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MOLECULAR AGING OF FRUCTOSE-BISPHOSPHATE ALDOLASE IN TISSUES OF RABBIT AND MAN

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SUMMARY

Electrofocusing permits the resolution of crude fructose-bisphosphate aldolase (EC 4.1.2.13) from various tissues of rabbit and man into five forms corresponding to the different tetrameric hybrids of native and post-translationally modified chains, Subunits α and α'. The comparison of the distributions of these hybrids in red cells from rabbit and man of different ages confirms that only one of the two types of chains is synthesized.

The rate of post-translational transformation of Subunits α into α' is not the same in different tissues and moreover is slowing down during the life of the red cells.

In all tissues, the distribution is nearly random.

INTRODUCTION

It has recently been shown that modifications of proteins during aging occur and it has, therefore, become of interest to distinguish errors in biosynthesis from post-translational changes. The enzyme, fructose-bisphosphate aldolase (EC 4.1.2.13) is a good model for such a study. Its tetrameric structure is well known. The existence of several isoenzymes, although making the research more difficult, allows interesting comparisons. Two ways of approach have been already used. An immunological technique, which was first employed by Mennecier [1] demonstrated the presence of inactivated molecules in aged cells and especially in red cells. It was later used by Gershon and Gershon [2, 3] for the study of molecular aging. Analytical techniques, initiated by Masters [4] in 1967, showed the existence of a sub-banding of fructose-bisphosphate aldolase (muscular type) in mouse. Susor et al. [5] in 1968 demonstrated that this microheterogeneity is the result of the existence in aldolase of two types of subunits, Subunits α and α', hybridized together to form five subcomponents α_4, $\alpha_3\alpha'$, $\alpha_2\alpha_2'$, $\alpha\alpha_3'$ and α_4'. Subunit α' was shown to derive from Subunit α through deamidation of a single asparagine residue [6, 7]. The present work deals with this analytical approach and tries to answer the following questions: (a) Does the transformation of Subunit α of aldolase into Subunit α', which has been described only in

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muscle of rabbit, also take place in other tissues of the same animal and of other species, especially man?

(b) Is the transformation related to the metabolism of the cell, regarding biosynthetic activity and turnover rate?

(c) Is it an active phenomenon, involving factors capable of catalyzing deamidation?

MATERIAL AND METHODS

Hemolysates were prepared from normal or reticulocyte rich blood of rabbits and men. Human reticulocytes came from a patient with hemolytic anemia with 27% reticulocytes. In rabbits reticulocytosis was induced by phenylhydrazine. Separation of old and young red cells of rabbits was obtained by the phthalate technique according to Danon and Marikovsky [8].

Red cells were lyzed by 3 vol. of distilled water. Aldolase had to be separated from hemoglobin before being submitted to analysis since the aldolase activity of crude hemolysates is low. In rabbit hemolysates the separation was performed by precipitation by $(NH_4)_2SO_4$ at 60% saturation, which leaves hemoglobin in solution. Aldolase of human hemolysates, whose hemoglobin is less soluble, was separated by a modification of the method of Penhoet et al. [9]: 50 ml of hemolysate dialyzed against 0.05 M Tris-HCl–0.005 M EDTA buffer, pH 7.5, were placed on a phosphocellulose column (Cellex P, BioRad) 50 cm $\times$ 2 cm, equilibrated with the same buffer. 500 ml of the same buffer eluted hemoglobin nearly completely. Aldolase was eluted as a very sharp peak with the same buffer, containing in addition 10^{-3} M fructose diphosphate, then concentrated on a Diaflo membrane CM 30.

White blood cells were prepared by decantation in the presence of Plasmagel, and the residual red cells were hemolysed in 0.8% NH_4Cl.

All extracts were dialyzed against a 0.5% Ampholine solution (LKB, No. 8044, pH gradient 7–10) before performing electrofocusing. The latter took place on a 110 ml LKB column. Electrode buffers were 0.2 M ethanolamine and 0.2 M H_2SO_4. The cathode was placed at the bottom of the sucrose gradient, containing 2% of the same Ampholine. The sample was deposited in the middle of the gradient. Electrofocusing took place under a voltage of 500 V for 100 h, at 4 °C. The column was then eluted in 1-ml fractions, in which aldolase activity was estimated by the optical test as described by Blostein and Rutter [10]. pH measurements were made at room temperature with a pH meter Radiometer pHM 52.

RESULTS

(1) Rabbit aldolase

(a) Elution profiles of crystalline rabbit muscle aldolase prepared according to Taylor et al. [11] showed a nearly symmetrical distribution with five major peaks. From the data of Susor et al. [5] these peaks can be characterized as the molecular forms α_4, $\alpha_3\alpha'$, $\alpha_2\alpha'_2$, $\alpha\alpha'_3$ and α'_4, with the following isoelectric points 8.45, 8.32, 8.15, 8.08 and 8.0. In addition, a small sixth peak, with an isoelectric point 7.88, might correspond to another type of modification of aldolase, comparable to that described by Susor et al. [12]. In this pure preparation, a comparison of protein concentration

(measured by absorbance at 280 nm) and enzymatic activity showed that the specific activity of all peaks is the same (Fig. 1a). Elution profiles after electrofocusing of crude rabbit muscle extracts are very similar to those of crystalline aldolase (Fig. 1b). Elution profiles of extracts from muscles of young rabbits (1 month old) showed only the α_4-tetramer, in agreement with the results of Koida et al. [6].

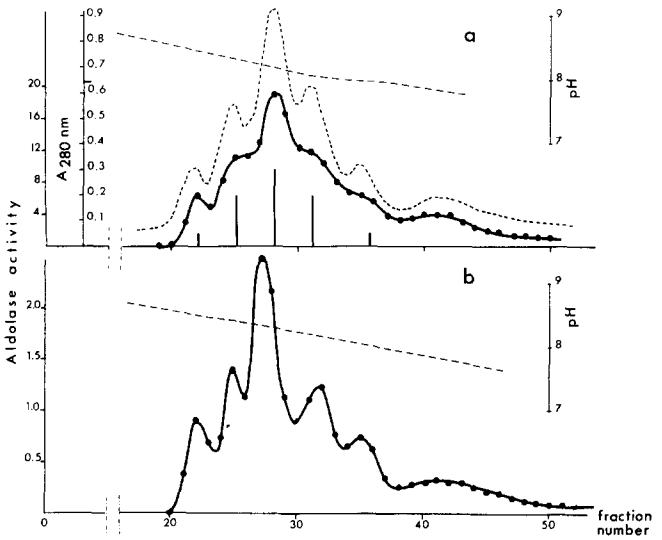

Fig. 1. Isoelectric fractionation of rabbit aldolase A. ———, aldolase activity (I.U. per ml in the fraction); · · · · · ·, absorbance at 280 nm; ---------, pH values of the fractions. (a) 15 mg of dialyzed crystalline rabbit muscle aldolase A of spec. act. 23 I.U./mg were electrofocused in an Ampholine gradient of pH 7–10, on a 110 ml column. Absorbance at 280 nm was continuously monitored with a Beckman DB-G spectrophotometer equipped with a 1 cm light-path flow cell; (b) 25 ml of a rabbit muscular extract (1/10, w/w) were deposited on the column. Bars indicate the theoretical random distribution of the five forms of aldolase A obtained with 50% of each of the two types of subunits α and α'.

(b) Electrofocusing of aldolase from red cells at different stages of maturation allows one to see directly the transformation of Subunits α into Subunits α'.

Elution profiles of preparations of aldolase from rabbit reticulocytes were clearly asymmetrical: the forms rich in α'-subunits are nearly absent (Fig. 2a).

By contrast, experiments made with hemolysates from normal rabbits displayed a prevalence of α'-subunits (Fig. 2b), while this prevalence increased in aldolase prepared from old red cells of the same rabbit (Fig. 2c). In this case the peak of Subunit α_4 disappeared completely, Subunit $\alpha_2\alpha'_2$ remained the major peak but Subunits $\alpha\alpha'_3$ and α'_4 became very noticeable.

In addition, and in agreement with previous results from our laboratory [1, 13], it is to be noted that aldolase activity decreases during the life of red blood cells: related to the amount of hemoglobin, reticulocytes show an activity four times higher than the mean of normal blood cells, while that of old cells is still 50% lower (Table I).

(2) Human aldolase

Electrofocusing profiles obtained from human tissue extracts showed a comparable repartition of enzymatic activity. Muscle extracts display five major peaks

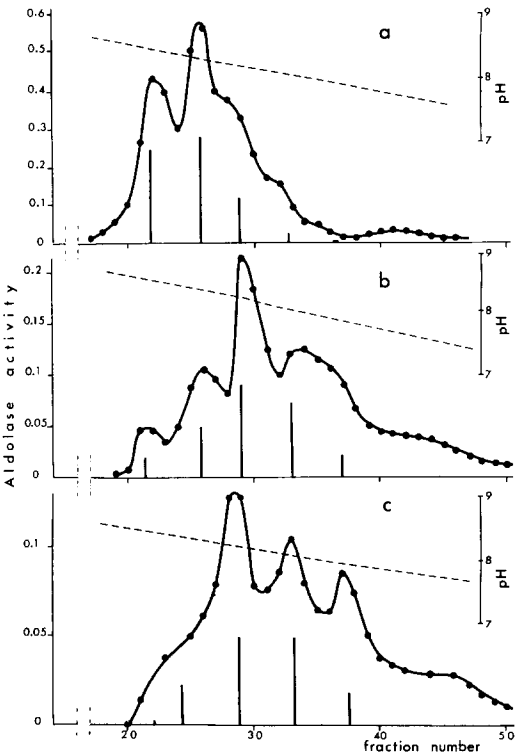

Fig. 2. Isoelectric fractionation of rabbit aldolase A from red cells. ———, aldolase activity (I.U. per ml in the fractions); ---------, pH values of the fractions. (a) 10 ml of packed reticulocytes were hemolyzed in 4 vol. of Tris 0.01 M–EDTA 0.001 M (pH 7.5) buffer. After removing the hemoglobin and dialyzing, the whole extract was submitted to electrofocusing; (b) and (c) 12 ml of packed normal erythrocytes, or 20 ml of packed old erythrocytes were prepared by the same method and submitted to electrofocusing. Bars indicate the theoretical random distributions of the five forms of aldolase A obtained with: (i) 78% of α- and 22% of α'-subunits; (ii) 45% of α- and 55% of α'-subunits; (iii) 40% of α- and 60% of α'-subunits.

TABLE I

ALDOLASE ACTIVITY IN RABBIT RED CELLS AT DIFFERENT STAGES OF MATURATION

	Aldolase activity (I.U./ml)	Hemoglobin concn (g/l)	Aldolase activity per g of hemoglobin (I.U./g)
Hemolysates from normal red cells	0.083	69.2	1.20
	0.060	68.5	0.875
Hemolysates from reticulocytes	0.294	71.3	4.14
	0.321	70.2	4.57
	0.237	68.4	3.46
Hemolysates from old red cells	0.041	69.3	0.59
	0.037	70.5	0.53

which can be assumed to represent the same forms as those of rabbit extracts, and also a sixth minor peak which might represent another form of aldolase heterogeneity (Fig. 3a). The isoelectric points have moved slightly towards alkaline pH values compared to rabbit aldolase. They are 8.51, 8.45, 8.39, 8.27 and 8.19, respectively. The isoelectric point of the sixth peak is too high to be accounted for by an hydrid between aldolases A and C.

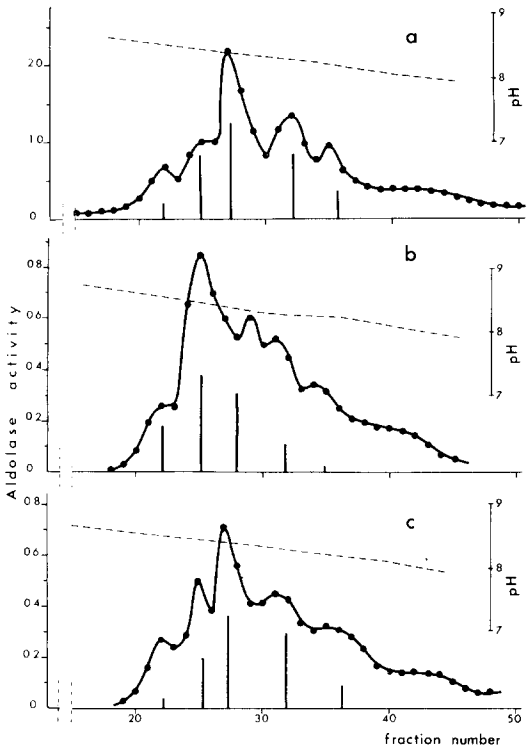

Fig. 3. Isoelectric fractionation of crude human aldolase A. ———, aldolase activity (I.U. per ml in the fractions); ---------, pH values of the fractions. (a) electrofocusing of 2 ml of a human muscular extract (1/10; w/v); (b) and (c) electrofocusing of hemolysates from (b), 20 ml of packed human red cells from a patient with an hemolytic anemia (27% reticulocytosis) and (c), 36 ml of packed human normal red cells. Bars indicate the theoretical random distributions of the five forms of aldolase A obtained with: (i) 50% of each of the two types of subunits; (ii) 65% of α- and 35% of α'-subunits; (iii) 45% of α- and 55% of α'-subunits.

As in the rabbit, a comparison between hemolysates from normal blood cells and from reticulocytes demonstrated a modification with the age of the cells: peaks rich in α'-subunits are smaller in aldolase from reticulocytes than from normal hemolysates (Figs 3b and 3c).

In order to know whether this heterogeneity can be observed in cells with a short life span, we made electrofocusing experiments with white blood cells from normal men. Results show that in aldolase from normal white blood cells (essentially granulocytes) the five peaks are visible, but with a predominance of forms rich in α-chains (Fig. 4).

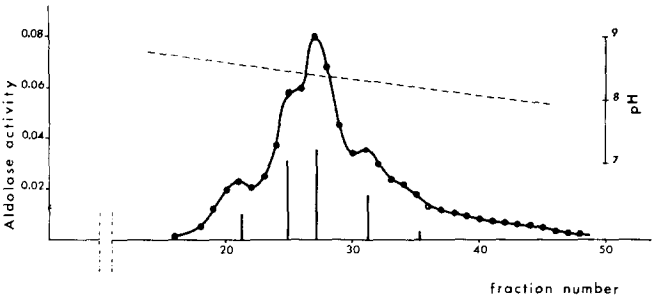

Fig. 4. Isoelectric fractionation of crude human white cells aldolase A. ———, aldolase activity (I.U. per ml) in the fractions; ---------, pH values of the fractions. Bars indicate the theoretical random distribution of the five forms of aldolase A obtained with 57% of α- and 43% of α'-subunits.

DISCUSSION

The microheterogeneity of fructose-bisphosphate aldolase (as a probable consequence of aging) has previously been described in the muscle of mouse [4] and rabbit [5] and also in beef and pork [14] and in rat hepatoma [15]. The present work extends this finding to the human species. This points to a conservation throughout mammalian species of the particular asparagine residue involved in the post-translational modification of aldolase A. Moreover, a similar microheterogeneity has also been found in liver aldolase B [16].

The major result of our investigations is a variation of the ratio of α- to α'-subunits according to the age of the cells. Muscle cells which synthesize proteins, but with a relatively slow turnover, contain an aldolase which shows a symmetrical distribution. The picture is quite different in red and white blood cells. In red cells, there is a progressive shift towards the forms with a lower pH_i. Molecular forms $\alpha\alpha'_3$ and α'_4 appear only after the reticulocyte stage. Since at that time there is no protein synthesis in the red cell, our results are a direct verification of the data of Midelfort and Mehler [17] obtained by radioactive labeling in vivo: only the α-subunits are synthesized, and the α'-subunits derive from them through a post-translational modification.

In very young cells, like white blood cells, the cathodic forms are widely predominant. One can, therefore, generally conclude that the older the cells, the richer the aldolase in α'-subunits. The speed of appearance of α'-chains, however, is not the same in all tissues. White blood cells from normal blood (cells of a few hours life span) possess about 40% of α'-subunits. In these cells, the rate of appearance of α'-subunits is very rapid. In the reticulocytes of rabbit as well as those of human origin, which are cells a few days old, accumulation of α'-subunits is about 25%, e.g. relatively rapid. In aged red cells, several weeks old, it does not go beyond 60–65%.

These results allow one to conclude that the rate of transformation of α- into α'-subunits is not the same in white and red blood cells, and that it decreases during the life of the red cells. They point to the existence of an active factor for the transformation. The results are in agreement with observations made in our laboratory by Kahn et al. [18], showing the presence in some tissues of an aging factor for human glucose-6-phosphate dehydrogenase. Moreover, Koida et al. [6] and ourselves ob-

served that crystalline muscle aldolase from young rabbits (1 month old) shows only α_4-form. This supports the hypothesis of an aging factor, which would be absent in muscle of rabbits younger than 3 months.

In all tissues we have studied, the distribution of tetrameric forms of aldolase is nearly random as shown by the comparison of theoretical random and observed distributions (Figs 1, 2, 3 and 4). So everything happens as if monomers α and α' would occur and then would be randomly reassociated. As aldolase is known to be a very stable tetramer (see ref. 19 for example), the significance of such a distribution is a matter of speculation.

Finally, the determination of the specific activity of the various peaks in crystalline aldolase (Fig. 1) failed to reveal any significant difference. The molecular aging as represented by a Form $\alpha \rightarrow$ Form α' shift does not explain our previous results obtained by an immunological technique [1]. We found that the "molecular specific activity" (ratio of enzymatic activity over immunological reactivity) of aldolase decreases in the following order: crystalline aldolase, crude muscle extract, hemolysate from reticulocytes, hemolysate from normal red cells, the amount of cross reacting material compared to crystalline aldolase being 30, 57 and 140%, respectively.

The two phenomena observed in aging, deamidation of aldolase and appearance of cross-reacting material, seem to be independent.

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