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Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism

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Mitochondrial aconitase is sensitive to oxidative inactivation and can aggregate and accumulate in many age-related disorders. Here we report that Lon protease, an ATP-stimulated mitochondrial matrix protein, selectively recognizes and degrades the oxidized, hydrophobic form of aconitase after mild oxidative modification, but that severe oxidation results in aconitase aggregation, which makes it a poor substrate for Lon. Similarly, a morpholino oligodeoxynucleotide directed against the *lon* gene markedly decreases the amount of Lon protein, Lon activity and aconitase degradation in WI-38 VA-13 human lung fibroblasts and causes accumulation of oxidatively modified aconitase. The ATP-stimulated Lon protease may be an essential defence against the stress of life in an oxygen environment. By recognizing minor oxidative changes to protein structure and rapidly degrading the mildly modified protein, Lon protease may prevent extensive oxidation, aggregation and accumulation of aconitase, which could otherwise compromise mitochondrial function and cellular viability. Aconitase is probably only one of many mitochondrial matrix proteins that are preferentially degraded by Lon protease after oxidative modification.

The selective removal of oxidatively modified proteins by proteolytic digestion is an important component of cellular defences against oxidative stress[1,2]. In the mammalian cell cytoplasm, nucleus and endoplasmic reticulum, the proteasome complex carries out this important function[3–7]. Although neither bacteria nor mammalian mitochondria express the proteasome, both can selectively degrade oxidatively damaged internal proteins[8–11].

The mitochondrial electron transport chain is probably the main source of intracellular free radicals[1,10,12–16], and mitochondrial proteins are highly subject to oxidative modification and inactivation[1,14,16–19]. Although various proteolytic systems have been identified in different mitochondrial compartments[20], the turnover of both normal and oxidatively modified mitochondrial proteins is understood poorly.

The ATP-stimulated proteolysis of caseine has been measured in the mitochondrial matrix from many different mammalian tissues[21–26]. The enzyme responsible was identified as the mammalian Lon protease[22,25,26], a homologue of bacterial protease La[21,27] and yeast PIM1 protease[28]. Yeast strains lacking a functional *pim1* gene have impaired ability to degrade mitochondrial matrix proteins[28] and to maintain functional mitochondrial DNA[29,30].

Aconitase, an essential mitochondrial enzyme, is particularly susceptible to oxidative damage[31,32] and is preferentially oxidatively modified and inactivated during ageing. Previous results have shown that mammalian mitochondria contain a proteolytic system that can recognize and degrade various oxidatively denatured proteins *in vitro*[10,11]. Here we have examined whether Lon protease is responsible for the degradation of both oxidized and native aconitase in bovine and human mitochondria, and whether downregulation of *lon* expression results in the accumulation of aconitase.

Results

Modification of aconitase by treatment with H_2O_2. The enzymatic activity of aconitase was decreased by treatment with H_2O_2 (Fig. 1a), but no simple relationship between activity and H_2O_2 concentration was observed. No substantial loss of aconitase activity occurred at H_2O_2 concentrations below 2 mM. Enzymatic activity decreased by 50% after treatment with 5 mM H_2O_2, but there was still 33% residual aconitase activity after incubation with 20 mM H_2O_2. Oxidation by even the lowest concentrations of H_2O_2 caused immediate and large increases in the overall hydrophobicity of aconitase, as measured by hydrophobic interaction chromatography (Fig. 1a). We have seen a similar oxidation-induced partial denaturation, followed by the exposure of hydrophobic 'patches' of (normally internally shielded) amino acids, in several other proteins[1,3,33].

Aggregation of aconitase was negligible at exposure to low concentrations of H_2O_2, but increased exponentially at H_2O_2 concentrations above 5 mM (Fig. 1a). Western blot analysis of the treated enzyme (Fig. 1b) showed bands migrating with a relative molecular mass (M_r) higher than 80,000 (80K) in SDS polyacrylamide gel electrophoresis (SDS–PAGE), indicating that substantial protein aggregation occurred at H_2O_2 concentrations as low as 2 mM. At 5 mM H_2O_2, both aggregation and protein fragmentation, as judged by an SDS–PAGE band at about 40K, occurred. Clearly, a significant proportion of the aconitase aggregation seen in Fig. 1a was noncovalent, because most of the aggregates were disrupted by SDS–PAGE. Finally, partial loss of antigenicity (loss of band density) was observed at the highest concentration of 20 mM H_2O_2, indicating that extreme oxidative modification of the protein had occurred.

A mitochondrial proteolytic system preferentially degrades oxidized aconitase. The proteolytic susceptibility of aconitase, when incubated with extracts of beef heart mitochondria, increased rapidly after *in vitro* treatment with H_2O_2 up to a concentration of 5 mM (Fig. 2a). At this exposure to oxidant, the rate of aconitase degradation was seven times higher than the breakdown of unoxidized

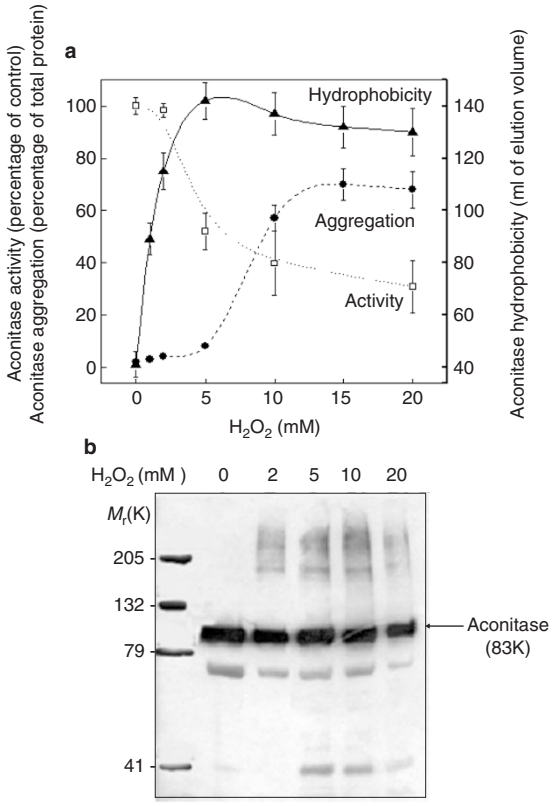

Figure 1 **Loss of aconitase activity and protein modification after H_2O_2 treatment.** Aconitase (0.33 mg ml^{-1}) was incubated with H_2O_2 at the concentrations indicated for 30 min at 37 °C. Residual peroxide was removed by dialysis, and both oxidized and control aconitase were studied. **a**, Effect of H_2O_2 on aconitase activity, reported as a percentage of the control activity, and on the hydrophobicity of the [^{3}H]aconitase protein, measured by butyl-Sepharose 4B hydrophobic interaction chromatography[33] and reported as the elution volume of [^{3}H]aconitase from the column in millilitres. Also shown is the overall aconitase aggregation measured by the percentage of total [^{3}H]aconitase ($M_r \approx$ 83K) retained by the 100,000 nominal molecular mass cut-off Ultracel membrane of a Centricon (Millipore) centrifugal filter. **b**, Western blot study of untreated aconitase and aconitase exposed to various concentrations of H_2O_2. Results in **a** are the means and standard errors of six independent determinations, and the blot shown in **b** was repeated several times with similar results.

Table 1 Inhibitors of ATP-dependent aconitase degradation (% inhibition)

	Aconitase		Oxidized aconitase	
	no ATP	5 mM ATP	no ATP	5 mM ATP
EDTA	11%	40%	30%	57%
PMSF	100%	83%	48%	75%
NEM	56%	64%	20%	45%
NLVS	0%	0%	0%	0%

Protein degradation was measured (see Fig. 2a) in the presence or absence of ATP (5 mM). The inhibitors EDTA (25 mM), PMSF (5 mM), NEM (1 mM) and NLVS (20 μM) were added as indicated. The uninhibited, absolute values for degradation of oxidized and control aconitase, both with and without ATP, are given in Table 2. Each value represents the mean of six independent determinations, for which the standard errors were always less than 10%.

Table 2 Proteolytic degradation of aconitase by mitochondrial extract and purified Lon protease

	Aconitase		Oxidized aconitase	
	no ATP	5 mM ATP	no ATP	5 mM ATP
Mitochondrial extract	0.2	1.0	1.8	3.6
Purified Lon Protease	91.5	327.5	574.0	1950.0

Values are in micrograms of degraded protein per milligram of mitochondrial extract protein or Lon protease protein per hour. Proteolytic specific activity was measured for the mitochondrial matrix fraction and for purified Lon protease against dialysed control and oxidized aconitase. Each value represents the mean of nine independent determinations for which the standards errors were always less than 10%.

aconitase. When aconitase was subjected to higher concentrations of H_2O_2, its proteolytic susceptibility actually decreased, and it returned to basal levels after treatment with 20 mM H_2O_2.

A comparison of Figs 1a, b and 2a shows that mildly oxidized aconitase is a good proteolytic substrate, whereas extreme oxidative modification of aconitase results in a loss of proteolytic susceptibility. We propose that the exposure of hydrophobic patches in mildly oxidized aconitase makes it a better proteolytic substrate, whereas increasing aggregation at higher H_2O_2 concentrations (Fig. 1) makes aconitase a progressively poorer proteolytic substrate (Fig. 2a). This interpretation is very much in agreement with our studies of the degradation of other oxidized proteins (and aconitase itself) by other proteases[1,3,6,9,32–34].

When ATP was added to the reaction mixture, the degradation of aconitase increased 2–4-fold, depending on the oxidation status of the substrate. We used 5 mM H_2O_2 treated (oxidized) aconitase—the substrate that achieved maximal degradation by the bovine heart mitochondrial extract—as a model substrate in subsequent studies and compared it with control (untreated) aconitase. With both substrates, the rate of proteolytic degradation was linear for the first 60 min and reached a plateau after 90 min (Fig. 2b).

To ensure that proteases in the mitochondrial extracts could not have been inhibited by any residual H_2O_2 (possibly remaining even after dialysis), we added increasing concentrations of H_2O_2 to a maximum of 20 mM to tubes containing buffer only and then dialysed the samples (as for Fig. 2a). These dialysed buffer samples were then added to mitochondrial extracts containing heat denatured [^{3}H]aconitase, which is an excellent substrate for proteolysis. Regardless of the initial H_2O_2 concentration before dialysis, these dialysed buffer samples had no measurable effect on mitochondrial extract proteolytic activity in the presence or absence of ATP, indicating that dialysis was very effective in removing H_2O_2 (data not shown).

Mitochondrial aconitase is degraded by an ATP-stimulated serine protease. Inhibitory trials, designed to characterize the proteolytic activity that selectively degrades oxidized aconitase, suggested that an ATP-stimulated serine protease may be involved (Table 1). Degradation of both oxidized and unoxidized aconitase was strongly inhibited by phenylmethylsulfonyl floride (PMSF), a serine protease inhibitor, and partially inhibited by *N*-ethylmaleimide (NEM), a cysteine protease inhibitor. Addition of ATP stimulated the proteolysis of oxidized and control substrates by about the same percentage (in the presence of 5 mM ATP the activity was increased 2–3.5-fold). EDTA decreased proteolytic activity in the absence of ATP only slightly, but blocked the ATP-stimulated portion of protein degradation much more effectively. NLVS, a recognized proteosome inhibitor, had no effect on degradation, thus excluding the

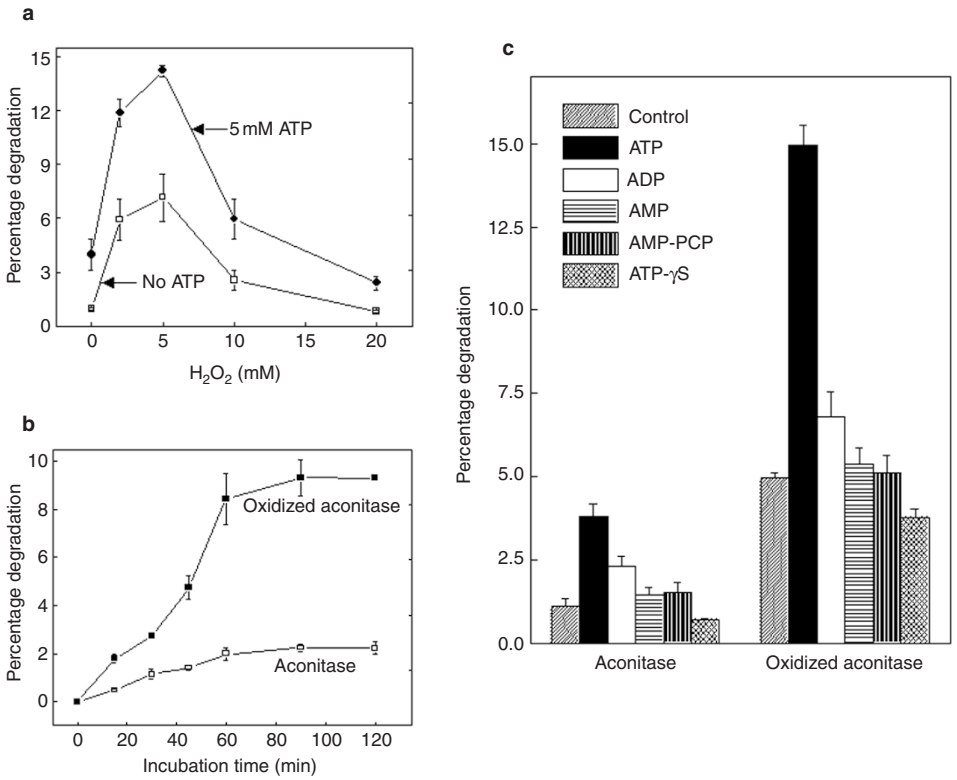

Figure 2 **Degradation of oxidatively-denatured aconitase during Incubation with bovine heart muscle mitochondrial extract.** Aconitase was tritium-labelled by reductive methylation[48] and exposed to increased concentrations of H_2O_2. In all panels, protein degradation was measured by increases in acid-soluble radioactivity after precipitation with trichloroacetic acid (see Methods)[32]. **a**, The oxidized substrates and the untreated control (5 μg) were dialysed and then subjected to proteolysis with bovine heart mitochondrial extract (200 μg) for 90 min. **b**, 5 μg of oxidized aconitase (5 mM H_2O_2 exposure) or control aconitase were incubated with bovine heart mitochondrial extract (200 μg) for the indicated times. Results shown in **a** and **b** are the means and standard errors of nine independent determinations. **c**, Oxidized and control aconitase proteolysis was measured in the presence of ATP, ADP, AMP, AMP-PCP and ATP-γS, all at a final concentration of 5 mM. All results are the means and standard errors of six independent determinations.

possibility of proteasome contamination. Limited experiments suggested that Lactacystin, another proteasome inhibitor, might also inhibit mitochondrial proteolysis (data not shown), but in a non-specific manner similar to the inhibition of lysosomal proteolysis by the same reagent[35].

Although ATP strongly stimulated degradation, ADP only produced a mild increase in aconitase proteolysis (Fig. 2c). Nonhydrolysable ATP analogues and AMP did not increase degradation at all (Fig. 2c), which suggests that the energy of ATP hydrolysis is used for this process. These data (Table 1 and Fig. 2c) match the reported inhibitor/activator profile of Lon protease[22,26].

Lon protease identification, purification and proteolytic activity. We purified the mitochondrial matrix from bovine heart muscle mitochondria and fractioned it using size-exclusion chromatography (Fig. 3a). The maximal proteolytic activity was identified in fraction 52, which corresponded to an M_r of roughly 600K (Fig. 3b). This value is similar to the molecular mass for Lon protease estimated by Kuzela and Goldberg[36] and slightly smaller than the one calculated by Watabe and Kimura[25]. After purifying this fraction further by affinity chromatography, silver staining of both the native and the SDS–PAGE gels revealed only one main band (Fig. 4), with a subunit M_r of 108K. An antibody against Lon recognized the purified Lon as two protein bands of roughly 100K and 80K. After purification, the specific proteolytic activity against aconitase increased more than 500-fold (Table 2).

The purified mitochondrial Lon protease preferentially degraded oxidized aconitase in an ATP-stimulated manner, with maximal activity against aconitase that had been exposed to 5 mM H_2O_2 (Fig. 5a). EDTA was able to block the ATP stimulation but not the ATP-independent baseline proteolysis, whereas PMSF was a potent inhibitor of the enzyme regardless of the presence or absence of ATP (Fig. 5b). These results (Fig. 5) clearly show the same pattern of proteolytic stimulation and inhibition seen with mitochondrial extracts (Fig. 2 and Table 1).

Several caveats must be considered when analysing the results shown in Fig. 5. First, Lon protease is stimulated by ATP, not dependent on ATP, therefore some baseline activity can be measured in the absence of any energy source. Second, all purified proteins contain small amounts of damaged molecules that are produced during isolation and/or storage. Thus, even our 'control' aconitase samples contained small quantities of denatured protein, which are good proteolytic substrates and artificially increase the apparent degradation values of control samples. Last, all normal proteins have an intrinsic proteolytic susceptibility depending on their amino acid sequence and the proteases with which they come into contact. It seems highly likely that aconitase is normally degraded by Lon protease, but that aconitase oxidation greatly facilitates this proteolysis (a viewpoint that is further supported below).

Downregulation of Lon impairs mitochondrial proteolysis. WI-38 VA-13 human lung fibroblasts were treated with oligomorpholines directed against the *lon* sequence, which produced a significant downregulation of *lon* expression when compared with cells treated with control oligomorpholines (Fig. 6a). Four days after this

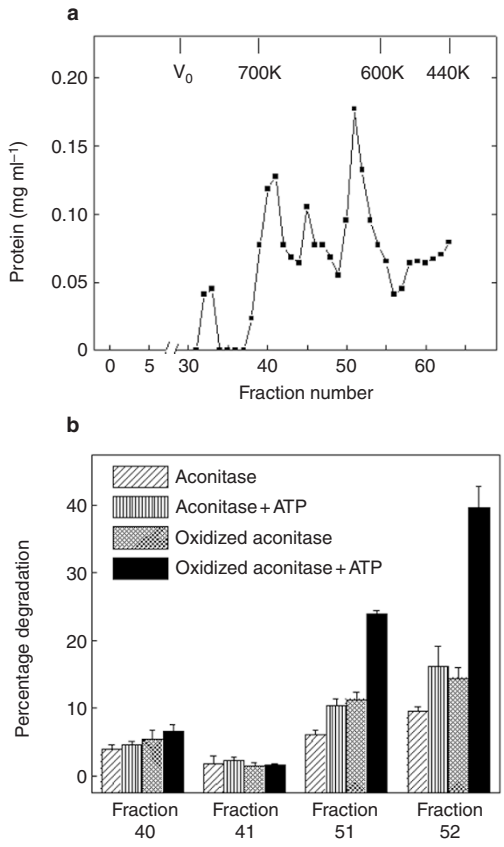

Figure 3 **Elution profile of Lon protease from size-exclusion chromatography and proteolytic activity of bovine heart mitochondrial matrix fractions.** **a**, Matrix fractionation. Mitochondrial matrix (~2 g of protein) prepared from bovine heart mitochondria was separated by size-exclusion chromatography[22,36]. **b**, Proteolytic activity in matrix fractions. Proteolytic activity was measured in each fraction against both oxidized and control aconitase (both dialysed), in the presence or absence of 5 mM ATP (only the four fractions with significant proteolytic activity are shown). Results shown are the means and standard errors of six independent determinations.

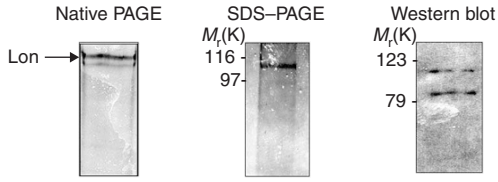

Figure 4 **Purification of mitochondrial Lon protease.** The bovine heart mitochondrial matrix fraction with maximum proteolytic activity (fraction 52 from Fig. 3) was dialysed overnight against the elution buffer supplemented with 0.5 mM ATP. Potassium phosphate was added to this fraction to a final concentration of 10 mM, and then the sample was applied to a 2-ml phosphocellulose column (P11, Whatman) and eluted with a 0.1–0.4 M potassium phosphate gradient[36]. The most active fraction was eluted with 250 mM potassium phosphate. Next, 2.5 μg of purified enzyme was loaded on a native gel and 2.5 μg was loaded on an SDS–PAGE gel. The gels were then fixed and stained with silver. Antibodies against Lon were used for the western blot. The gels were repeated several times with very similar results.

treatment, Lon protein could not be detected by western blot in cells treated with the *lon* antisense oligonucleotides.

We then isolated mitochondria from the cells and prepared matrix fractions. We incubated these mitochondrial matrix fractions with *in vitro* oxidized and native purified aconitase. The mitochondrial matrix purified from cells treated for 4 d with *lon* antisense oligonucleotides had lost more than 87% of its ability to degrade both oxidized and control aconitase (Fig. 6b). The remaining 13% of proteolysis may have been due to a very small amount of residual activity of Lon protease (even though we could not detect the enzyme by western blot) or to other mitochondrial proteases, some of which are also stimulated by ATP[20].

We also examined the intracellular accumulation and degradation of aconitase in intact WI-38 VA-13 human lung fibroblasts that had been treated with the *lon* antisense oligonucleotides. We detected an increase in the amount of total aconitase protein in cells treated with *lon* antisense oligonucleotides. Four days after oligonucleotide treatment, the *lon*-deficient cells contained up to three times more aconitase protein than did controls (Fig. 7a, b). To quantify the amount of aconitase that was oxidized, we examined the protein carbonyl content of the cells. There was a marked increase in carbonylated aconitase in the cells treated with *lon* antisense oligonucleotide (Fig. 7c), and this increase was inversely related to the decrease in quantities of Lon and Lon activity observed in the same samples (Fig. 6a, b).

The accumulation of aconitase protein had more complex effects on the total enzymatic activity of aconitase inside the cells treated with *lon* antisense oligonucleotide. After 2 d, aconitase activity increased inside the *lon*-deficient cells (Fig. 7d), concurrent with the increase in the amounts of native and moderately oxidized aconitase (Fig. 7b, c). But after 4 d, the aconitase present inside the cells treated with *lon* antisense oligonucleotide was already heavily damaged (carbonylated), and its activity had decreased by twofold, even though the cells contained at least double the total amount of aconitase protein that they had on the second day after treatment (Fig. 7d).

Discussion

The essential mitochondrial Kreb's cycle enzyme, aconitase (citrate/*cis*-aconitate/isocitrate) contains four iron–sulphur centres that make the enzyme especially sensitive to oxidative inactivation, both *in vitro* and *in vivo*[31,32,37–39]. Oxidized mitochondrial proteins, especially aconitase, can accumulate during oxidant stress, during ageing and in age-related diseases[40–43]. We have shown here that oxidized aconitase, generated by exposure to H_2O_2, is preferentially degraded (as compared with a nonoxidized control) in both mitochondrial extracts and mitochondrial matrix fractions from beef heart muscle.

Notably, mild oxidative modification of the protein resulted in increased exposure of hydrophobic 'patches' and increased susceptibility to proteolysis, even at oxidant concentrations that caused only a minor loss of aconitase enzymatic activity. At exposure to high concentrations of oxidant, aconitase was strongly modified, becoming aggregated and even losing antigen-recognition sites, and enzymatic activity was compromised more severely. But this strongly oxidized form of the protein was a poor proteolytic substrate. We propose that early recognition and selective degradation of oxidized (hydrophobic) aconitase in the mitochondrial matrix is important in preventing aggregation and accumulation of the damaged protein (and probably of other oxidized mitochondrial matrix proteins).

The matrix proteolytic activity responsible for selective degradation of oxidized aconitase was strongly stimulated by ATP, mildly stimulated by ADP, and unaffected by AMP or nonhydrolysable ATP analogues. The matrix activity was strongly inhibited by PMSF, and partially inhibited by NEM and EDTA. These characteristics match the known activator/inhibitor profile of mammalian

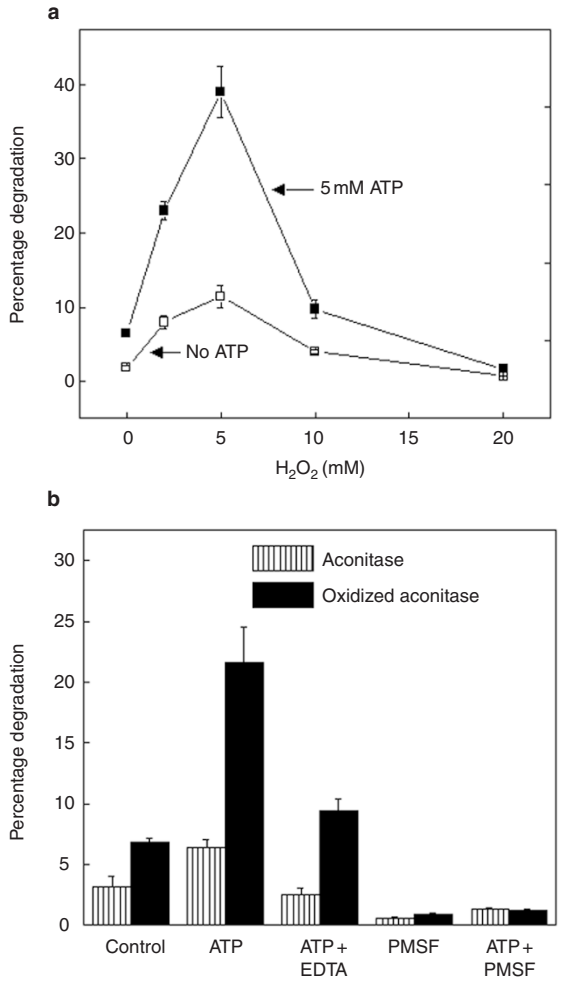

Figure 5 **Purified Lon protease preferentially degrades oxidized aconitase.** **a**, ATP stimulation of Lon protease. Radiolabelled aconitase was treated with the H_2O_2 concentrations indicated, dialysed and then subjected to degradation by Lon protease in the absence or presence of 5 mM ATP. The variously oxidized aconitase proteolytic substrates (each at 5 μg) were incubated with purified Lon protease (2 μg) in the presence or absence of ATP (5 mM) and proteolysis was measured by the increase in acid-soluble radioactivity. **b**, Inhibitors and activators of Lon protease. Untreated aconitase and aconitase oxidized by exposure to 5 mM H_2O_2 were dialysed and then subjected to proteolysis by the purified Lon protease, as in **a**. The effects of the proteolytic activator ATP (5 mM) and the inhibitors EDTA (25 mM), and PMSF (5 mM) were then tested. The values reported represent the means and standard errors from six independent determinations.

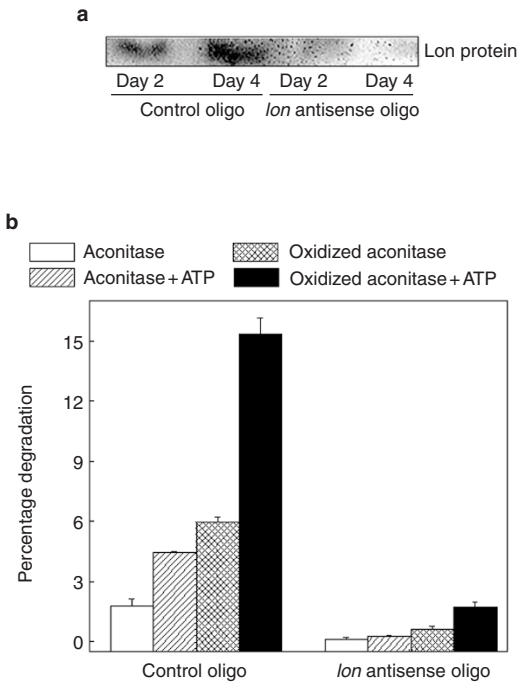

Figure 6 **Diminished ATP-stimulated degradation of aconitase in intact WI-38 VA-13 human fibroblasts after treatment with *lon* antisense oligonucleotides.** **a**, The amount of Lon protease protein decreases after treatment with *lon* antisense oligonucleotides. WI-38 VA-13 human lung fibroblast cells were treated with a *lon* antisense morpholino oligonucleotide, or a matched control morpholino oligonucleotide, and the amount of Lon protease was determined 2 and 4 d after treatment by western blot analysis. These experiments were repeated three times with very similar results. **b**, Downregulation of *lon* reduces aconitase proteolysis in mitochondrial extracts. Mitochondrial matrix was isolated from the two groups of cells shown in a and incubated with oxidized aconitase (5 mM H_2O_2 treatment) or control aconitase to measure proteolytic susceptibility. The ATP concentration was 5 mM when used. All results shown are the means and standard errors of six independent determinations.

mitochondrial Lon protease[23–26,36], the homologue of bacterial protease La[21,27]. In addition, the same proteolytic activity co-purified with Lon protease after size-exclusion chromatography and affinity chromatography of the mitochondrial matrix fraction, and purified Lon protease had the same activator/inhibitor profile (ATP, PMSF and EDTA) as that seen in mitochondrial extracts and matrix fractions.

In WI-38 VA-13 human lung fibroblasts, experimental downregulation of Lon mediated by morpholino oligodeoxynucleotides directed against the *lon* sequence substantially decreased the intracellular amount of Lon protein and decreased the degradation of aconitase, in particular the selective degradation of oxidized aconitase. Although H_2O_2 was not included in these experiments, growth in 19.9% oxygen (that is, standard cell-culture conditions with 5% CO_2) was sufficient oxidative stress to cause the intracellular formation of oxidatively modified (carbonylated) aconitase. Initially, treatment with *lon* antisense oligonucleotides resulted in elevated mitochondrial aconitase activity, but 4 d after this treatment the accumulating aconitase protein became so severely modified (by carbonylation) that enzymatic activity declined. We conclude that Lon protease is essential in minimizing the aggregation and intracellular accumulation of oxidized mitochondrial aconitase. Thus, in defences against oxidized proteins in the mitochondria, Lon protease may have a very similar role to that conducted by the proteasome in the cell cytoplasm and nucleus[1,3,4,7].

Oxidative inactivation of aconitase has been associated with decreased life span in *Drosophila*[40]. A decline in aconitase activity has also been described in other abnormalities, such as increased oxidative stress caused by MnSOD deficiency[41], and in several neurodegenerative diseases including progressive supranuclear palsy[44], Friedreich's ataxia[45] and Huntington disease[46]. In yeast, loss of Lon function results in irregularly shaped mitochondria in which the matrix space becomes filled with electron-dense inclusion bodies, which are thought to be aggregated mitochondrial proteins[28,30]. The only mammalian system in which reduced expression of Lon protease has been reported is ageing murine muscle[47], in which an accumulation of oxidized protein aggregates occurs[42]. We propose that ATP-stimulated Lon protease may be an essential component of mitochondrial (and cellular) defences against the stress imposed by life in an oxygen environment. By recognizing minor oxidative

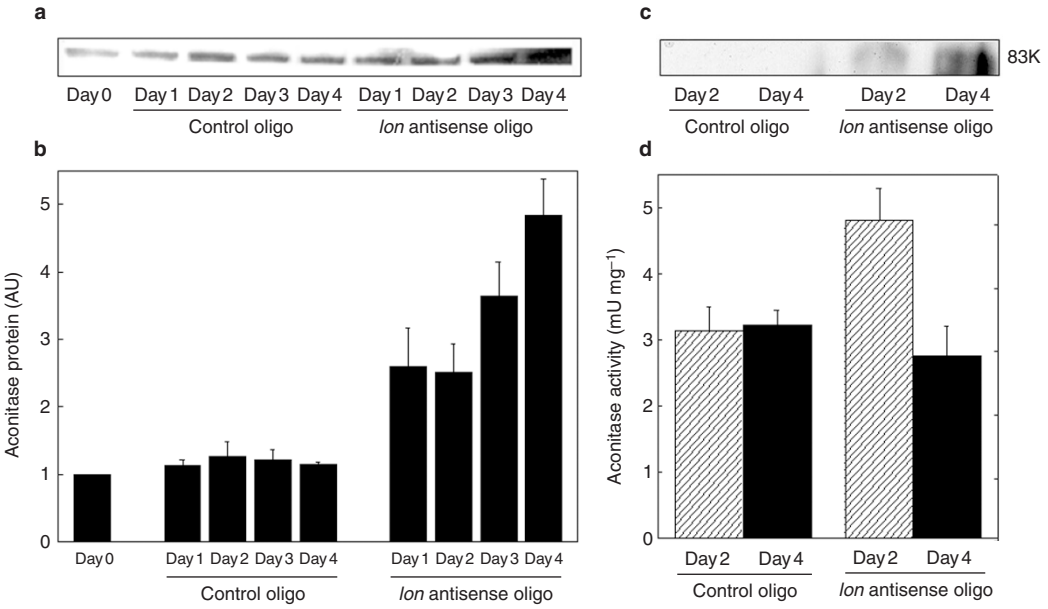

Figure 7 **Aconitase protein quantity, activity and oxidative modification in WI-38 VA-13 human lung fibroblasts treated with *lon* antisense oligonucleotides.** Cell extracts were prepared from WI-38 VA-13 human lung fibroblasts exposed either to a *lon* antisense morpholino oligonucleotide or to a control morpholino oligonucleotide for 0–4 d. Total aconitase protein was visualized using a primary polyclonal antibody against human aconitase, and carbonyl groups (oxidized proteins) were identified using antibodies against DNP. **a**, Representative total aconitase western blot. **b**, Summary of means and standard errors of total aconitase from three different experiments in which total amounts of aconitase, visualized by western blotting as in **a**, were quantified by densitometry and are expressed in arbitrary units. **c**, Representative carbonylated aconitase western blot detection, 2 and 4 d after treatment with morpholino oligonucleotides. **d**, Aconitase activity was measured in whole-cell extracts. Results are means and standard errors of three independent determinations.

changes to aconitase structure and rapidly degrading the mildly modified protein, Lon protease may prevent more extensive oxidation, aggregation and accumulation, which might otherwise compromise mitochondrial function and cell viability. Given the large accumulation of oxidized protein aggregates that occurs with loss of Lon protease activity[28,30,42,47], it seems likely that the oxidized forms of several mitochondrial matrix proteins are preferred substrates for the protease.

Methods

Materials

All chemicals and reagents were obtained from Sigma unless otherwise specified. Porcine heart mitochondrial aconitase (A5384, Sigma) was radiolabelled by reductive methylation using [^{3}H]formaldehyde (NEN)[48]. Radiolabelled aconitase was exposed to increasing concentrations of H_2O_2 at 37 °C and pH 7.4 as described[32], except that a shorter incubation time (30 min) was used. We dialysed both the oxidized aconitase and the controls extensively before using them as proteolytic substrates.

Isolation of mitochondria

Bovine heart mitochondria were isolated as described[49] in a homogenizing buffer (0.21 M mannitol, 70 mM sucrose, 1 mM EDTA and 5 mM HEPES, pH 7.4).

Human WI-38 VA-13 lung fibroblasts (ATCC) were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). The cultures were grown in a humidified incubator containing 19.9% O_2 and 5% CO_2 at 37 °C. For isolating mitochondria, we collected 10^9 cells for each experiment[50]. The homogenizing buffer was the same as that used for heart mitochondrial purification.

Mitochondrial pellets from both sources above were incubated with digitonin (50 μg per mg protein) to disrupt any remaining contaminating lysosomes[10]. Finally, mitochondria were suspended in TMGD buffer (10 mM Tris (pH 8), 5 mM $MgCl_2$, 1 mM dithiothreitol and 20% glycerol) and frozen at –70 °C until used. For proteolysis assays, we ruptured the mitochondria by sonication with a VWR sonicator (Branson 450) at maximum intensity and separated the matrix fraction by centrifugation for 1 h at 120,000*g* (ref. 36).

Matrix fractionation and Lon protease purification

The matrix fraction from bovine heart mitochondria was subjected to gel filtration on a XK 26/100 column (Amersham) packed with Sephacryl S-300 HR (Amersham) and equilibrated with an elution buffer comprising 10 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 0.5 mM EDTA and 20% glycerol[22,36]. Thyroglobulin (700K), ferritin (440K), catalase (232K) and aldolase (158K) were used to calculate the calibration curve by regression analysis. The flow rate was maintained at 1 ml min^{-1} and each 2-ml fraction was collected. The column was calibrated with known marker proteins and the fractions were assayed for their proteolytic activities against both native and oxidized aconitase. We further purified the fraction containing the maximum proteolytic activity by phosphocellulose affinity chromatography on a P11 column (Whatman) with a 0.1–0.4 M potassium phosphate gradient containing ATP[36]. After this step, the enzyme preparation showed only one principal band when run on the nondenaturing, native gels.

Aconitase degradation

Untreated and oxidatively denatured mitochondrial aconitase (5 μg) was incubated with sonicated mitochondria (200 μg) or purified Lon protease (2 μg) in TMGD buffer, for 90 min at 37 °C with vigorous shaking. For experiments with purified Lon protease, we also added 200 μg of unlabelled bovine serum albumin as a carrier. After 90 min, trichloroacetic acid was added to a final concentration of 10%, and the tubes were incubated on ice for 30 min, followed by centrifugation at 3,000*g* for 15 min. The supernatants were collected, and the pellets solubilized and neutralized in 1 N NaOH. The degradation of ^{3}H-labelled aconitase was monitored by the increasing production of acid-soluble radioactivity (from previously acid-insoluble aconitase protein) after precipitation with trichloroacetic acid. To calculate the percentage of protein degradation, we used liquid scintillation counting[32] to measure the acid-soluble radioactivity relative to the initial acid-insoluble radioactivity.

Aconitase activity

Mitochondrial aconitase activity was determined both in cell extracts and with purified aconitase (Sigma) using the Bioxytech Aconitase-340 assay (Oxis Research), which measures the formation of NADPH from $NADP^+$ concomitant with the conversion of citrate to isocitrate through *cis*-aconitate. We monitored the reaction at 340 nm for 5 min at 37 °C (ref. 51).

Preparation of antibodies and western analysis

The polyclonal antibody against Lon was raised in rabbits against a synthetic peptide corresponding to amino acids 593–609 of intact human Lon protease, and it crossreacts with bovine Lon. In bovine heart mitochondrial extracts and WI-38 VA-13 human lung fibroblast cell extracts, the polyclonal antibody against Lon recognized mainly one protein band with an estimated M_r of about 100K, which corresponds to the molecular mass reported for human and bovine Lon protease[21,25,36]. With purified Lon protease a second band of M_r 80K was detected, consistent with a previously reported fragmentation product[36].

The polyclonal antibody against aconitase was targeted to residues 767–780 of the carboxy terminus of human mitochondrial aconitase and crossreacts with both porcine and bovine mitochondrial aconitase. This antibody specifically recognized an band of 83K, corresponding to the M_r of intact mitochondrial aconitase in whole-cell and mitochondria extracts. When oxidized aconitase (Sigma) was used, a few fragmentation and aggregation products were also detected. ProSci produced both antibodies.

For western blot analysis, we loaded 25 μg of purified oxidized aconitase (Sigma), 2.5 μg of purified Lon protease and 5 μg of cell extracts per lane. After separation, the proteins were transferred onto BioRad PVDF membranes. The membranes were incubated with the antibody against Lon at a dilution of 1:1,000, or with the antibody against aconitase at a dilution of 1:2,000. The secondary antibody in both cases was a goat anti-rabbit IgG (Jackson ImmunoResearch) used at a dilution of 1:20,000. Antigens were detected by chemiluminesce with Amersham's ECL substrates.

To detect oxidized aconitase, we used the Oxyblot kit from Intergen Company, which is based on the derivatization of carbonyl groups in the presence of 2,4-dinitrophenylhydrazine (DNPH) and uses a primary antibody against dinitrophenylhydrazone (DNP) moieties for detection. We loaded 10 μg of protein in each lane. The blots were stripped and reprobed with the antibody against aconitase to confirm the aconitase identity.

Oligonucleotides and delivery

We used a custom (Gene Tools, LLC) antisense morpholino oligonucleotide against the *lon* gene to downregulate *lon* in WI-38 VA-13 cells. This oligonucleotide (5′-CGTAGCCAGTGCTCGCCGC-CATAGC-3′) is complementary to bases 31–55 of *lon* exon 1. A standard control sequence (5′-CCTCT-TACCTCAGTTACAATTTATA-3′) that has no target and no significant biological activity (Gene Tools) was also tested. Delivery was achieved by using a weakly basic delivery reagent, ethoxylated polyethylenimine (EPEI). The cells were incubated in the presence of the delivery system for a total of 3 h, as suggested by the manufacturer. The final oligonucleotide concentration in the delivery medium was 1 μM. We then removed the delivery media and grew the cells in fresh EMEM medium. The antisense morpholino oligonucleotide system used by us has high and predictable activity inside the cell and is immune to nucleases, which meant that only a single treatment was required[52].

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.