previously described [6–9]. ^{3}H-labelled superoxide dismutase was oxidatively denatured by exposure to 30 mM-H_2O_2 for 30 min at pH 7.4 [2]. Both the oxidatively-denatured proteins and their untreated controls were extensively re-dialysed before further use.

Untreated proteins and oxidatively-denatured proteins were incubated with beef heart, beef liver or rat liver mitochondrial preparations, disrupted mitochondria or the mitochondrial matrix for 60 min at 37 °C (with vigorous shaking) to measure protein degradation. The degradation of ^{3}H-labelled proteins was monitored by release of acid-soluble radioactivity, following precipitation of the remaining intact proteins with trichloroacetic acid [6–9]. Degradation measurements over time (10, 20, 40 and 60 min) revealed linear rates of proteolysis for at least 60 min (see Tables 4, 5 and 6, and Fig. 1).

In some experiments, unlabelled intact mitochondria were exposed to various oxidative stresses. Aggregated proteins and protein fragments or degradation products were then assessed by SDS/polyacrylamide-gel electrophoresis [46]. The release of free amino acids (from previously intact proteins) was taken as a measure of proteolysis in such experiments.

RESULTS AND DISCUSSION

Degradation of newly-synthesized proteins in rat liver mitochondria

Since mitochondrial preparations are typically contaminated [37; see also below] with lysosomes, sarcoplasmic/endoplasmic reticulum and mast cell granules, our studies began by examining the degradation of newly-synthesized mitochondrial proteins. Mitochondrial proteins were labelled (*in vitro*) by incorporation of [^{3}H]leucine in a pulse-chase procedure, in the presence of 0.36 mM-cycloheximide [37]. Under such conditions mitochondria are capable of limited protein synthesis, but contaminating organelles cannot conduct protein synthesis *de novo*. The mitochondria were then exposed to various forms of oxidative stress, and protein degradation was measured by release of radioactivity in acid-soluble forms. Doxorubicin (Adriamycin) and menadione (2-methyl-1,4-naphthoquinone or vitamin K_3) stimulated the degradation of newly-synthesized mitochondrial proteins during incubation in an air atmosphere (Table 1). A similar stimulation of proteolysis was observed with xanthine oxidase plus xanthine. Proteolysis was unaffected, however, by incubation with the same agents under an N_2 atmosphere. Doxorubicin is reduced by mitochondrial NADH dehydrogenase to produce the doxorubicin semiquinone radical, superoxide, H_2O_2 and $^{\cdot}OH$ [30–32]; similar reactions occur with menadione. Xanthine oxidase produces both superoxide and H_2O_2 directly [47] and may also generate $^{\cdot}OH$ by a transition metal-catalysed Haber Weiss reaction [48]. Thus, the N_2 results in Table 1 provide evidence for the role of activated oxygen species in mitochondrial protein degradation.

The stimulatory effects of menadione and doxorubicin on protein degradation in Table 1 (air atmosphere) also depended on the presence of a reduced substrate for the electron-transport chain. In contrast, proteolysis induced by xanthine oxidase (+xanthine) was identical both in the presence and absence of electron-transport chain substrates (results not shown). These findings further strengthen the conclusion that proteolysis can be initiated by activated oxygen species.

With higher concentrations of doxorubicin or menadione (200 μM and above) and using the conditions outlined in Table 1, we observed a decrease in proteolysis

Table 1. Degradation of newly-synthesized proteins in mitochondria exposed to oxidative stress

Rat liver mitochondria were prepared and [^{3}H]leucine was incorporated into newly-synthesized proteins as described under Experimental procedures. The mitochondria actively oxidized succinate or pyruvate plus malate and were intact, as shown by high respiratory control ratios (ADP stimulation). The labelled mitochondria were then incubated (37 °C) for 1 h with 10 mM-pyruvate and 2.5 mM-malate, under either an atmosphere of air or 100% N_2. For the permeant molecules doxorubicin and menadione (which undergo reduction inside the mitochondrion), a buffered medium consisting of 90 mM-KCl, 50 mM-Hepes, 1 mM-EDTA, 5 mM-KH_2PO_4, 10 mM-$MgCl_2$, 15 mM cold leucine and 0.36 mM-cycloheximide (pH 7.4) was used. For xanthine oxidase and xanthine (which generate radicals outside the mitochondrion), the Hepes and EDTA were omitted, and the KCl concentration was raised to 175 mM. Following incubation, the mitochondria were placed on ice, disrupted and precipitated with trichloroacetic acid, and centrifuged as described under Experimental procedures. The % degradation of newly-synthesized proteins was calculated as the percentage of incorporated (acid-precipitable) radioactivity which appeared as acid-soluble counts in the supernatants. Values are means ± S.E.M. of three independent experiments. Maximal (State III) rates of oxygen consumption decreased after 60 min incubation with doxorubicin or menadione (under air), but no increase in State IV respiration occurred, indicating that the mitochondria were still coupled. We also tested for leakage of malate dehydrogenase from the matrix and adenylate kinase from the inter-membrane space, but found no evidence for significant mitochondrial disruption.

Addition	Incubation atmosphere...	Degradation (%) Air	N_2
None		16.6±2.1	15.2±1.9
Doxorubicin		85.3±8.5	11.8±1.3
Menadione		72.3±9.1	10.5±0.9
Xanthine		15.9±1.9	16.3±1.7
Xanthine oxidase		16.2±2.3	15.7±1.7
Xanthine oxidase +xanthine		83.2±7.9	16.8±2.3

(results not shown). Such results could either be caused by an ATP limitation [49] or by severe protein damage, which can actually decrease proteolytic susceptibility [3,6–11]. Alternatively, proteolytic enzymes may be damaged by high fluxes of activated oxygen species. To test for an ATP limitation to proteolysis, we incubated mitochondria with 10 μM- or 200 μM-doxorubicin or menadione in the presence of an ATP-regenerating system. As shown in Table 2, 10 μM-quinone stimulated proteolysis, but 200 μM-quinone was inhibitory even in the presence of excess ATP.

Table 2. Effects of doxorubicin and menadione concentration on the degradation of newly-synthesized proteins in the presence of an ATP-regenerating system

Rat liver mitochondria were isolated and proteins were labelled (*in vitro*) by incorporation of [^{3}H]leucine, as reported in the legend to Table 1. Mitochondria were then incubated (in air) with doxorubicin or menadione (or with no additions) as described for Table 1, except that an ATP-regenerating system was added. The ATP-regenerating system consisted of 5 mM-phosphoenolpyruvate, 3 mM-ATP and 6 i.u. of pyruvate kinase. Following a 1 h incubation (37 °C), the percentage degradation of newly-synthesized proteins was measured as described in Table 1. ATP concentrations after the 1 h incubation (±doxorubicin or menadione) ranged from 2.84 to 2.97 mM. Values are means of three independent experiments with S.E.M. < 10%.

Concn. (μM)	Degradation (%) Doxorubicin	Menadione
0	22.6	22.6
10	61.3	76.4
200	15.9	14.6

Generalized protein damage and degradation in rat liver mitochondria

The results in Tables 1 and 2 describe the effects of activated oxygen species on incomplete, mitochondrially-encoded polypeptides. To determine if these results might also apply to complete mitochondrial proteins, we exposed unlabelled mitochondria to various forms of oxidative stress (Table 3). Previous work [3,6–11,22–24] indicated that covalently cross-linked protein aggregates can form as a result of exposure to oxidative stress: such aggregates generally exhibit decreased proteolytic susceptibility. Protein fragments can also be generated by direct chemical scission, following reaction of proteins with oxidants and oxygen [3,6–9,22–24].

Doxorubicin, menadione and xanthine oxidase plus xanthine increased the formation of protein aggregates and protein fragments or peptides (Table 3). Peptides generated by proteolysis could not be distinguished from protein fragments by the techniques employed. These same agents also caused a generalized increase in the concentrations of free amino acids, indicating that more rapid rates of proteolysis occurred. Succinate + antimycin A + carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and pyruvate + malate + dicyclohexylcarbodi-imide (DCCD) also increased the formation of protein aggregates, protein fragments or peptides, and free amino acids (Table 3). Antimycin A stimulates the production of superoxide and H_2O_2 by respiring mitochondria, and FCCP further augments this response [28]. In the presence of oxidizable substrate, DCCD stimulates mitochondrial superoxide and H_2O_2 generation, possibly by increasing the steady-state levels of ubisemiquinone [29].

Only highly-modified proteins are reported in Table 3, i.e. proteins so highly cross-linked that they would not enter the polyacrylamide-separating gel or fragments/peptides so small that they ran with the dye front. Several more subtle alterations in electrophoretic banding were also observed. These changes, however, could not be carefully quantified (due to band slurring and uneven staining). A 10–20% decrease in overall staining intensity was observed following exposure to the various forms of oxidative stess.

Table 3 reports overall average increases in the concentrations of the 13 amino acids studied. The ratios of each amino acid to all others remained fairly constant with each incubation condition. It is important to note

Table 3. Protein aggregation, fragmentation and degradation following exposure of mitochondria to oxidative stress

Rat liver mitochondria were prepared as described under Experimental procedures. The mitochondria were then incubated (37 °C) in a buffer consisting of 175 mM-KCl plus 30 mM-potassium phosphate (mono- and di-basic mixed to pH 7.4), and an ATP-regenerating system (5 mM-phosphoenolpyruvate, 3 mM-ATP, 5 mM-$MgCl_2$ and 11.5 μg of pyruvate kinase). Incubation time was 2 h for electrophoresis studies and 1 h for amino acid measurements. Polyacrylamide-gel electrophoresis studies utilized a discontinuous (Tris/glycine/SDS) system consisting of an 8% T-separating gel and a 3% T-stacking gel [46]. Aggregated products were those proteins which would not enter the separating gel (i.e. which remained in the stacking gel). Fragmented or degraded products were those protein fragments or peptides which exhibited molecular sizes of less than 5000 Da (i.e. which ran with the gel front). Electrophoretic bands were quantified by scanning densitometry [6–9], and the results are expressed as percentage of total protein staining intensity (results from a representative gel are shown). Amino acid concentrations were measured with a Beckman 121 MB analyser using sulphosalicylic acid (1.5%) extracts of supernatants from centrifuged mitochondria. The acid extracts were diluted with an equal volume of 0.15 M-LiOH, 0.15 M-citric acid, 1% dithiodiglycol and 0.1% phenol (at pH 2.2). The pre-incubation concentrations and post-incubation concentrations of 13 amino acids were measured (Ala, Arg, Asp, Cys, His, Ile, Leu, Lys, Phe, Ser, Thr, Tyr, Val). The amino acid results above are expressed as [post-incubation concentrations/pre-incubation concentrations] and represent the average means ± S.E.M. for all 13 amino acids.

	Gel electrophoresis		
Addition	Aggregated products	Fragmented or degraded products	Amino acids
None	0	2	2±1
Succinate	0	3	10±2
Succinate + antimycin A + FCCP	3	6	46±5
Pyruvate + malate	0	3	4±1
Pyruvate + malate + DCCD	2	5	38±4
Pyruvate + malate + doxorubicin	5	15	52±8
Pyruvate + malate + menadione	7	12	56±6
Xanthine oxidase	0	3	4±2
Xanthine oxidase + xanthine	12	18	42±5

that each of the agents/conditions (except xanthine oxidase) which increased protein damage or proteolysis in Table 3 had also been found to decrease maximal rates of electron transport in both liver and muscle mitochondria, and to diminish respiratory control [29, O. Marcillat, Z. Yin & K. J. A. Davies, unpublished results]. Furthermore, in an extension of previous work [29], we found that doxorubicin and antimycin A + FCCP damaged mitochondrial dehydrogenase activities, and DCCD diminished transmembrane electrical potentials following incubation. Although these agents or conditions induce both protein damage and lipid peroxidation, our investigations with the lipid-soluble antioxidant butylated hydroxytoluene indicated that lipid peroxidation can be inhibited by 70–90% without significant protection of functional activities (K. J. A. Davies, unpublished results).

Degradation of oxidatively-denatured proteins by mitochondrial proteases

We next tested the ability of mitochondrial (soluble) proteases to discriminate between untreated and oxidatively-denatured proteins. Many previous studies of mitochondrial protease activities have been greatly influenced by contaminating lysosomal (and other) proteases with acidic pH optima (see [37] for a discussion of this problem). Feasibility studies were conducted with mitochondria from rat liver, beef liver and beef heart using various mitochondrial isolation techniques. The lowest (lysosomal) acid phosphatase [50] and acid protease ([^{3}H]casein degradation at pH 4) activities were found in beef heart mitochondria. Careful isolation followed by digitonin treatment reduced the acid phosphatase contamination to 0.03% of that originally present. We, therefore, decided to use beef heart mitochondria for all further experiments. [The data in Fig. 1 and Tables 4–6 were obtained with highly purified and digitonin-treated beef heart mitochondria. In several mitochondrial preparations we observed much higher contaminating acid protease and acid phosphatase activities (results not shown). Great care must be taken at all stages of mitochondrial isolation to obtain preparations with this little lysosomal contamination. Since lysosomal protease activity can also be extremely high in the hearts of certain individual cows, other investigators are advised to test for inter-animal variations.]

A distinct pH 8-proteinase activity clearly discriminated between untreated and ·OH-modified [^{3}H]Hb, whereas the smaller residual pH 4 activity exhibited no preferential degradation (Fig. 1*a*). The ·OH-treated [^{3}H]Hb used in these studies was an excellent proteolytic substrate for neutral/alkaline proteases in erythrocytes, reticulocytes and *Escherichia coli* [3,6–11]. Several of the Hb modifications induced by ·OH exposure have also been described [6–8]. It should also be noted that the pH 8 activity was greatly enhanced by sonic disruption of mitochondrial preparations, whereas the contaminating pH 4 activity was unaffected by such treatment (Fig. 1, compare *a* and *b*). The minor pH 8 activity seen with intact mitochondria and ·OH-modified [^{3}H]Hb was probably due to a slight leakage of mitochondrial enzymes induced by digitonin treatment. A small leakage of adenylate kinase [51] from the intermembrane space, and of malate dehydrogenase [52] from the matrix was found to occur with digitonin treatment (results not shown). Similar results to those in Fig. 1 were also obtained with intact and disrupted rat liver and beef liver mitochondria, but the contaminating pH 4 protease activities were much higher in these preparations (results not shown). These results suggest the existence of an intra-mitochondrial proteolytic system or pathway which can preferentially degrade oxidatively-denatured proteins.

Hb, albumin and superoxide dismutase (as well as many other proteins) are subject to oxidative denaturation by ·OH [2,3,6–11]. Superoxide dismutase is also oxidatively damaged by its product H_2O_2 [53,54]. These (and other) oxidatively-modified proteins are excellent substrates for degradation by neutral/alkaline proteo-

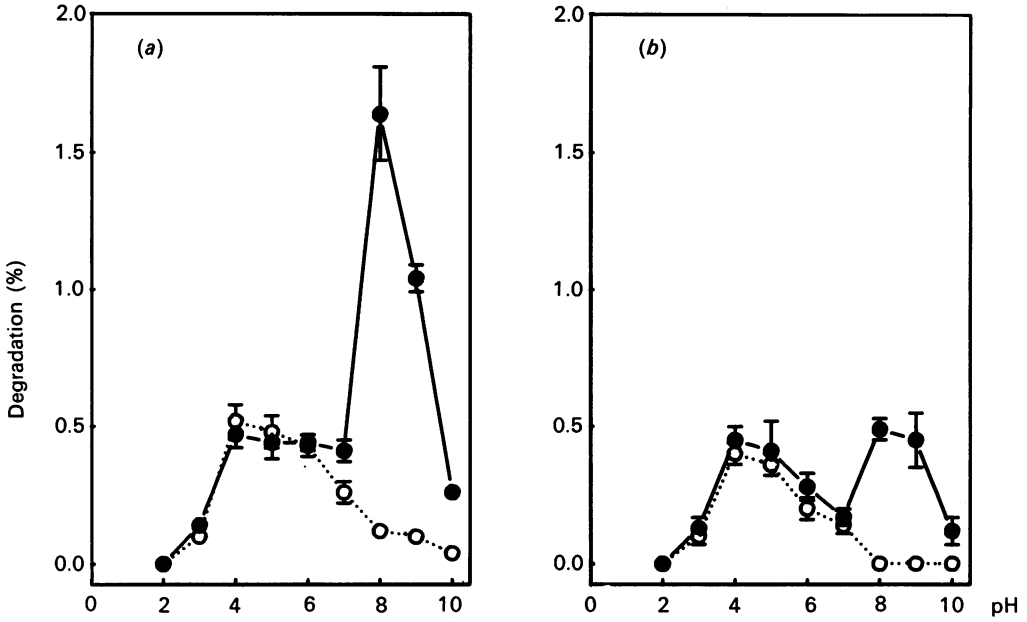

Fig. 1. Degradation of oxidatively-denatured haemoglobin during incubation with intact and disrupted beef heart mitochondria

Experiments were conducted with beef heart mitochondria treated with digitonin (50 μg/mg of protein). Where indicated, mitochondrial disruption was achieved by sonication at 4 °C [30–32]. [^{3}H]Hb was prepared and exposed to the hydroxy radical (10.0 nmol $^{\cdot}$OH/nmol Hb) as described under Experimental procedures. All preparations were incubated (for 1 h at 37 °C) at an osmolarity of approx. 0.25 to preserve the integrity of intact mitochondria. The buffers used were as follows: pH 2–3, 250 mM-formic acid; pH 4, 150 mM-formic acid; pH 5, 100 mM-acetic acid plus 67 mM-Tris; pH 6, 85 mM-acetic acid plus 82 mM-Tris; pH 7, 80 mM-acetic acid plus 87 mM-Tris; pH 8, 56 mM-acetic acid plus 87 mM-Tris; pH 9, 125 mM-boric acid; pH 10, 62.5 mM-boric acid. All incubations involved 0.01 ml of untreated or $^{\cdot}$OH-treated [^{3}H]Hb substrates (0.33 mg of protein/ml); 0.06 ml of buffer solution (pH 2–10); and 0.06 ml of intact or disrupted bovine heart mitochondria (1.0 mg protein/ml). Incubations were terminated by the addition of 0.85 ml of 12.0% (w/v) trichloroacetic acid and 0.06 ml of 3.0% (w/v) bovine serum albumin (as carrier). Percentage [^{3}H]Hb degradation was measured as follows; [acid-soluble counts − background]/[total counts − background] × 100. (*a*) Shows results from sonically-disrupted mitochondria, whereas (*b*) shows the results from intact mitochondria. In both (*a*) and (*b*) the proteolytic substrates were as follows: (●) $^{\cdot}$OH-treated [^{3}H]Hb; and (○) untreated [^{3}H]Hb. Values are means ± S.E.M. of three independent determinations.

lytic systems in erythrocytes from cows, rabbits and humans, rabbit reticulocytes and *E. coli* [2,3,5–11]. As shown in Table 4, mitochondrial proteases also preferentially degraded the oxidatively-denatured forms of Hb, albumin and superoxide dismutase. Some pH 8 protease activity was found with intact, digitonin-treated mitochondria (probably due to leakage of enzymes as discussed above). Disruption of mitochondria greatly increased protease activity, however, and the highest rates of degradation were observed with mitochondrial matrix preparations (Table 4). Thus the mitochondrial proteolytic system or pathway which can preferentially degrade oxidatively-denatured proteins would appear to reside in the matrix. Furthermore, this proteolytic system/pathway can recognize various different proteins and more than one form of oxidative denaturation.

Characteristics of the mitochondrial proteolytic pathway for oxidatively-denatured proteins

Although a detailed analysis of this novel proteolytic pathway is beyond the scope of the present report, it was important to determine if the previously described ATP-dependent neutral protease [37,38] was involved. It was also decided that the inhibitor profile of this proteolytic pathway should yield important clues for future studies, and provide further potential evidence to discriminate between true mitochondrial proteases and contaminating activities.

An ATP-stimulated endoprotease in the mitochondrial matrix has been shown to initiate the degradation of added proteins such as casein and globin [38]. Such stimulation has been reported in sonically-disrupted mitochondria, as well as with the isolated protease [38]. We measured the effects of ATP (and Mg^{2+}) on the degradation of $^{\cdot}$OH-modified [^{3}H]Hb and [^{3}H]albumin during incubation with sonicated mitochondria. Instead of stimulating proteolysis, ATP actually caused a 28–30% decrease in degradation (Table 5). Such data argue against the participation of ATP-stimulated or -dependent proteases in the degradation of oxidatively-denatured proteins. Similar results were previously obtained when studying the degradation of oxidatively-denatured proteins in cell-free extracts of red blood cells and *E. coli* [2,3,5,6–11], and the degradation of oxidatively-damaged glutamine synthetase in bacterial extracts has also been reported to occur as an ATP-independent activity [12].

Although no full explanation can yet be provided for the above findings, it appears probable that the activities of various proteolytic pathways within a cell or organelle may be determined by the substrate (and co-factor) specificities of a few initial cleavage enzymes (proteinases). The products of initial cleavage may be further

Table 4. Degradation of oxidatively-denatured proteins during incubation with intact beef heart mitochondria, disrupted mitochondria and the mitochondrial matrix, at pH 8

Digitonin-treated beef heart mitochondria (50 μg digitonin/mg of protein) were used for all experiments. Where indicated, mitochondria were disrupted by sonication [30–32]. The mitochondrial-matrix fraction consisted of the supernatants of sonically-disrupted mitochondria, following centrifugation at 100000 *g* for 1 h. The degradation of untreated and ·OH-modified [^{3}H]Hb and [^{3}H]-bovine serum albumin, as well as untreated and H_2O_2-treated [^{3}H]superoxide dismutase, was measured as described in the legend to Fig. 1, except that all measurements were at pH 8.0. Values are means ± S.E.M. of three independent determinations.

	Degradation (%)		
Substrate	Intact mitochondria	Disrupted mitochondria	Matrix
Haemoglobin			
Untreated	0.00	0.12 ± 0.02	0.12 ± 0.05
·OH-treated	0.13 ± 0.01	1.76 ± 0.05	5.32 ± 0.17
Serum albumin			
Untreated	0.19 ± 0.09	0.25 ± 0.09	0.35 ± 0.11
·OH-treated	0.98 ± 0.19	1.65 ± 0.14	2.72 ± 0.24
Superoxide dismutase			
Untreated	0.05 ± 0.10	NT*	0.23 ± 0.05
H_2O_2-treated	1.73 ± 0.12	NT	5.27 ± 0.22

*NT, not tested.

Table 5. Effects of ATP on the degradation of oxidatively-denatured proteins in sonically-disrupted beef heart mitochondria, at pH 8

Digitonin-treated beef heart mitochondria (50 μg digitonin/mg of protein) were used for all experiments. Sonically-disrupted mitochondria were incubated with ·OH-modified [^{3}H]Hb or with ·OH-modified [^{3}H]bovine serum albumin, as described in the legend to Fig. 1, except that all measurements were performed at pH 8.0 in the presence of 5 mM-$MgCl_2$. Where indicated, 5 mM-ATP was added to the samples prior to the start of incubation. Values are means ± S.E.M. of three independent experiments.

	Degradation (%)		ATP-effect on degradation (%)
·OH-modified substrate	Without ATP	With ATP	
Haemoglobin	2.46 ± 0.11	1.77 ± 0.21	−28
Albumin	1.97 ± 0.14	1.38 ± 0.12	−30

degraded by a series of proteases and peptidases, several of which may actually be shared by various proteolytic pathways. Thus, it is possible that stimulation of one proteolytic pathway may actually inhibit the operation of other proteolytic pathways. We do not yet know if the degradation of oxidatively-denatured proteins is conducted by a previously unknown system of proteolytic enzymes, or if known enzymes participate in a novel pathway. It must also be emphasized that our results in no way question the existence or significance of the ATP-stimulated endoprotease previously reported to function in the mitochondrial matrix [37,38].

Table 6. Potential inhibitors of the degradation of oxidatively-denatured Hb

Digitonin-treated beef heart mitochondria (50 μg of digitonin/mg of protein) were used for all experiments. The degradation of ·OH-treated [^{3}H]Hb during incubation with intact mitochondria and sonically-disrupted mitochondria was measured, at pH 4 and pH 8, as described in the legend to Fig. 1. The degradation of ·OH-modified [^{3}H]Hb in the absence of any potential inhibitor is also shown in Fig. 1. Pepstatin and leupeptin were used at a concentration of 20 μg/ml, haemin at 0.1 mM and EDTA at 10 mM. Values are means ± S.E.M. of three independent experiments.

	Inhibition of protein degradation (%)			
	Intact mitochondria		Disrupted mitochondria	
Additions	pH 4	pH 8	pH 4	pH 8
Pepstatin	98	0	99	0
Leupeptin	25	0	43	8
Haemin	0	100	0	94
EDTA	0	100	4	88
1 mM-PMSF	0	100	0	55
5 mM-PMSF	2	50	9	51

We next incubated mitochondria with various potential protease inhibitors, using ·OH-modified [^{3}H]Hb as substrate (Table 6). The contaminating pH 4 protease activity of both intact and disrupted mitochondria was sensitive to the lysosomal protease inhibitors pepstatin and leupeptin, but little or no inhibition was exerted by these agents at pH 8. In contrast, haemin, EDTA and phenylmethylsulphonyl fluoride (PMSF) were strongly inhibitory at pH 8 (both with intact and disrupted mitochondria) but exerted little or no inhibition at pH 4 (Table 6). Inhibition by PMSF (a serine reagent) may indicate the presence of a serine protease. Although PMSF inhibition was complete with intact mitochondria (which had low activity) no more than 55% inhibition could be achieved with disrupted mitochondria (which had much higher activity). No satisfying interpretation of these results can yet be proposed. Strong inhibition by EDTA may be taken as initial evidence for the operation of a metalloprotease. The mechanism of inhibition by haemin (which affects many proteases) is unknown.

Inhibition of the degradation of oxidatively-denatured proteins by serine reagents such as PMSF and diisopropyl fluorophosphate; by transition metal chelators such as EDTA, *trans*-1,2-diaminocyclohexane-*NNN'N'*-tetra-acetic acid, diethylenetriaminetetra-acetic acid, 8-hydroxyquinoline, and *o*-phenanthroline); as well as by haemin, has been observed in cell-free extracts of red blood cells and *E. coli* [2,3,6–11]. Thus, serine proteases and metalloproteases may be widely employed for the degradation of oxidatively-denatured proteins.

Since mitochondria are one of the major sources of intracellular oxygen radical and H_2O_2 production [28,29],

mitochondrial proteins must be subjected to a relatively high level of oxidative stress. This condition may be further compounded in the presence of various quinonoid substances which are redox cycled by the electron-transport chain to generate large fluxes of activated oxygen species [30–32]. Since mitochondrial proteins can clearly be damaged or denatured by such conditions [21,22,29, Y. Zhang, O. Marcillat & K. J. A. Davies, unpublished results] the existence of a mitochondrial proteolytic system or pathway which can recognize and remove oxidized polypeptides may be of great importance. Given that the inner membrane would exclude any cytoplasmic proteases (even should they penetrate the outer membrane) an intra-mitochondrial proteolytic system would provide the only means of editing post-translational oxidative modifications during the organelle's life.

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