Selectivity of protein oxidative damage during aging in *Drosophila melanogaster*

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The purpose of the present study was to determine whether oxidation of various proteins during the aging process occurs selectively or randomly, and whether the same proteins are damaged in different species. Protein oxidative damage to the proteins, present in the matrix of mitochondria in the flight muscles of *Drosophila melanogaster* and manifested as carbonyl modifications, was detected immunochemically with anti-dinitrophenyl-group antibodies. Aconitase was found to be the only protein in the mitochondrial matrix that exhibited an age-associated increase in carbonylation. The accrual of oxidative damage was accompanied by an approx. 50 % loss in aconitase activity. An increase in ambient temperature, which elevates the rate of metabolism and shortens the life span of flies, caused an elevation in the amount of aconitase carbonylation and an

accelerated loss in its activity. Exposure to 100 % ambient oxygen showed that aconitase was highly susceptible to undergo oxidative damage and loss of activity under oxidative stress. Administration of fluoroacetate, a competitive inhibitor of aconitase activity, resulted in a dose-dependent decrease in the life span of the flies. Results of the present study demonstrate that protein oxidative damage during aging is a selective phenomenon, and might constitute a mechanism by which oxidative stress causes age-associated losses in specific biochemical functions.

Key words: aconitase, hyperoxia, mitochondria, protein carbonylation, protein oxidation, oxidative stress.

INTRODUCTION

A variety of studies have shown that the steady-state amounts of the products of oxygen free radicals that attack macromolecules tend to increase with age, especially in long-lived, post-mitotic cells [1–4]. Among the various types of damage that occur to macromolecular species, oxidative modification of intracellular proteins has been suggested to have a crucial role in the aging process, because the oxidized proteins often lose catalytic function and undergo selective degradation [2,5–8]. Oxidative damage to a specific protein, especially at the active site, can thus result in the progressive loss of a particular biochemical function.

A well-characterized oxidative modification of proteins involves the formation of carbonyl groups in the side chains of certain amino acid residues [2]. Age-related increases in the amount of carbonylated proteins have been reported in the homogenates of a variety of tissues [8–10], but see [10a], [11]). Because the free radical attacks on macromolecules tend to be uncatalysed events, it was originally widely believed that protein carbonylation was random and ubiquitous. Notwithstanding, it is well documented that activities of most of the enzymes that have been examined to date do not decline during aging, which is contrary to what might be expected if protein carbonylation was indeed indiscriminate [12,13]. The basis of this apparent inconsistency has been investigated previously by us using Western blot analysis for the identification of specific proteins exhibiting carbonylation. Employing mitochondria from the flight muscles of the housefly as a model, it was found that only aconitase [14,15] and adenine nucleotide translocase [16] exhibited a detectable age-associated increase in carbonylation

and a corresponding loss in functional activity, suggesting that protein carbonylation during aging is a highly selective, rather than a random and ubiquitous, phenomenon.

The purpose of the present study was to understand further the nature of the relationship between protein oxidative damage and aging by addressing a set of corollary questions, including: (a) whether the same or a different set of proteins are damaged during aging in other animal species, particularly in an example for which the genome has been sequenced; (b) whether the proteins exhibiting age-related increases in oxidative damage are relatively more susceptible to damage induced by reactive oxygen species; and (c) whether the rate of aging or life expectancy of the organisms is related to the loss of catalytic activity of the target proteins.

Accordingly, protein oxidative damage was examined in the matrix of the flight-muscle mitochondria of *Drosophila melanogaster* at different ages. Variations in the susceptibility of different proteins to oxidative stress was determined following exposure of the flies to hyperoxia, which is known to increase the rates of mitochondrial generation of superoxide and hydrogen peroxide [17,18]. Effects of alterations in ambient temperature, which is directly related to metabolic rate and inversely related to life span [19,20], on aconitase oxidative damage and enzymic activity were examined at different ages. To determine whether aconitase activity was essential for survival, the effect of administration of fluoroacetate, which is a competitive inhibitor of aconitase activity [21], on the median life span of the flies was examined. Oxidative damage to the proteins in *D. melanogaster* was found to be both highly selective and inversely proportional to the life span of the flies.

Abbreviations used: IEF, isoelectric focusing; DNP, dinitrophenyl; DNPH, dinitrophenyl hydrazine.

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EXPERIMENTAL

Materials

Acrylamide/bisacrylamide (40 %) and ammonium sulphate (enzyme grade) were obtained from Life Technologies, Gaithersburg, MD, U.S.A. Isoelectric focusing (IEF) ampholyte (pH 3–10), SDS and glycine were from Bio-Rad, Hercules, CA, U.S.A. Coomassie Brilliant Blue R250 solution, *N,N,N',N'*-tetramethylethylenediamine ('TEMED'), ammonium persulphate, sodium fluoroacetate, BSA Fraction V, 2,4-dinitrophenyl hydrazine (DNPH), Tris base and EDTA were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Bicinchoninic acid ('BCA') protein assay reagent and Gelcode® Blue Stain reagent were from Pierce Laboratories, Rockford, IL, U.S.A. Reagent alcohol, polyoxyethylene-20-sorbitan monolaurate (Tween 20; enzyme grade), potassium chloride (enzyme grade), ethyl acetate and methanol were from Fisher Scientific Co., Fair Lawn, New Jersey, U.S.A. Polyclonal rabbit anti-dinitrophenyl (DNP) and horseradish peroxidase-conjugated goat anti-rabbit antibodies were purchased from Zymed, San Francisco, CA, U.S.A. Immobilon™-P was purchased from Millipore, Bedford, MA, U.S.A. Protein standard was obtained from Novex, California, U.S.A. ECL™-Plus was purchased from Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.

Maintenance of flies

Male *D. melanogaster* of γw (yellow body, white eyes) strain were used in all experiments. After emergence from pupae, adult male flies were segregated by sex and maintained in groups of 25 in standard glass vials at 25 °C and 50 % relative humidity. Fresh vials containing a cornmeal/agar/yeast medium with 0.7 % (v/v) propionic acid/phosphoric acid (10:1) and methyl paraben as a mould inhibitor were provided on alternate days for the first 20 days; subsequently, the food was changed each day.

Isolation of flight-muscle mitochondria and preparation of submitochondrial particles

All procedures were conducted at 4 °C unless stated otherwise. Mitochondria were prepared by differential centrifugation using a modification of the procedure described by Van Den Bergh [22]. Briefly, the flies were immobilized on ice and their thoraces were severed with a razor blade. Thoraces were then pounded gently in a chilled mortar containing mitochondrial isolation buffer consisting of 154 mM KCl, pH 7.0, 0.16 mM KHCO₃, 1 mM EDTA, 1 μ M butylated hydroxytoluene and 1 μ M diethylenetriaminepenta-acetic acid. The resulting brei was filtered through two layers of Spectrum SpectraMesh® nylon filters (10 μ m mesh pores) under suction. Filtrate was centrifuged at 300 *g* for 3 min in an Eppendorf 5403 centrifuge to sediment the cell debris. The supernatant was gently siphoned off, and then centrifuged at 3000 *g* to obtain the mitochondrial pellet, which was suspended in chilled hypotonic buffer containing 50 mM NaPO₄, pH 7.0, and disrupted by four sonications of 30 s each, with the duty cycle set at 20 % and the output control at 5 in a Branson 250 sonifier. After centrifugation at 8200 *g* for 10 min, the supernatant was centrifuged at 80000 *g* for 40 min in a Beckman Ultracentrifuge TL-100 to separate the mitochondrial matrix from the sedimented membrane fraction.

DNPH treatment of the mitochondrial matrix proteins

Mitochondrial matrix proteins were treated with DNPH as described by Levine et al. [23]: of the mitochondrial matrix

homogenate, 1 ml was treated with 200 μ l of 10 mM DNPH (prepared in 2 M HCl) at room temperature for 1 h. Proteins were precipitated with 10 % (w/v) ice-cold trichloroacetic acid, and washed three times with ethanol/ethyl acetate (1:1, v/v) to remove excess DNPH. The washed pellet was dissolved in 20 mM Tris/HCl buffer, pH 6.8/0.2 % (w/v) SDS. A blank was prepared by the treatment of a mitochondrial matrix sample with 2 M HCl.

PAGE

SDS/PAGE of the proteins was performed under reducing conditions using a Mini-PROTEAN II electrophoresis cell (from Bio-Rad) with 10 % (w/v) polyacrylamide resolving gel, as described by Laemmli [24]. Gels were stained with Coomassie Brilliant Blue R250, or processed immediately for immunoblotting by the Western blotting technique.

Immunoblotting

Following SDS/PAGE, proteins were transferred to PVDF (Immobilon™-P) membrane using the method of Towbin et al. [25] in a Mini-PROTEAN II Trans-blot electrophoretic transfer cell (Bio-Rad). Transfer was performed at 100 V for 1 h with buffer containing 25 mM Tris/HCl, pH 8.3, 192 mM glycine and 20 % (v/v) methanol. Immunochemical detection of carbonylated proteins was performed as described by the methods of Keller et al. [26] and Shacter et al. [27]. The membrane was blocked for 1 h with 5 % (w/v) non-fat milk in Tris-buffered saline containing Tween 20 (TBST) and washed thrice with TBST. The membrane was incubated with polyclonal rabbit anti-DNP primary antibody diluted 1:10000 in TBST/0.2 % (w/v) BSA. The membrane was then treated with horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:25000 dilution in TBST/0.2 % BSA. Carbonylated proteins were visualized using the ECL™-Plus Western blotting detection kit. Comparisons of the extent of carbonylation were made by densitometric scanning of the immunostained bands in X-ray film using an AlphaImager 2000, manufactured by Alpha Innotech Corp. (San Leandro, CA, U.S.A.).

Purification and identification of the carbonylated proteins

Unless stated otherwise, all steps were performed at 4 °C. The matrix of the flight-muscle mitochondria of *Drosophila* was fractionated with 70 % (w/v) ammonium sulphate, and the supernatant, containing most of the protein of interest, was desalted by repeated centrifugations at 2000 *g* in an Ultrafree®-15 centrifugal device (from Millipore, with 10 kDa cut off for biomolecules), with successive additions of buffer. The proteins were incubated with IEF loading buffer containing 4 M urea, 3 % ampholyte, pH 3–10, 2 % (v/v) Nonidet P40 and 1 % 2-mercaptoethanol for 30 min, and loaded on a 5 % IEF vertical gel. The IEF run was performed as described previously by Bollag et al. [28] using a Mini-PROTEAN II electrophoresis cell. Gels were fixed with 20 % (w/v) trichloroacetic acid for 30 min, and overnight with 1 % trichloroacetic acid. Gels were then washed with deionized water for 30 min and stained with Gelcode® Coomassie Blue reagent for 1 h, followed by equilibration with deionized water, according to the manufacturer's instructions. The discernible protein bands were excised, incubated in 62.5 M Tris/HCl, pH 6.8, 2.3 % (w/v) SDS, 10 % (v/v) glycerol and 5 % 2-mercaptoethanol for 30 min at room temperature, and then run on an SDS/10 % polyacrylamide gel. The protein was identified on the basis of its molecular mass and

Figure 1 Immunoblotting detection of carbonylated proteins in the matrix of mitochondria from flight muscles of *D. melanogaster* at different ages

DNPH-treated proteins were resolved on an SDS/polyacrylamide gel, transferred on to Immobilon[®]-P membrane, and the carbonylated proteins were detected by reaction with anti-DNP polyclonal antibodies. Left panel: proteins were stained with Coomassie Blue R250. Right panel: lane 1 contains the negative control where the protein sample from 10-day-old flies was not treated with DNPH. Lanes 2–5 contain mitochondrial matrix proteins from 10, 22, 35 and 45 days of age respectively. An 85 ± 5 kDa protein exhibits carbonylation, which tends to increase with age.

immunoblotting pattern. The N-terminal amino acid sequence of this protein was determined by both automated Edman degradation and peptide-mass analysis of the LysC digests.

Effect of fluoroacetate on the life span of flies

Flies at the age of 10 days were housed 25 per shell vial, containing fly food (ready-mix *Drosophila* dried food obtained from Philip Harris International, Shenstone, Lichfield, Staffs, U.K.) in the absence or presence of 0.1, 0.5, 1, 5 and 10 mM sodium fluoroacetate. A total of 150 flies constituted each experimental set.

Exposure to hyperoxia

Drosophila at the age of 10 days, confined in the normal glass vials (25 flies in each vial) with regular food, were placed in a sealed Plexiglas[®] chamber and connected via a manometer to a cylinder containing 100% oxygen. Oxygen was bubbled through water and then passed into the chamber under a low, steady positive pressure. The flies were maintained at 25 °C, and fresh food was provided daily.

Measurement of aconitase activity

The activity of mitochondrial aconitase was measured on the basis of the conversion of citrate into α -oxoglutarate coupled with the reduction of NADP, as described by Kennedy et al. [29]. Mitochondrial pellets were sonicated four times in a Branson 2200 sonicator for 30 s each in buffer containing 154 mM Tris/HCl, pH 7.4, and 5 mM citrate. The reaction mixture contained 27 mM Tris/HCl, pH 7.4, 5 mM sodium citrate, 0.2 mM NADP, 0.6 mM MnCl_2 and 1 unit of isocitrate dehydrogenase (where one unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of isocitrate from citrate per min at pH 7.4 at 30 °C). The formation of NADPH was followed over time at 30 °C at a wavelength of 340 nm using a Beckman DU 640 spectrophotometer.

Measurement of protein content

The protein content was measured using a bicinchoninic acid protein assay kit and BSA as a standard [30].

RESULTS

Localization of carbonylated proteins

To identify specific proteins exhibiting age-associated carbonyl modifications, the mitochondrial matrix fraction of the thoracic flight muscles of the flies, ranging from 10 to 64 days of age, was reacted with DNPH and the proteins were resolved by SDS/PAGE. Total protein was visualized with Coomassie Blue staining, and carbonylated proteins were detected by Western blotting. The gels, stained with Coomassie Blue, showed a spectrum of protein bands; however, immunostaining indicated that only a single protein band, with a molecular mass of 85 ± 5 kDa, exhibited a positive reaction for carbonylation (Figure 1). The negative control (mitochondrial matrix fraction not treated with DNPH) did not exhibit any positively immunostained band, confirming both the specificity of the antibody and the selectivity of the carbonylation of proteins.

Purification and identification of the 85 kDa protein showing selective carbonylation

The 85 kDa protein, exhibiting carbonylation, was purified and its N-terminal sequence was determined. Towards this end, the mitochondrial matrix fraction from 10-day-old *Drosophila* was subfractionated with ammonium sulphate; the proteins remaining in the supernatant were resolved by IEF. All the bands from the IEF gel were excised and run on an SDS/10% polyacrylamide gel. The protein of interest was localized by its molecular mass (85 kDa) and its positive immunostaining (results not shown). The N-terminal sequence of the protein was determined by Edman degradation, which identified the protein as aconitase (Figure 2). This identification was confirmed by reversed-phase HPLC mass spectrophotometric mapping of a LysC digest of the purified protein. The measured masses were submitted to the ProFound web site at Rockefeller University, which implements a Bayesian algorithm for identifying proteins in the non-redundant database of the National Centre for Biotechnology Information [31]. The purified protein was identified as the product of the *CG9244* gene from *D. melanogaster* with a probability of > 98%. Because *CG9244* had not been linked with its protein product, the deduced amino acid sequence from the nucleotide sequence was submitted to the ProSite program at Basel, which

<u>A</u>	<u>KVAMSHFE</u> ANEYV	<i>Xenopus laevis</i> oocytes aconitase
<u>A</u>	<u>KVAMSHLE</u> PSQYI	<i>Mus musculus</i> aconitase
<u>A</u>	<u>KVAMTHFEP</u> NEYI	<i>Homo sapiens</i> mitochondrial aconitase
<u>A</u>	<u>KVAMSHFEP</u> HEYI	<i>Sus scrofa</i> heart mitochondrial aconitase
<u>A</u>	<u>KVAMSHFEP</u> HEYI	<i>Bos taurus</i> heart mitochondrial aconitase
S	<u>KVA</u> I <u>SKFEPK</u> SYL	<i>Caenorhabditis elegans</i> mitochondrial aconitase
<u>A</u>	<u>KVALSKFD</u> NDVYL	<i>Musca domestica</i> mitochondrial aconitase
[AV]	KVALSKFD?DV?L	85 kDa protein from mitochondrial matrix of <i>Drosophila melanogaster</i>

Figure 2 N-terminal amino acid sequence of aconitase from different sources

A computer-assisted search of the protein database showed sequences from different sources after alignment. The underlined amino acids are those showing homology with mitochondrial aconitase from *D. melanogaster*. [AV], either Ala or Val; '?' denotes an undetermined amino acid.

Figure 3 Immunochemical quantification of the age-associated increase in the amount of aconitase carbonylation (left panel), and comparison of aconitase activity in the mitochondria of flight muscles of *D. melanogaster* at different ages (right panel)

Left panel: values are derived from data from three separate experiments. A section of the film without any protein was set as the blank. Right panel: aconitase activity was measured with a coupled assay using citrate as the substrate. Values are the means $\pm$ S.D. for 10 determinations in two separate experiments. The *P* values for enzymic activity were significantly different from each other ($P < 0.0005$), except for 25 cf. 35 days, 35 cf. 46 days, 35 cf. 60 days and 46 cf. 60 days (pairwise mean comparison was performed by using the post-hoc Tukey test, following one-way ANOVA in Systat 7.0.1 software).

identified the 85 kDa protein as the *D. melanogaster* mitochondrial aconitase.

Quantification of aconitase carbonylation and loss of enzyme activity during aging

The effect of age of the flies on the amount of carbonylation of aconitase was determined by densitometric scanning of the Western blots. Quantification of the density of the bands revealed an age-related linear increase in carbonylation (Figure 3, left panel). In comparison with the 10-day-old flies, the amount of

carbonylation increased 1.7-, 2.4-, 2.8- and 5.1-fold at 22, 35, 45 and 64 days of age respectively. In a separate study, the molar ratio of carbonylated aconitase was quantified using oxidized BSA as a standard, as described previously [23,32]. Briefly, BSA, exposed to aerial oxidation, was derivatized with DNPH and the carbonyl content was determined spectrophotometrically, assuming the molar absorbance coefficient (ϵ) of the hydrazone complex to be $22000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The carbonyl content of aconitase, measured densitometrically by comparison with oxidized BSA standards, was 0.02, 0.068 and 0.157 mol/mol of aconitase protein in 14-, 36- and 75-day-old flies respectively

Figure 4 Effect of ambient temperature on mitochondrial aconitase activity and carbonylation in flight muscles of *D. melanogaster*

Immediately after emergence from the pupae, the flies were housed at 17 °C and 29 °C. The amount of carbonylation and activity of mitochondrial aconitase were compared at 10, 20 and 30 days of age. To measure carbonylation, the immunostain intensity of aconitase was quantified with reference to an arbitrary value of 100, assigned to the amount present in 10-day-old flies kept at 17 °C. The blank was set with sections of the film without protein. Values are an average of two independent experiments. The amount of aconitase protein varied with age in flies housed at 29 °C; therefore the immunostain intensity of aconitase was normalized to its relative protein concentration at each age in order to compensate for this variation. The amount of aconitase protein present in 10-day-old flies, kept at 17 °C, was assigned an arbitrary value of 100. Values for aconitase activity are means $\pm$ S.D. for 10 determinations in two experiments. Aconitase activity was significantly different at the two temperatures at all ages examined (pairwise mean comparison was performed by post-hoc Tukey test, following one-way ANOVA in Systat 7.0.1 software).

(results are on the basis of the average of two replicated measurements).

To determine whether the age-related increase in carbonyl content of aconitase was accompanied by a loss in its catalytic function, the enzyme activity was measured with a coupled assay using citrate as a substrate. A sharp age-related decline in specific activity of aconitase was observed until 46 days of age, when approx. 50 % of the activity had been lost compared with the 10-day-old flies (Figure 3, right panel). Subsequently, the loss in enzyme activity with age (up to 60 days) was relatively minor. Thus age-related increases in aconitase carbonylation, after approx. 46 days of age, were not reflected in significant losses in enzymic activity.

Effect of varied ambient temperature on carbonylation and catalytic activity of aconitase

The purpose of this experiment was to determine if alterations in ambient temperature, which has a direct effect on the metabolic rate and an inverse effect on life span of the flies [19,20], cause variations in the amount of carbonylation and activity of aconitase. A comparison was made among flies maintained at 17° and 29 °C at 10, 20 and 30 days of age. At all three ages, for flies kept at 29 °C, the amount of aconitase carbonylation was higher, and the activity lower, than that for those kept at 17 °C (Figure 4). The amount of aconitase carbonylation increased in a parallel fashion in the two groups between 10 and 20 days, after which the increase was relatively slower at 17 °C. In contrast, the

Figure 5 Effect of 100% ambient oxygen on carbonylation of mitochondrial aconitase (top panel), quantification of aconitase carbonylation under 100% ambient oxygen (middle panel), and effect of 100% ambient oxygen on mitochondrial aconitase activity (bottom panel)

Top panel: flies at the age of 10 days were exposed to 100% oxygen for 1–4 days, and the carbonylated proteins in the mitochondrial matrix were detected by immunostaining. Lanes 2–6 contain mitochondrial aconitase at 0, 1, 2, 3 and 4 days respectively, following exposure to hyperoxia. Lane 1 is a negative control (protein sample without DNPH treatment). Middle panel: values are based on data from two separate experiments. Immunostain intensity at day zero was assigned an arbitrary value of 100. A section of the film without protein was used as a blank. Bottom panel: aconitase activity was measured in mitochondria isolated from the flight muscles of flies exposed to 100% ambient oxygen for 1–3 days. Values are means $\pm$ S.D. for 10 determinations in two separate experiments. Pairwise mean comparisons (performed by post-hoc Tukey tests, following one-way ANOVA in Systat 7.0.1 software) indicated a significant effect of hyperoxia on enzymic activity.

age-related losses in aconitase activity between the two groups narrowed progressively with age (Figure 4). Overall, the results of this experiment indicated that elevation in ambient tem-

Figure 6 Effect of fluoroacetate on the life span of *D. melanogaster*

Flies at the age of 10 days were exposed to various concentrations of sodium fluoroacetate mixed in their food. It should be noted that the 'instant' *Drosophila* food was used in order to minimize human exposure to fluoroacetate. Previous studies in our laboratory have indicated that the life span of flies fed instant fly food is somewhat shorter than those fed the usual agar-cornmeal mixture prepared in the laboratory. A total of 150 flies were used, with 25 flies in each vial containing a different concentration of fluoroacetate.

perature increases the accrual of aconitase carbonylation, and accelerates the loss in its catalytic activity.

Effect of hyperoxia on aconitase carbonylation and enzyme activity

Exposure to hyperoxia is known to provide a highly effective means of inducing oxidative stress in flies *in vivo*, because air is carried directly to the interior of the cells by an amastomosing tracheolar network. Accordingly, 10-day-old flies were exposed to 100% ambient oxygen for 4 days (a duration that results in > 50% mortality). Immunoblotting studies, carried out in order to localize carbonylated proteins, showed that aconitase was the most intensely carbonylated protein in hyperoxia-exposed flies (Figure 5, top panel), with three other protein bands exhibiting relatively faint positive reaction after 3 days of exposure to hyperoxia (results not shown). There was an approximate 2-fold increase in the intensity of aconitase carbonylation after 4 days of hyperoxic exposure (Figure 5, middle panel). Aconitase activity decreased quite sharply, to approx. 45%, after the first day of exposure to hyperoxia, and to approx. 15% after three days of exposure (Figure 5, bottom panel). Flies kept under normoxic conditions did not exhibit any significant changes in aconitase activity during this period (results not shown).

Effect of fluoroacetate on life span

The purpose of this experiment was to determine whether inactivation of aconitase activity is linked to the life span of the flies. The diet of the flies was supplemented with various concentrations of fluoroacetate, an inhibitor of aconitase. Increasing concentrations of fluoroacetate were found to have a progressively more negative effect on the median life span of the flies (Figure 6), suggesting that aconitase activity is essential for

the survival of the flies, which is consistent with the known lethality of the knock-out for aconitase (unpublished work).

DISCUSSION

The results indicate that only a single protein, aconitase, in the matrix of the flight-muscle mitochondria of *D. melanogaster* exhibits a detectable age-related increase in carbonylation. Aconitase also displays relatively high susceptibility to carbonylation under conditions of experimentally induced oxidative stress by exposure to 100% ambient oxygen. The increase in aconitase carbonylation with age and under hyperoxia was accompanied by a loss in its enzymic activity. Elevation in the ambient temperature at which adult flies were reared resulted in a relatively greater degree of aconitase carbonylation and loss of enzyme activity. Administration of fluoroacetate, an inhibitor of aconitase activity, resulted in a dose-dependent decrease in life span, suggesting that loss of aconitase activity may be linked to the survival of the flies.

The present finding that aconitase is the only discernible protein in the mitochondrial matrix of *Drosophila* to exhibit an age-related increase in carbonylation is similar to that made previously in the housefly [14], which suggests that the selectivity of protein oxidative damage during aging is not limited to a single species, and the same protein may be damaged in related species. It should, however, be pointed out that it is possible that the threshold sensitivity of the immunostaining procedure employed here may have precluded the detection of some proteins with a relatively minor degree of oxidative damage. Notwithstanding, selectivity of protein carbonylation during aging is demonstrated by the fact that the relative amount of a specific protein is not a factor in the detection of oxidative damage. For instance, other proteins in the mitochondrial matrix, which were as abundant as aconitase (i.e. with molecular masses of approx. 52 and 40 kDa in Figure 1) did not exhibit detectable carbonylation at any age. Malate dehydrogenase, which co-purifies with aconitase, exhibits no age-associated increase in carbonylation in the housefly mitochondria [15]. The molecular mass of cytochrome *c*, a highly abundant mitochondrial protein, was found to remain totally unaltered during aging in the housefly [33]. Such findings clearly refute the notion that protein carbonylation during aging is a random phenomenon, which accords with the extensive biochemical data that has revealed that the vast majority of enzymes do not lose catalytic activity during aging.

The present finding that the amount of aconitase carbonylation increases from 0.02 mol carbonyl/mol of aconitase protein in the young flies to 0.157 mol/mol in the aged ones raises the question of whether the magnitude of such an elevation in carbonylation can lead to an approx. 50% loss in the aconitase enzyme activity during aging. Results of a previous study [34] have indicated that the enzyme activity of a protein such as glutamine synthetase, exposed to metal-catalysed oxidation, is completely lost when its carbonyl content approaches 0.25 to 0.3 mol/mol. The magnitude of aconitase carbonylation and loss in its activity during aging are thus quite comparable with those observed in the studies on glutamine synthetase. An explanation of such apparent discrepancies between the amounts of carbonylation and losses in enzyme activity might be that carbonylation is only one among several structural modifications caused by metal-catalysed oxidation. Uncarbonylated protein molecules in such a system might have sustained other types of oxidative damage, which also contribute to the loss in enzyme activity. It is thought that carbonyl groups provide a convenient generic marker for the identification of oxidized proteins.

There are several intersecting lines of reasoning supporting the suggestion that aconitase carbonylation is a possible marker of physiological age. The present finding that an increase in ambient temperature, which elevates the rate of oxygen consumption in poikilotherms such as insects and decreases their life span [19,20], also accelerates the accrual of aconitase carbonylation and loss in its catalytic activity accords with this concept. In a previous study on houseflies, an increase in flying activity was found to increase simultaneously: (a) the rate of oxygen consumption of the flies; (b) the rate of hydrogen peroxide production by flight-muscle mitochondria; and (c) the amount of aconitase carbonylation and the loss in its catalytic activity [35]. The findings in the housefly and *Drosophila*, taken together, suggest that aconitase carbonylation is associated directly with the rate of metabolism and is inversely proportional to the life span of these insects.

An obvious question arising from this study is why aconitase is the only protein in the mitochondrial matrix that exhibits a detectable age-associated increase in carbonylation. Stadtman (reviewed in [2,10], but see [10a]) has demonstrated that the presence of a metal-binding site in a protein is a key feature in terms of predicting its susceptibility to oxidation and introduction of a carbonyl group. Aconitase is believed to be particularly sensitive to attacks by superoxide anion radical because of the presence of a $[4\text{Fe-4S}]^{2+}$ cluster at its active site, which can result in the release of an iron [36–39]. Reaction between iron and hydrogen peroxide, a stoichiometric product of superoxide dismutation, can potentially generate the hydroxyl free radical via the Fenton reaction, initiating a cascade of oxidative modifications in cellular macromolecules. Incidentally, it may be argued that, although a loss of iron might account for some of the observed damage to aconitase, there are no reports of comparable damage to other iron–sulphur-containing proteins. Adenosine nucleotide translocase, an inner-mitochondrial-membrane protein, also undergoes extensive carbonylation during aging of the housefly [16]. Although this protein does not have an iron–sulphur centre, it does bind bivalent cations: one of the essential requirements for metal-catalysed oxidation of proteins. Factors such as molecular conformation, rate of turnover and the relative abundance of amino acid residues susceptible to metal-catalysed oxidation have also been suggested to be involved in the selectivity of protein oxidative damage [40,41].

Another pertinent question raised by the present study concerns the mechanism by which loss of aconitase activity has a role in the aging of the fly. Several lines of evidence suggest that a decrease in aconitase activity can potentially result in a myriad of secondary deleterious alterations. One such effect would be to slow down glycolysis and the citric acid cycle, thereby blocking the normal flow of electrons to oxygen, leading to an accumulation of reduced metabolites. An age-related increase in citrate concentration has been demonstrated in the housefly [42], as well as in rats [43]. Citrate can bind Fe^{2+} , which in turn can catalyse the scission of hydrogen peroxide to generate the highly reactive hydroxyl free radical ([10], but see [10a]). In the rat lung, fluoroacetate-induced loss of aconitase activity has been shown to limit the overall activity of the mitochondrial tricarboxylic acid cycle, and therefore the mitochondrial capacity for oxidative phosphorylation [44]. The neurotoxic effects of fluoroacetate or fluoroacetate are also hypothesized to be caused by the accumulation of citrate, and the consequent chelation of free calcium ions [45]. It should be noted that, while fluoroacetate has been considered widely to be a specific competitive inhibitor of aconitase activity, it has also been reported to cause a decrease in the activities of pyruvate dehydrogenase kinase and adenylate cyclase [46]. Whether such non-specific effects have an impact on

the life span of flies cannot be resolved on the basis of the present study.

In conclusion, the results of this study demonstrate that protein oxidative damage during aging is selective, and that similar proteins might be affected in related species. Damage to selective proteins can result in an array of secondary deleterious effects.

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