AGE-ASSOCIATED ACCUMULATION OF ALTERED FDP ALDOLASE B IN MICE

Conditions of detection and determination of aldolase half life in young and old animals

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1. Introduction

Inactive enzyme molecules in aging animals have been described for mouse aldolase B [1] and aldolase A [2], nematode aldolase [3,4], and other enzyme systems [5–8]. We have postulated that the accumulation of inactive enzyme molecules in cells of aging organisms could be due, at least in part, to their inefficient disposal by the protein degradation system [7,9]. In [10] no difference in the activity of aldolase B from young or old mice was observed. It was suggested that our results with aldolase B [1], and possibly other results concerning age-related inactive enzyme molecules, were due to an artifact emanating from increased proteolysis and possible removal of the COOH-terminal tyrosine in enzyme preparations from tissues of old animals.

Here we show that when liver homogenates are carefully prepared in isotonic buffers and lysosomes are removed (as is routinely done in our laboratory) no loss of enzyme activity due to proteolysis occurs.

Under these conditions, freshly prepared homogenates of livers of old animals contain aldolase with reduced catalytic activity/unit of enzyme antigen. This observation fully corroborates [1]. We also describe here an increased $t_{1/2}$ of aldolase B in livers of old animals. This increase at least can partially explain the basis for the accumulation of inactive enzyme molecules in livers of old animals.

2. Materials and methods

Animals were C57B1/6J female mice aged 5–6 months (young) and 27–28 months (old).

Livers of individual C57B1/6J female mice were homogenized in 12 ml of either TEM buffer as routinely used by us [1] or the phosphate buffer in [10]. Where indicated, phenylmethylsulfonyl fluoride (PMSF) and leupeptin were added to either buffer to give a final concentration of 1 mM and 15 μg/ml, respectively. Homogenates prepared according to [10] were centrifuged at 12 000 × *g* for 15 min and those prepared in TEM were centrifuged at 24 000 × *g* for 3 h as in [1]. In certain experiments both preparations were centrifuged for 1 h at 100 000 × *g*. Aldolase activity was assayed as in [1].

Double-label experiments for $t_{1/2}$ determinations were done as follows: Individual mice were injected intraperitoneally with 50 μCi [^{35}S]methionine (380 Ci/mM, New England Nuclear). After 24 h each mouse was injected with 200 μCi [^{3}H]methionine (2 Ci/mmol, New England Nuclear) and killed after 2 h. Individual livers were removed to cold TEM, weighed, washed and homogenized in 3 vol. (w/v) cold TEM. Each crude homogenate was brought to a final volume of 12 ml and centrifuged for 1 h at 100 000 × *g*. Aldolase was isolated from each liver by incubating 1 ml supernatant with 2 ml monospecific rabbit antimouse aldolase antibody at 4°C for 16–18 h. Control samples were incubated with normal rabbit serum. The aldolase–antibody complex was collected by centrifugation at 10 000 × *g* for 10 min, washed once with 10 mM Tris buffer (pH 8.0) containing 100 mM NaCl and 0.5% Triton X-100, twice with 10 mM Tris–100 mM NaCl (pH 8.0), then dissolved at 37°C overnight in 0.01 M phosphate buffer (pH 7.0) containing 1% SDS and 1% β-mercaptoethanol. Samples were then boiled for 5 min and run on 10% SDS polyacrylamide gels according to

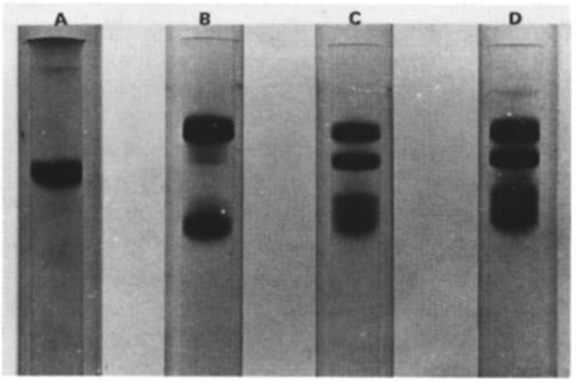

Fig.1. SDS–polyacrylamide gel electrophoresis of aldolase–antibody precipitates: (A) purified aldolase; (B) purified rabbit immunoglobulin (Sigma); (C) 'young' aldolase–antibody complex; (D) 'old' aldolase-antibody complex.

[11]. 70% of the radioactive label initially placed on the gel was recoverable and all of this label was found in the band corresponding to aldolase (fig.1). This band was sliced out of the gel, dissolved in soluene and ^{35}S and ^{3}H levels were determined by scintillation counting. The $t_{1/2}$ of mouse liver aldolase was calculated according to [12].

3. Results

To repeat our studies and compare them to those in [10], freshly excised livers were divided into 4 parts and homogenized in either phosphate buffer or TEM with or without PMSF and leupeptin. These homogenates (12 000 × *g* supernatants for the phosphate buffer and 24 000 × *g* supernatants for TEM or 100 000 × *g* for both preparations) were adjusted to the same initial activity (fig.2) and used to compare specific activity/antigenic unit of aldolase between young adult (5–6 months) and old (27–28 months) mice. Considerably more antibody is required to precipitate 'old' than 'young' enzyme in all the preparations, thus corroborating our results. However, when the enzyme is prepared according to the procedure in [10], in phosphate buffer, there is a measurable loss of activity/unit antigen in both 'young' and 'old' preparations. PMSF and leupeptin prevent this loss but have no effect on enzyme prepared in TEM buffer which maintains its activity in buffer alone. This difference is most probably due to the fact that the homogenization in hypotonic phosphate buffer [10] releases lysosomal proteolytic activity which damages aldolase molecules. Also, centrifugation at 12 000 × *g* may not be sufficient to remove any intact lysosomes which remain after the hypotonic treatment of the tissue [13]. Our aldolase preparations in isotonic TEM, are post-lysosomal homogenates which are stable over extended periods of time (table 1). This stability is evident in homogenates from mice of all ages despite the fact that the initial activity in liver homogenates of old mice is low.

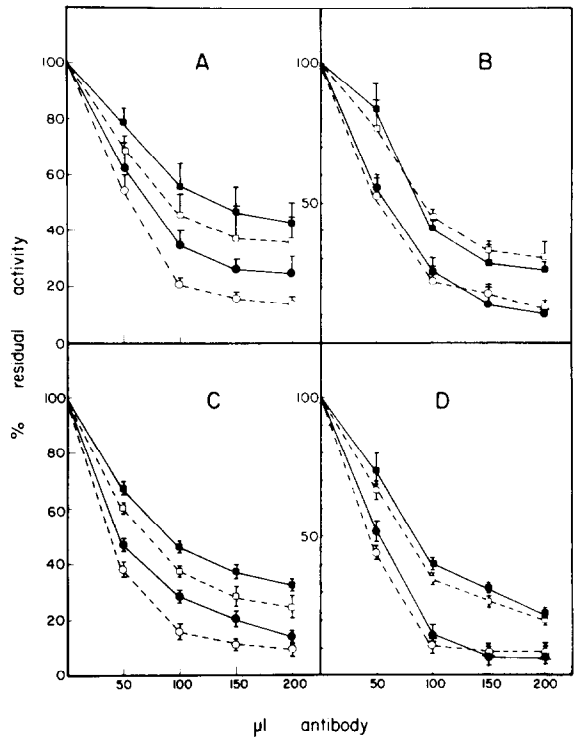

Fig.2. Immunotitration of aldolase B in liver homogenates of young (5–6 months) and old (27–28 months) mice. (A,B) Aldolase activity was determined in the 100 000 × *g* supernatant of the homogenates of 3 individual livers from each age group. (C,D) The activity of 2 livers of each age group was determined in the 12 000 × *g* supernatant of the homogenates in phosphate buffer and 24 000 × *g* supernatant of the homogenates in TEM. Each liver was examined individually after being divided into 4 parts, 2 of which were homogenized in phosphate buffer with or without PMSF and leupeptin; the remaining 2 parts were homogenized in TEM buffer with or without PMSF and leupeptin. The bars represent standard deviation in (A,B) and the range of activity in (C,D). (A,C) Homogenates in phosphate buffer; (B,D) homogenates in TEM buffer; (○) homogenates from young animals containing PMSF and leupeptin; (●) homogenates from young animals without PMSF and leupeptin; (□) homogenates from old animals containing PMSF and leupeptin; (■) homogenates from old animals without PMSF and leupeptin.

Table 1
Activity of aldolase (units/mg protein) from young and old mice in liver homogenates prepared in TEM buffer after various periods of storage at −20°C

Animal age (months)	Fresh prep.	3 weeks	5 months	15 months
2	0.056	0.060	0.060	0.059
31	0.029	0.029	0.028	0.027

The possibility was explored that the age related accumulation of inactive-aldolase molecules, as attested by reduced catalytic activity/unit of enzyme antigen, may be related to a decreased rate of turnover of the enzyme in livers of old animals. The $t_{1/2}$ of aldolase was determined in livers of young (4 months) and old mice (25 months) and indeed $t_{1/2}$ of the enzyme increases from 25.6 h in livers of young animals to 37 h in livers of 25 month old animals (table 2).

4. Discussion

The above results fully corroborate our previous results. These studies, performed on freshly prepared liver homogenates, demonstrate that under the hypotonic preparative conditions used in [10], aldolase from livers of both young and old animals is damaged. This damage can be averted by PMSF and leupeptin. These protease inhibitors had no effect on aldolase prepared under isotonic conditions (fig.2) which is stable even after freezing at −20°C (table 1). Nevertheless, in both isotonic and hypotonic preparations the catalytic activity per unit of antigen was reduced in old animals.

The lack of difference in specific activity of the phosphocellulose purified enzyme reported in [10] can be best explained by the fact that modified enzyme molecules may not bind to phosphocellulose. A catalytically inactive form of aldolase has been found in livers of fasted rabbits which is not retained on phosphocellulose columns but is absorbed to anti-aldolase coupled to cyanogen bromide-activated Sepharose [14]. This form is reversibly activated upon feeding of the rabbits, thus the removal of the COOH-terminal tyrosine as the cause of aldolase inactivation in livers of fasted rabbits is rendered very unlikely.

These findings should be related to other work which has indicated accumulation of inactive enzyme molecules in tissues of senescent organisms. It was assumed that removal of short peptides from protein molecules would result, at least in some cases, in net charge changes and discernible loss of molecular mass. These have not been observed in a considerable number of cases which have been examined (review [7,8]), including nematode aldolase and rat liver superoxide dismutase, in which only one form could be discerned by isoelectric focusing and SDS gel electrophoresis [5,15]. End group analysis of enolase from old nematodes, with reduced catalytic activity did not reveal any changes at either the carboxy- or amino-terminus [16]. This evidence and the present report strongly support our views that the accumulation of inactive

Table 2
^3H:^{35}S ratios[a], K_d- and $t_{1/2}$-values for liver aldolase isolated from young and old mice

Animal age (months)	No. of animals (pooled)	^3H (dpm)	^{35}S (dpm)	$^3H/^{35}S$	K_d/day	$t_{1/2}$ (h)
4	10	6070	3197	1.9	0.64	25.6
25	6	5168	3228	1.6	0.45	37

[a] The ^3H and ^{35}S values shown in this table are total counts found in the thrice-washed (section 2) precipitates of aldolase–antibody complexes formed after incubation of 1 ml liver homogenate with 2 ml antiserum. A sample from each precipitate was run on SDS–polyacrylamide gel electrophoresis to verify that all the radioactivity was in aldolase (section 2). The same ratios with, albeit, lower counts were found in the aldolase bands in these gels by the procedure in section 2.

enzyme molecules in cells of senescent organisms is a widespread phenomenon. The cause of inactivation is post-translational and in most cases does not involve simple cleavage of peptides from the enzyme molecules [7,8].

The accumulation of inactive enzyme molecules in cells of aging animals may be due to a decrease in the rate of protein disposal from cells. The slowdown in murine liver aldolase degradation is significant and may account for the 35–40% of inactive molecules we have found with age (see fig.2). A substantial slowdown in the rate of protein degradation in senescent nematodes [17,18] and in the inducible enzyme tyrosine amino transferase in aged mouse liver [19] has been reported. Recent work in our laboratory also indicates that the rate of degradation of proteins of all cellular fractions of murine hepatic cells, with the exception of the microsomal fraction, slows down considerably. This slowdown is physiologically expressed in a considerable age-related decline in the capacity to dispose of modified protein molecules such as puromycyl peptides and analog-laden proteins ([17], in preparation).

The susceptibility of enzymes from old animals to proteolysis by trypsin, chymotrypsin, subtilisin and pronase is indistinguishable from that of enzymes derived from young cells (cf. [19]). This observation taken together with the above findings have led us to propose that the cellular proteases involved in protein degradation may themselves be increasingly altered as a function of age in the same manner as do other cellular enzymes [9]. This hypothesis is being investigated.

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