Deamidation *In Vivo* of an Asparagine Residue of Rabbit Muscle Aldolase

(isozymes/electrofocusing/subunits/protein structure)

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ABSTRACT Microheterogeneity of rabbit muscle aldolase is caused by deamidation *in vivo* of an asparagine residue near the C-terminus of each subunit. Isotopic labeling of a peptide containing the asparagine residue at various time intervals before isolation of aldolase permits estimation of the half-time for the deamidation as about 8 days, which is about the time estimated for the half-life of the enzyme *in vivo*. It is concluded that the aldolase as genetically determined is a tetramer, designated α_4 , that undergoes random deamidation to form $\alpha_3\beta$, $\alpha_2\beta_2$, and $\alpha\beta_3$ species as intermediates in the formation of β_4 , the species in which all of the specific asparagine has been deamidated. Isoelectric focusing data indicate that the subunits do not exchange appreciably *in vivo*.

Microheterogeneity in supposedly homogeneous protein preparations is a well documented phenomenon (1). Sometimes this is due, in multimeric proteins, to subunits with different primary structures that associated randomly. Sometimes it is due to the covalent attachment of nonprotein groups (e.g., phosphate or carbohydrate) or to partial proteolytic hydrolysis. However, in certain cases, no differences in covalent structure can be detected among the various species. If the species are interconvertible, as in the case of horse liver alcohol dehydrogenase (2), they are called conformational isozymes; if they are not, an explanation involving progressive deamidation of asparagine or glutamine residues is usually invoked. A mechanism of this last type is difficult to prove in practice because of the large number of amide residues in most proteins and the possibility that deamidation at any exposed residue might produce the same effect on electrophoretic mobility. In one example (3), direct evidence was obtained that *in vitro* deamidation at pH values around 12 led to the production of electrophoretic isozymes.

There are several examples in the literature of microheterogeneity arising *in vivo* in which the more cathodic isozyme seems to be the first one synthesized and the more anodic species arise from it in a time-dependent process. Isozymes of phosphoglucosmutase in erythrocytes (4) and of nucleoside phosphorylase in erythrocytes and other tissues (5) are generated in this way. The cathodic isozyme of rat kidney cytochrome *c* becomes labeled with ^{59}Fe more rapidly than the other isozymes and decays into the anodic forms when incubated with an electrolyte solution *in vitro* (6). Although other mechanisms have not been ruled out in the above examples, slow deamidation seems the most likely explanation.

The question of deamidation has been raised in the case of rabbit muscle aldolase (7). The enzyme is thought to be a tetramer with an $\alpha_2\beta_2$ structure (8). Tryptic maps of separated

α and β subunits were first reported to be quite different (9), but more recent studies on tryptic maps of separated cyanogen bromide peptides showed extensive regions of homology (7). The only difference found to date is an asparagine-aspartic acid substitution at a position near the carboxyl terminus (10). The incompleteness of the primary sequence work makes it impossible to say that there is no other difference between the α and β subunits, but it seems reasonable to speculate that aldolase is genetically an α_4 tetramer.

We have shown (11) that two carboxyl-terminal hexapeptides, one containing asparagine and the other with an aspartic acid residue, are released by limited chymotryptic hydrolysis of native muscle aldolase. Their structures are as follows:

Ile-Ser-His-Asn-Ala-Tyr (α subunit)

Ile-Ser-His-Asp-Ala-Tyr (β subunit)

The simple isolation of the two peptides made it possible for us to approach the question of whether the latter arises from the former by *in vivo* deamidation. In this paper, we report the analysis of the aldolase subunits by an experimental approach similar to that used with cytochrome *c* (6), namely, injection of a radioactive tracer to label the peptides for comparison of specific radioactivities at various times.

MATERIALS AND METHODS

Rabbit muscle aldolase was prepared by the method of Taylor *et al.* (12), and was recrystallized three times. The preparations yielded 0.8-1.5 g of aldolase and had specific activities of 15-18 units/mg by the fructose diphosphate (FDP) cleavage assay at 25° (13). Protein concentrations were determined spectrophotometrically from an assumed extinction coefficient of $E_{1\%}^{1\text{cm}} = 9.38$ (11).

Aldolase was labeled in the C-terminal hexapeptide with isoleucine. A 100- μCi sample of [^{14}C]isoleucine was injected into the hip muscle of a 1.4-1.8 kg rabbit, age 10-12 weeks. At a specified time afterwards, the animal was killed by decapitation and the aldolase was isolated. No attempt was made to prevent reutilization of labeled amino acid during protein turnover *in vivo* by administration of excess unlabeled isoleucine.

The carboxyl-terminal hexapeptides from the α and β subunits were isolated after limited chymotryptic digestion (11).

Concentrations of radioactive label were determined by pipeting an aliquot into 10 ml of Bray's solution and counting in a Packard Tricarb Scintillation Counter. Counts per minute were converted to disintegrations per minute by the channels-ratio method. Concentrations of the chymotryptic peptides were determined by hydrolysis of aliquots in 5.7 N HCl for

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TABLE 1.

Time of <i>in vivo</i> labeling	Whole protein (dpm/mg)	Isoleucine† (dpm/μmol)	Asparagine peptide		Aspartate peptide		Relative total dpm, Asn/Asp
			μmol	dpm/μmol	μmol	dpm/μmol	
8 hr	520	970	2.12	1485	1.17	169	16.3
20 hr*	120	220	1.31	420	1.23	40	10.5
3 days	600	1130	0.98	2040	0.68	491	6.0
7 days	540	1000	0.84	1400	0.60	573	3.4
25 days	270	520	0.94	570	0.89	440	1.4
85 days	145	270	0.94	210	0.92	294	0.73
125 days	55	94	2.12	46	1.71	134	0.42

* From a mature, 3.6-kg rabbit.

† Calculated on the basis of 86 mol of isoleucine per tetramer of aldolase.

22 hr at 105°; the amount of free isoleucine was measured in an amino-acid analyzer. Norleucine was added before the hydrolysis step as an internal standard.

Isoelectric focusing separations were done in a 110-ml LKB Productor column with the LKB pH 7–10 Ampholite mixture. The pH 7–9 Ampholite solution used by Susor *et al.* (14) for the separation of aldolase A isozymes was inadequate, since the pH range from 8 to 9 covered only 15% of the length of the column.

RESULTS

In the choice of an appropriate amino acid and radioactive dosage for labeling experiments, two factors were considered: (i) The amino acid must be present in the hexapeptides liberated by chymotrypsin, and (ii) the amino acid should be incorporated with as high an efficiency as possible. Work from Velick's laboratory (15, 16) showed that essential amino acids were incorporated into aldolase about 100 times more efficiently than nonessential amino acids and that 100 μCi was an adequate dose. We therefore used 100-μCi injections of isoleucine. The efficiency was also increased by the use of young, under 2 kg, animals.

The results of a series of labeling experiments in which the animals were killed at various times after injection are shown in Table 1. The data indicate that the asparagine-containing peptide picks up label more rapidly than the aspartate-containing peptide. In addition, the radioactive label appears to be chased from the former into the latter after long periods of survival. Possible explanations for these results are either that the aspartate peptide is a deamidation product of the asparagine peptide, or that the asparagine peptide is synthesized and turned over 15- to 20-times more rapidly than the aspartate peptide. The second possibility is unlikely, since the α and β subunits exist as a set of five isozymes (14), presumably hybrids of α and β subunits. The α subunit in the α₃β, α₂β₂, and αβ₃ hybrids would have to be preferentially degraded without destruction of the β subunit.

The occurrence of isozymes that appear to contain various ratios of α and β subunits provides an opportunity to test the postulated deamidation mechanism of interconversion of subunits. If it is assumed that deamidation is a random process, newly synthesized α₄ aldolase will be gradually converted first to α₃β₁; this product will be converted at the same rate to α₂β₂, etc. Thus, the α₄ aldolase should be composed of the most recently synthesized material and the β₄ material should be the oldest. If the aldolase is pulse-labeled, the specific

radioactivity of α₄ should be greater than the average specific activity of all α subunits, and the β₄ should have a lower specific activity than the average of all β subunits.

This type of analysis was done by analysis of aldolase separated by isoelectric focusing. The aldolase was labeled with isoleucine 20 hr before the rabbit was killed. The average specific activities of α and β subunits, calculated from the isoleucine content of aldolase (17) and the specific activities of the isolated chymotryptic peptides, were 230 dpm/mg and 20 dpm/mg, respectively. As shown in Fig. 1, the isozymes were partially resolved. Enzyme specific activity was similar in all peaks, but the radioactivity was not equally distributed; the pH 8.9 peak (presumably α₄) had a specific activity of 320 dpm/mg and the peak below pH 8.0 (presumably β₄) had no detectable radioactivity. These results fit the model proposed above, and do not appear to be compatible with a mechanism involving *de novo* synthesis of α and β subunits by independent genetic determinants.

A quantitative evaluation of the turnover rate of muscle aldolase is attractive in view of the recent report by Poole

FIG. 1. Isoelectric focusing of muscle aldolase (rabbit labeled for 20 hr with [¹⁴C]Ile before death). 18 mg of dialyzed aldolase was placed in the center of a 0–40% sucrose gradient in a 110-ml LKB electrofocusing column. Ethylenediamine was put in the heavy, bottom electrode solution and phosphoric acid was put in the light, top electrode solution. Electrofocusing was started at 400 V, and was gradually increased to 800 V over 24 hr. The column was eluted after >3.5 days. Specific radioactivities of fractions were determined after dialysis against water. 1-ml Fractions were collected.

FIG. 2. A plot of log of dpm/mg against days of incubation. The poor fit of the points to the straight line is as predicted by Poole (18).

(18). As he points out, reutilization of radioactive label after degradation of the protein to amino acids will make the half-life for turnover of various proteins appear to be much longer than it really is. Fig. 2 shows a semilog plot of specific radioactivity of aldolase against time. The half-life is about 38 days. Some uncertainty in this value for the apparent half-life is introduced by the use of young, growing animals, whose protein was obviously being synthesized faster than it was being broken down. However, this factor might be counterbalanced by a more efficient reutilization of labeled amino acids for protein synthesis.

One can calculate the true turnover rate of aldolase from the $\alpha \rightarrow \beta$ conversion data in Table 1. A scheme for the protein turnover and $\alpha \rightarrow \beta$ conversion is as follows:

$$\frac{d(\alpha)}{dt} = K_1 - (k_2 + k_3)(\alpha) = 0$$

$$\frac{d(\beta)}{dt} = k_2(\alpha) - k_3(\beta) = 0$$

$$(\alpha) = (\beta) \text{ (in mature rabbits)}$$

Therefore,

$$k_2 = k_3$$

The assumption that the α and β subunits are degraded at the same rates is based on the conclusion drawn above that they have identical amino-acid sequences, with the exception of one residue, and on data from our previous paper (11) that shows that this residue is in an area of the protein exposed to the solvent in both types of subunits. From the data of Heimberg and Velick (15) and those of Poole (18), 80–90% of the free radioactive amino acid is incorporated into protein within 2–3 hr after injection. Therefore, one can assume that the relative amount of radioactivity in the Asn and Asp peptides, $(\alpha)^*/(\beta)^*$, in the 8- and 20-hr samples is a measure of the total α converted to β during the *in vivo* incubation time. Using $\ln[(\alpha)^*/(\alpha)^*t - (\beta)^*/(\beta)^*t] = kt$, one obtains values for $t_{1/2}$ of 8.3 and 7.9 days for the 8- and 20-hr samples, respectively. These values are less than 25% of the $t_{1/2}$ calculated from the total radioactivity decay, but the discrepancy correlates well with

the theoretical plot of Poole (18) for the effect of reutilization of label. Therefore, we conclude that aldolase has a half-life of about 8 days in rabbit muscle.

DISCUSSION

Although deamidation has been suggested often as a mechanism for the modification of proteins, and has been demonstrated *in vitro*, to our knowledge this phenomenon has not been demonstrated previously *in vivo*. It is possible that other examples of deamidation of asparagine or glutamine residues will be found as other proteins that exhibit microheterogeneity are examined. The peculiar features of aldolase that enable us to isolate a fragment containing a single asparagine or aspartic acid residue may be sufficiently unusual that the identification of the labile amide may be difficult in other proteins. The kinetics of deamidation of proteins in general, however, should be susceptible to analyses by electrofocusing of isotopically labeled proteins.

We have no basis for preferring an enzymatic or nonenzymatic mechanism for the deamidation. The fact that the susceptible amide is located in the C-terminal region that is also accessible to proteolytic digestion is compatible with attack by either a hypothetical deamidase or an ion in the environment. The existence of protein deamidases is suggested by the work of Lane, Irwin, and Neuhaus (19), who described a urinary protein from male rats with electrophoretic properties that are modified with a concomitant release of ammonia. The changes in isoelectric point reported after incubation of the protein with kidney homogenates cannot be correlated simply with deamidation. The *in vitro* modification of cytochrome *c* (6) offers a nonenzymatic precedent. It is also possible, as pointed out to us by Dr. Arie Berger, that the imidazole group of the adjacent histidine residue catalyzes the deamidation.

The data reported in this paper have some bearing on the actual subunit structure of muscle aldolase. The finding that the enzyme could not be resolved into isozymes by available chemical methods, but that it contained two kinds of subunits, was originally interpreted to mean that aldolase had an $\alpha_2\beta_2$ structure (20). However, the resolution of the enzyme into five species by Susor *et al.* (14) showed this model to be incorrect, and led to the speculation that the isozymes arose by the random association of nonidentical subunits (21). Recent efforts directed at determining the total primary structure of aldolase have shown sequence identity between the subunits over about 67% of the polypeptide chains (C. Y. Lai, personal communication), with the only difference being the asparagine-aspartic acid substitution near the carboxyl terminus (10). This structural information, along with the results of labeling experiments reported here, lend support to a model for aldolase isozymes based on specific, progressive deamidation of an asparagine residue of a single type of subunit. This model means that the α_4 , $\alpha_3\beta$, $\alpha_2\beta_2$, $\alpha\beta_3$, and β_4 species represent sequential stages during the lifetime of the aldolase protein *in situ*. The findings that the specific radioactivity of the presumed α_4 species isolated from an electrofocusing column is higher than the value calculated for the average α subunit, and that the presumed β_4 species contained no detectable radioactivity, indicate that recombination of subunits *in vivo* is slow compared with the rate of deamidation and destruction of aldolase. There is no evidence that dissociation and recombination occur under physiological conditions.

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