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Kinetic study of racemization of aspartyl residues in synthetic elastin peptides

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Summary. We previously reported that biologically uncommon D-aspartyl residues are present in sun-damaged skin from elderly people, possibly in elastin. Here, we report the kinetics of Asp racemization in model peptides corresponding to elastin sequences from exons 6 and 26. We estimated the activation energy (E) of racemization of Asp residues, the racemization rates (RR) at 37°C and the time (t) required for the D/L ratio of Asp to approximate to 1.0 (D/L ratio of Asp = 0.99) at 37°C. For an exon 6 peptide, E = 29.0 kcal/mol, RR = 2.59×10^{-2}/yr and t = 101.0 yr. For an exon 26A peptide E = 26.2 kcal/mol, RR = 4.27×10^{-2}/yr and t = 61.3 yr; and for a second exon 26A peptide E = 25.7 kcal/mol, RR = 5.55×10^{-2}/yr and t = 47.0 yr. These results suggest that racemization of Asp residues in elastin could occur within a human life span. We propose that D-Asp could be a useful molecular indicators of aging.

Keywords: Aging – D-Aspartic acid – Elastin – Kinetics – Racemization – Racemization rate constant – Skin

1 Introduction

In the past, proteins were believed to consist exclusively of L-amino acids in living tissues. However, biologically uncommon D-aspartyl (Asp) residues have now been reported in various proteins of the tooth (Helfman et al., 1975), eye lens (Masters et al., 1977), aorta (Powell et al., 1992), brain (Fisher et al., 1986; Roher et al., 1993), bone (Ohtani et al., 1998), and skin (Fujii et al., 2002) in elderly humans. Aspartic acid is the most easily racemizable amino acid (Bada, 1984) and D-Asp may be formed by racemization in metabolically inactive tissues during the chronological aging process. However, the specific sites in which racemization of Asp residues in proteins proceeds were not described in the above reports, except in the case of alpha A-crystallin (Asp-58 and Asp-151) (Fujii et al., 1994a) and alpha B-crystallin (Asp-36 and Asp-62) (Fujii et al., 1994b) in the lens, and a beta-amyloid protein (Asp-1, 7 and 23) in brain (Roher et al., 1993). Apart from these studies, D-Asp was simply reported to be present in whole tissues. We have previously shown that the aspartic acid in lens protein is not uniformly racemized, in that residues at specific sites vary in susceptibility to racemization, depending on the primary structure (Fujii et al., 1996) and higher order structure (Fujii et al., 1999) of the protein. D-Asp formation was also accompanied by isomerization from the natural alpha-Asp to the biologically uncommon beta-Asp (isoaspartate) form (Fujii et al., 1999).

Recently, we found protein containing D-beta-Asp in elastic fibers from sun-exposed skin of elderly people and suggested that the protein containing this isomer may be elastin (Fujii et al., 2002). In a more recent study, Ritz-Timme et al. (2003) prepared insoluble elastin from human skin and ligament, and analyzed the D/L ratio of the total Asp residues in hydrolyzed samples. They showed that the racemization rate of the Asp residues in elastin increased with age. The racemization rates in protein are both influenced by the primary and the higher order structures surrounding the individual Asp residues. However, the higher order structure of elastin has not yet been determined. Therefore, we estimated the racemization rate of individual Asp residues using the model peptides in this study. The elastin molecule in human skin has one Asp residue in exon 6, two Asp residues in exon 26A, and one Asn in exon 23A, on the basis of the amino acid sequence deduced from the cDNA sequence (Indik et al., 1987; Fazio et al., 1988). Because the racemization rate of Asp residues in elastin may differ in different sites, we

synthesized three Asp-containing model peptides corresponding to the exons 6 and 26A of the elastin cDNA sequence and analyzed the kinetics of Asp racemization in these peptides. The present study shows that these Asp residues in the elastin molecule are susceptible to racemization under physiological conditions.

2 Materials and methods

2.1 Peptides and heating experiments

To determine the racemization rates of Asp residues in the model peptides, we subjected GVADAAAA (exon 6), REGDPSSS (exon 26A-1), AGADEGVR (exon 26A-2) to heating. The analysis of exon 23A was excluded in this study because the peptides is highly spliced out (Indik et al., 1987; Fazio et al., 1988). These peptides each correspond to sequences from human elastin and were synthesized and purified by reversed-phase high performance liquid chromatography (RP-HPLC) by Asahi Techno Glass Corporation (Funabashi, Japan). They were dissolved in distilled water and incubated at 50, 60, 70, 80, 90°C for 1–32 days.

2.2 Determination of D/L ratio of amino acid

Amino acid contamination was prevented by baking all glassware at 500°C for 4 h. The peptides were dried and then hydrolyzed with gas-phase 6 N HCl in a vacuum at 108°C for 7 h (Pico Tag Work Station, Waters Tokyo, Japan). After hydrolysis, the samples were dried again *in vacuo* prior to derivatization. The hydrolyzed samples were then dissolved in 0.13 M borate buffer (pH 10.4) and incubated briefly with *o*-phthaldialdehyde (OPA) and N-tert-butyloxycarbonyl-L-cysteine (Boc-L-Cys) to form diastereoisomers. The D/L ratio of the amino acids was determined using RP-HPLC (Shimazu, LC-9A) with a Nova-Pak ODS column (3.9 mm × 300 mm: Waters, Tokyo) and fluorescence detection (344 nm excitation wavelength and 433 nm emission wavelength). Elution was carried out with a linear gradient of 7–47% acetonitrile plus 3% tetrahydrofuran in 0.1 M sodium acetate buffer (pH 6.0) in 120 min at a flow rate of 0.8 ml/min, at 30°C. The D/L values were determined from the mean of duplicate measurements.

2.3 Kinetic measurement

Racemization of aspartic acid in peptide is a reversible first-order reaction and can be expressed as follows:

$$-d[L]/dt = k[L] - k[D] \quad (1)$$

where [L] and [D] represent the concentrations of the L- and D-Asp, respectively, and k is the rate constant for the racemization reaction. Integration of Eq. (1) gives

$$\ln[(1 + D/L)/(1 - D/L)] = 2\,kt + \ln[(1 + D/L)/(1 - D/L)]t_0 \quad (2)$$

where t is the time of the reaction and t_0 stands for $t = 0$. The t_0 term in Eq. (2) is due to racemization induced by acid hydrolysis. We determined the rate constants of racemization of Asp residues in the three peptides at five temperatures (50, 60, 70, 80 and 90°C) by using Eq. (2). On the other hand, the Arrhenius equation below gives the activation energy of racemization of amino acids in the peptides.

$$\ln k = \ln A - E/RT \quad (3)$$

where E is the activation energy of racemization, R is the gas constant, A is the frequency constant, and T is the absolute temperature. From Eq. (3), we calculated the activation energy of racemization and the time required for the Asp D/L ratio to approximate to 1.0 (0.99) at body temperature (37°C).

3 Results

Rate constants k for the racemization of Asp residues in the three peptides at five different temperatures were estimated as 1/2 of the slope of the linear regression least-squares line, according to Eq. (2). Results of the plot at 60°C are shown in Fig. 1, as an example. The straight lines of the peptides ($r^2 = 0.971$–0.999) indicate that the kinetics of racemization in the model peptides usually represent a first-order reaction. There was little difference in the rate constants among the three peptides. The Asp residue in the exon 26A-2 peptide was the most susceptible to racemization, while that in the exon 6 peptide was the least susceptible. Similar experiments were also performed at the other temperatures. The rate constants of the three peptides at 50–90°C are listed in Table 1. The temperature dependence of the rate constants for the three peptides is shown in an Arrhenius plot (Fig. 2). The activation energies for the racemization of Asp residues in

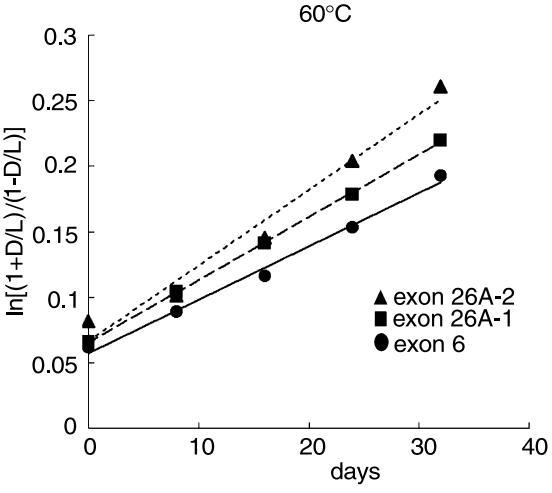

Fig. 1. Racemization of the Asp residues in exon 6 (GVADAAA), exon 26A-1 (REGDPSSS), and exon 26A-2 (AGADEGVR) peptides in distilled water at 60°C

Table 1. Comparison of racemization rate constants (k) of Asp residues in elastin-mimic peptides at 50–90°C

	$k \times 10^2$ (/day)		
°C t	Exon 6	Exon 26A-1	Exon 26A-2
50	0.05	0.07	0.09
60	0.21	0.24	0.29
70	0.68	0.56	0.75
80	1.78	1.87	2.33
90	7.72	7.04	7.47

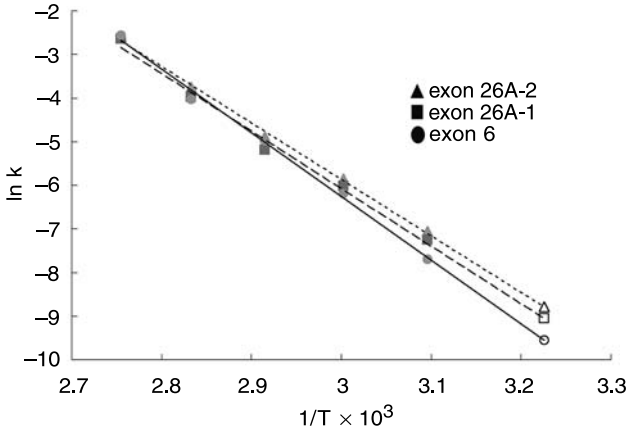

Fig. 2. Arrhenius plot of the rate constants derived from incubation of exon 6 (GVADAAA), exon 26A-1 (REGDPSSS) and exon 26A-2 (AGADEGVR) peptides in distilled water at 50–90°C. The racemization rates of Asp in the three peptides at 37°C were estimated by the Arrhenius equation

Table 2. Summary of racemization of Asp residues in elastin-mimic peptides

Peptide	Sequence	E (kcal/mol)	$k_{37} \times 10^2$ (/year)	$Year_{37}$
Exon 6	GVADAAAA	29.0	2.59	101.0
Exon 26A-1	REGDPSSS	26.2	4.27	61.3
Exon 26A-2	AGADEGVR	25.7	5.55	47.0

these peptides were determined from the slope of the plot; the results are listed in Table 2. The activation energy of 25.7 kcal/mol of the exon 26A-2 peptide was the lowest of the three. From the Arrhenius equation, we estimated the rate constants and the time required for the Asp D/L ratio to approximate to 1.0 (0.99) at body temperature (37°C) by using Eq. (2). These results are also shown in Table 2. The data consistently indicate that the Asp residue in the exon 26A-2 (AGADEGVR) peptide is the most susceptible to racemization, while the Asp residue in the exon 6 (GVADAAAA) peptide is least susceptible. At 37°C, the time required for the Asp D/L ratio in the exon 26A-2 peptide to approximate to 1.0 (0.99) was calculated to be relatively short (47.0 years, Table 2).

4 Discussion

The present study shows that the racemization rate constants of Asp are different in the three different peptides tested. The rate constants in exon 26A-2, 26A-1 and 6 at body temperature were estimated at 5.55×10^{-2} year^{-1}, 4.27×10^{-2} year^{-1}, and 2.59×10^{-2} year^{-1}, respectively. Thus, the rate constant in exon 26A-2 is twice that of exon 6 (Table 2). In a recent study, Ritz-Timme et al. (Ritz-Timme et al., 2003) estimated the racemization rate constants of the Asp residues in the insoluble elastin fraction extracted from human skin or yellow ligaments. The values they reported were 3.6×10^{-3} year^{-1} and 4.1×10^{-3} year^{-1}, respectively, i.e. lower than our estimate by a whole order of magnitude. The reason for their lower values lies in the evaluation of 4–5 Asp residues to yield an average value for the entire elastin molecule. We predict that all the Asp residues in a protein would not in fact be racemized uniformly, but heterogeneously according to site. Consistent with this hypothesis, we have previously demonstrated that certain Asp residues in lens proteins had a greater tendency to racemization than others. In aged human lenses, the D/L ratio of total Asp residues in alpha A-crystallin was 0.19 (Fujii et al., 1991). This value is the average of 15 Asp residues and 2 Asn residues in alpha A-crystallin. Subsequently, we determined the D/L ratio of individual Asp and Asn residues in alpha A-crystallin. We found that two Asp residues, Asp-151 (D/L = 5.7) and Asp 58 (D/L = 3.1) were highly inverted to the D-form, whereas the other 15 Asp/Asn residues were not so markedly racemized (Fujii et al., 1994a). Similarly, two specific racemized Asp sites (Asp-36 (D/L = 0.92) and Asp-62 (D/L = 0.57)) in alpha B-crystallin were identified, and the other 15 Asp/Asn residues were not racemized at all (Fujii et al., 1994b). Therefore, the D/L ratio calculated from total Asp in a protein will be an underestimate.

The present study indicated that the time required for racemization to reach equilibrium at 37°C is 47, 61, and 101 years for exon 26A-2, exon 26A-1 and exon 6, respectively (Table 2). If the racemization of Asp residues in elastin of skin proceeds at the same rates as with these model peptides, this implies that they could undergo significant racemization during a human lifetime. Previous studies showed that the racemization of Asp residues in peptides occurs via a succinimide intermediate, which results in the formation of D- and beta-Asp. Therefore, the racemization rate of Asp depends on the rate of succinimide formation, which is increased when the size of the residue to the carboxyl side of the Asp residue is small (Geiger et al., 1987; Stephenson et al., 1989; Tyler-Cross et al., 1991). Therefore, we expected that the racemization of Asp in exon 6 would be the most susceptible of the three peptides. However, the results obtained were contrary to our expectations. On the other hand, we previously reported racemization of the D-Asp sites in alpha B-crystallin despite the pres-

ence of bulky neighboring amino acids such as leucine or threonine (Fujii et al., 1994b). Therefore, the rule that racemization of Asp occurs more readily when the neighboring amino acid is small may not always apply. In fact, the present study clearly indicated that the Asp in exon 26A-2 was the most susceptible to racemization. However, differences between the three peptides were not large and the time required to reach equilibrium (D/L ratio of Asp = 1.0) was 50–100 years. These results indicate that each of these Asp residues in elastin is very susceptible to racemization during a normal human life span.

In human elastin, exon 26A is particularly intriguing because this segment is highly hydrophilic and atypical for elastins in amino acid sequence (Arg, Asp, Glu, Ser). The segment contains a stable beta-turn with chemotactic activity for monocytes (Basaccia et al., 1998), possesses strong immunogenicity (Debelle et al., 1992), and is expressed in limited areas such as the human epidermis (Hirano et al., 2001) and the neointima of hypertensive pulmonary arteries (Botney et al., 1992). The presence of a D-amino acid in the protein will result in the loss of normal immunogenicity and chemotactic function, and the structure of elastic fibers.

The racemization of Asp in elastin has been reported in different tissues such as human skin (Fujii et al., 2002), yellow ligaments (Ritz-Timme et al., 2003), aorta (Powell et al., 1992), and lung parenchyma (Shapiro et al., 1991). Elastin is predominantly distributed in lung, aorta, ligament, and skin. Because the turnover of elastin in these tissues is slow, the amount of D-Asp in the elastin increases and accumulates in aged tissues. Abnormal elastin molecules containing D-Asp may contribute to some pathological conditions, arteriosclerosis, and ossification of the posterior longitudinal ligament, and lung emphysema. We have recently shown that peptide-immunoreactive fibers in the dermis containing D-beta-Asp are present in the sun-protected skin of the aged as well as in sun-exposed skin (Fujii et al., 2002; Miura et al., 2004). These results suggest that the formation of D-beta-Asp in the elastic fibers of skin is closely related to chronological skin aging. We propose that the presence of D-Asp could be a useful molecular indicator for chronological aging as well as UV-induced ageing of the skin.

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