Short Communication

Aging Effects on the Liver Aldolase of Rabbits

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A chemical method is used to determine the amount of aldolase sequence in rabbit livers. It is demonstrated that the aldolase sequences of the livers of young rabbits are more efficient catalytically than are those from the livers of old rabbits. The presence of altered residues in the aldolase sequences of old animals may account for these observations.

Recent evidence, based on data obtained by immunological techniques, suggests that an accumulation of inactive forms of enzymes, or of forms of low catalytic activity, occurs in older organisms. The ratio of catalytic activity to material that cross-reacts with rabbit antiserum has been found to decrease with age in enzymes from fibroblasts (Lewis & Tarrant, 1972), nematodes (Zeelon *et al.*, 1973) and mouse liver (Gershon & Gershon, 1973). These results suggest that protein that is catalytically less active but is still immunologically cross-reactive occurs in older organisms. However, they do not indicate what changes in the protein cause the decrease in catalytic activity or whether proteins that do not react with the antiserum are being formed.

Measurement of proteins within tissues or cells by determination of their enzymic (or other biological) activity indicates the number of functional molecules present but not the number of non-functional molecules. However, comparison of a measured amount of amino acid sequence unique to an individual protein with the amount of biological or catalytic activity should indicate whether inactive forms of the protein are formed under certain conditions. A relatively small amount of sequence is required to define uniquely the protein from which it originates, since the random occurrence of a sequence is 1 in 20^n , where n is the number of amino acids in the sequence. Homologous proteins contain regions of sequence in common, but portions of the polypeptide chains should contain sufficient unique sequences to define them. Therefore measurement of the amount of peptide can be used to quantify the amount of protein sequence.

An isotope-dilution method can be used to measure the quantity of individual sequences within cells or tissues if a functional group that can be quantitatively modified is present in the sequence and if the protein from which the sequence is derived is available in pure form. The pure protein, which has certain of its functional groups modified with a radioactive reagent, is added to a mixture of proteins in which the same functional groups have been modified with a non-radioactive form of the reagent. Comparison

of the specific radioactivity of peptides containing modified functional groups generated in and isolated from these mixtures with the specific radioactivity of the same peptide generated in and isolated from the pure protein will indicate how much the radioactive peptides have been diluted by peptides arising from proteins in the mixture. If peptides of sufficient size are isolated, there is a very high probability that they are produced from a unique protein, and hence the dilution of the specific radioactivity of these peptides can be used to estimate the amount in the mixture of the protein from which they are generated.

The enzyme fructose 1,6-diphosphate aldolase can be obtained in a pure form from rabbit liver with relative ease (Penhoet *et al.*, 1969a). The sequence of an 18-residue carboxymethylcysteine-containing peptide, produced by CNBr cleavage of rabbit liver aldolase that had been carboxymethylated with iodoacetic acid, has been determined (Forcina & Perham, 1971). Rabbit liver aldolase therefore contains a functional group, the thiol of cysteine, that can be quantitatively modified and isolated in a peptide large enough to identify it as being uniquely derived from liver aldolase. The muscle isoenzyme has six replacements in this region of the sequence compared with liver enzyme (Forcina & Perham, 1971; Gibbons *et al.*, 1970) and therefore does not contain this sequence. The ratio of aldolase activity to material cross-reacting with aldolase antiserum has been found to decrease with age in nematodes (Zeelon *et al.*, 1973) and mouse liver (Gershon & Gershon, 1973). It was therefore decided to examine the livers of old and young rabbits to determine whether the ratio of aldolase catalytic activity to aldolase sequences decreased with age.

Materials and methods

Two New Zealand rabbits, one old and one young, were used in each experiment. The young animals were 6-8-week-old females and the old animals were discarded breeder females of approx. 3 years of age which had refused to mate after having had 10-15 litters. The rabbits were killed by a blow on the head

and the livers were quickly removed. Samples (5g) of liver were homogenized in 10ml of 0.01M-Tris-HCl buffer, pH7.5, containing 1mM-EDTA, 10mM-2-mercaptoethanol and 0.25M-mannitol and centrifuged at 40 000g and 5°C for 1h. Portions of the supernatant were taken for the determination of aldolase activity (Gracy *et al.*, 1970) and protein concentration (Itzhaki & Gill, 1964).

The remainder of the supernatant was carboxymethylated with iodoacetic acid for 1h in 0.1M-Tris-HCl, pH8.0, containing 8M-urea and purified as previously described (Anderson & Perham, 1970). Known amounts of protein from liver supernatants (usually 100–200mg) were then mixed with known amounts of purified rabbit liver aldolase (usually 4–8mg) that had been carboxymethylated under identical conditions except that iodo[2-¹⁴C]acetic acid (obtained from Amersham-Searle, Arlington Heights, Ill., U.S.A., and diluted to a specific radioactivity of 1.0mCi/mmol) was used. The rabbit liver aldolase was purified from the livers of 3-month-old rabbits (Penhoet *et al.*, 1969a) to a specific activity of 1.7 units/mg of protein and homogeneity on polyacrylamide gels. The mixtures containing no free thiol groups, as determined by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) in 8M-urea, were then treated with CNBr and fractionated by passage through Sephadex G-75 (superfine) and paper electrophoresis and chromatography as previously described (Anderson *et al.*, 1969; Gibbons *et al.*, 1970). Radioactive peptides were located in column effluents by liquid-scintillation counting of small samples in Aquasol (New England Nuclear Corp., Boston, Mass., U.S.A.) and on paper by radioautography. *N*-Terminal residues were identified by the dansyl method (Gray, 1967). Amino acid analysis and peptide determination were carried out with a Technicon TSM Amino Acid Analyzer, with norleucine as internal standard, after hydrolysis at 106°C with 6M-HCl for 24h in sealed evacuated tubes.

Results and discussion

Mean values (six experiments) of 0.049 ± 0.005 and 0.033 ± 0.006 unit/mg of protein were obtained for aldolase activity from the livers of young and old rabbits respectively. A Student's *t* test indicated that this difference was significant ($P < 0.01$). The reason for the presence of more aldolase activity/mg of protein in the livers of young rabbits compared with old rabbits may be that there is either an increased rate of synthesis or a decreased rate of breakdown of aldolase relative to other proteins in the young animals. The presence in the livers of old animals of some aldolase that is less catalytically efficient may also account for these findings. To distinguish between these possibilities, the amount of aldolase sequence present in the livers of individual rabbits was determined. From a mixture of the carboxymethylated liver proteins and [¹⁴C]carboxymethylated rabbit liver aldolase which had been treated with CNBr, a peptide was isolated having an *N*-terminal valine residue and a typical amino acid composition of CmCys* (1.0), Thr (3.0), Glu (2.0), Pro (1.0), Gly (1.0), Ala (3.0), Val (1.9), Tyr (1.0), His (1.0), Lys (2.0), Hse (0.9). The content of serine, the contaminating amino acid present in largest amount, was less than 0.1 mol/mol of peptide. From these data, the isolated peptides were judged to be pure samples of the peptide Val-Thr-Ala-Gly-His-Ala-CmCys-Thr-Lys-Lys-Tyr-Thr-Pro-Gln-Glu-Val-Ala-Hse, which had previously been isolated from rabbit liver aldolase (Forcina & Perham, 1971). The specific radioactivity of this peptide was calculated for peptides isolated from mixtures of the [¹⁴C]carboxymethylated rabbit liver aldolase and carboxymethylated liver proteins. By comparison with the specific radioactivity of the peptide isolated from purified aldolase, the amount of sequence contributed by the liver proteins was calculated. Table 1 shows the amount of sequence per

* Abbreviations: CmCys, carboxymethylcysteine; Hse, homoserine.

Table 1. *Quantities and specific activities of aldolase in the liver supernatants of young and old rabbits*

Quantities are given in nmol of aldolase sequence/mg of protein. Specific activities are given as μ mol of fructose 1,6-diphosphate cleaved/min per nmol of aldolase sequence. A Student's *t* test indicates that the specific activities of the liver aldolase of young and old rabbits are significantly different.

Expt. no.	Quantity (nmol/mg)		Specific activity (unit/nmol)	
	Young	Old	Young	Old
1	0.57	0.35	0.100	0.062
2	0.66	0.78	0.092	0.077
3	0.61	0.51	0.095	0.049
4	0.40	0.40	0.110	0.088
5	0.59	0.34	0.097	0.072
6	0.36	0.36	0.091	0.081
Mean $\pm$ S.E.M.	0.53 ± 0.05	0.46 ± 0.07	0.098 ± 0.003	0.072 ± 0.005

mg of protein obtained for the carboxymethylated proteins of liver supernatants. By using these data and the specific-activity measurements of supernatant aldolase in units/mg of protein, the specific activity of aldolase in units/nmol of aldolase sequence was calculated (Table 1). Aldolase activity per unit of sequence is significantly higher in the livers of young rabbits than in those of old rabbits ($P < 0.01$). The liver preparations used were found to cleave the substrates fructose 1,6-diphosphate and fructose 1-phosphate at equal rates, which indicated that other isoenzymes of aldolase were not present (Penhoet *et al.*, 1969b). It therefore appears that either some or all of the liver aldolase sequence becomes less efficient in older rabbits. It cannot be determined from these experiments whether the loss of catalytic efficiency is due to the loss of certain essential residues or whether environmental effects such as the presence of inhibitors are responsible. It seems unlikely that competitive inhibitors could be responsible, since the activity is measured *in vitro* under conditions where substrate must be in large excess. If alterations of certain specific residues are responsible for the decreased catalytic efficiency, it is clear that none of these residues is present in the segment of sequence examined, since in this case the amount of activity would be directly proportional to the amount of this sequence and it clearly is not. The 18 residues examined represent only 5% of the total polypeptide chain and therefore the occurrence, in other regions of the sequence, of specific residues that are particularly susceptible to an alteration causing catalytic inactivation cannot be discounted. It is also possible that alterations may be randomly distributed throughout the polypeptide chain. Examination of the correlation between amounts of other regions of aldolase sequence and aldolase activity may be expected to indicate the extent and nature of altera-

tions in the aldolase molecule. Determination of the catalytic efficiency of aldolase sequences as a function of age in other rabbit tissues and in the tissues of other species as well as studies of age-related catalytic efficiencies of other enzymes may be expected to indicate whether the phenomenon is general and directly associated with aging. It may also be noted that the isotope-dilution method of protein determination used in these studies may prove to be a means of determining proteins that do not have an easily measurable biological activity, such as collagen and histones.

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