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Conformation-Dependent Racemization of Aspartyl Residues in Peptides

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Abstract: Biologically uncommon D-aspartyl (D-Asp) residues have been detected in proteins of various tissues of elderly humans. The presence of D-Asp has been explained as a result of the racemization of L-Asp (denoted as Asp) in the protein of inert tissues. We have previously suggested that the racemization of Asp may depend on the conformation of the peptide chain. However, the nature of the peptide conformation that affects the D-Asp formation has not yet been examined. Here we report the kinetics of Asp racemization in two model peptides, (Asp-Leu)$_{15}$ and (Leu-Asp-Asp-Leu)$_8$-Asp, which form β-sheet structures and α-helical structures, respectively. For the β-sheet structures, the activation energy of racemization of Asp residues was 27.3 kcal mol^{-1}, the racemization rate constant at 37 °C was 2.14×10^{-2} per year and the time required to reach a D/L ratio of 0.99 at 37 °C was 122.6 years as estimated from the Arrhenius equation. For the α-helical structures, the activation energy of racemization was 18.4 kcal mol^{-1}, the racemization rate constant 20.02×10^{-2} per year and the time 13.1 year. These results suggest that Asp residues inserted in α-helical peptides are more sensitive to racemization than Asp residues inserted in peptides adopting β-sheet structures. The results clearly indicate that the racemization rate of Asp residues in peptides depends on the secondary structure of the host peptide.

Keywords: conformation analysis · kinetics · peptides · racemization · secondary structure

Introduction

It was previously believed that proteins consisted exclusively of L-amino acids in living tissues. However, biologically uncommon D-aspartyl (D-Asp) residues have now been reported in various proteins of the tooth,[1] eye lens,[2] aorta,[3] brain,[4–5] bone[6] and the skin[7–8] of elderly humans. Aspartic acid is the most easily racemizable amino acid[9] and D-Asp may be formed by racemization in metabolically inert tissues during the aging process, thus providing a tool for the dating of samples, especially fossils.[10–13] Earlier studies suggested that all L-Asp (denoted as Asp hereafter) residues were uniformly racemized in metabolically inert protein such as the above-mentioned tissues. However, we found that Asp58 and Asp151 were specifically highly inverted to D-isomers in alpha A-crystallin,[14] and that Asp36 and Asp62 residues were racemized to a considerable extent in alpha B-crystallin obtained from the lens of an 80 year-old human.[15] The alpha A-crystallin has 15 Asp and two Asn residues, and alpha B-crystallin has 11 Asp and two Asn residues. Other Asp residues were not racemized. This result clearly showed that the Asp residues in protein are not uniformly racemized but inverted to the D-isomer at specific sites. Why is D-Asp formed at specific sites in protein?

The racemization rate is considered to depend on the primary structure[16] and higher order structures of protein.[17] D-Asp formation occurs via isomerization of the natural α-Asp to the biologically uncommon β-Asp (isoaspartate) form.[17] Previous studies[17] have shown that racemization of Asp residues in proteins or peptides occurs via a succinimide intermediate, which results in the formation of D-β-Asp, as shown on Scheme 1. Therefore, the racemization rate of Asp depends on the rate of the succinimide formation; the rate is increased when the size of the amino acid following Asp, that is, at its carboxyl side, is small. In this manner, the amino acids following Asp58 and 151 in alpha A-crystallin being Ser59 and Ala152, the racemization of Asp via succinimide occurs easily. By contrast, the amino acids following Asp36 and 62 in alpha B-crystallin, that is, Leu37 and Thr63, have a large steric hindrance and should impede the succinimide formation and thus the racemiza-

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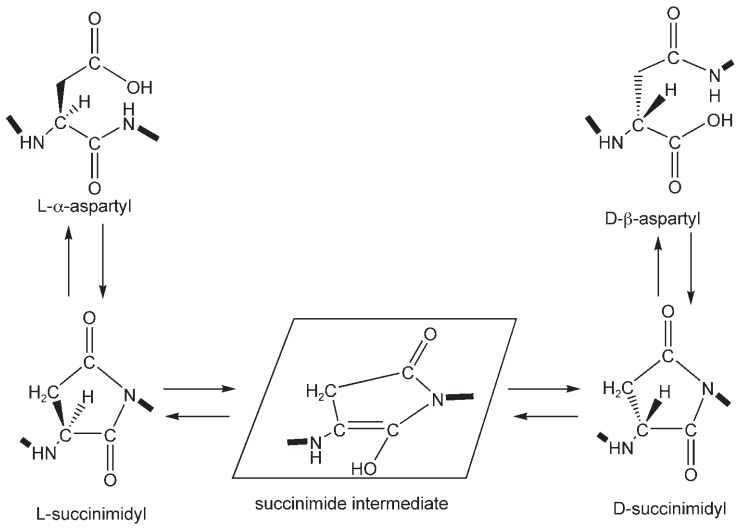

Scheme 1. Reaction pathways for spontaneous stereoinversion and isomerization of aspartyl residues in protein via a succinimide intermediate.

tion, which is not the case. This result suggests that the racemization rate of Asp not only depends on the steric hindrance of the amino acids next to Asp but also on the geometry of the chain hosting the Asp residues.[15] We have recently shown that racemization of Asp in model peptides of elastin may also depend on other factors than the primary structure.[18]

However, the influence of the chain conformation on the formation of D-Asp in peptides or protein has never been studied. In order to establish such a relationship, we examined the kinetics of the racemization rates in the peptides with α-helix and β-sheet structures.

Brack and co-workers[19,20] have reported that peptides with Asp-Leu repeats adopt a β-sheet structure in the presence of metal salts and that, under the same conditions, peptides with Leu-Asp-Asp-Leu repeats adopt an α-helix. In the following we will analyze the kinetics of Asp racemization in such peptides and propose that the racemization of Asp in peptides depends on the secondary structure.

Abstract in French: *Les acides aminés non biologiques* D-*aspartyl (*D-*Asp) ont été trouvés dans les protéines de divers tissus âgés. La présence de* D-*Asp a été attribuée à la racémisation de* L-*Asp dans les tissus inertes. Précédemment, nous avons suggéré que la racémisation de* L-*Asp dépendait de la conformation de la chaîne peptidique, sans toutefois avoir examiné la nature de la chaîne peptidique affectant la racémisation. Ici, nous décrivons la cinétique de racémisation de résidus* L-*Asp insérés dans deux peptides modèles, (Asp-Leu)$_{15}$ et (Leu-Asp-Asp-Leu)$_8$-Asp, qui adoptent respectivement une structure en feuillets-β et en hélice-α. L'énergie d'activation de la racémisation de* L-*Asp est de 27.3 kcal mol^{-1} pour (Asp-Leu)$_{15}$ et de 18.4 kcal mol^{-1} pour (Leu-Asp-Asp-Leu)$_8$-Asp. La constante de vitesse de racémisation à 37 °C est de 20.02 × 10^{-2} par an pour (Asp-Leu)$_{15}$ et de 2.14 × 10^{-2} par an pour (Leu-Asp-Asp-Leu)$_8$-Asp. Enfin, le temps nécessaire pour atteindre un rapport* D/L *de 0.99 à 37 °C est de 122.6 ans pour (Asp-Leu)$_{15}$ et de 13.1 ans pour (Leu-Asp-Asp-Leu)$_8$-Asp. Les résultats montrent que les résidus* L-*Asp insérés dans des peptides α-hélicoïdaux sont racémisés plus facilement que les mêmes acides aminés insérés dans des feuillets-β. Ils montrent ainsi que la racémisation des résidus Asp dans une chaîne peptidique dépend étroitement de la conformation du peptide.*

Results

According to Equation (2) (see Experimental Section, kinetic measurements), the rate constants k for the racemization of Asp residues in the two peptides at five different temperatures (50, 60, 70, 80, 90 °C) was estimated as $^1/_2$ of the slope of the linear regression least-squares line. The results of the plot at 80 °C are shown in Figure 1. The straight lines of the peptides (r^2 = 0.96–0.98) indicate that the kinetics of racemization in the model peptides usually represent a first-order reaction.[16,18] There was significant difference in the rate constants between the two peptides at 80 °C. The Asp residue in (Leu-Asp-Asp-Leu)$_8$-Asp was much more susceptible to racemization than that in (Asp-Leu)$_{15}$. Similar experiments were also performed at other temperatures. The rate constants of the two peptides at 50–90 °C are listed in Table 1. The temperature dependence of the rate constants for the two peptides is shown as an Arrhenius plot in Figure 2. The activation energies for the racemization of Asp residues in these peptides were determined from the slope of the plot (the results are listed in Table 2). The activation energy of racemization of the Asp residues in (Leu-Asp-Asp-Leu)$_8$-Asp was 18.4 kcal mol^{-1}; this was significantly lower than that in (Asp-Leu)$_{15}$ where the activation energy was 27.3 kcal mol^{-1}. From the Arrhenius equation,

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

T [°C]	$k \times 10^2$ per day	
	(Asp-Leu)$_{15}$	(Leu-Asp-Asp-Leu)$_8$-Asp
50	0.03	0.175
60	0.145	0.485
70	0.455	0.9
80	1.37	1.985
90	3.37	4.46

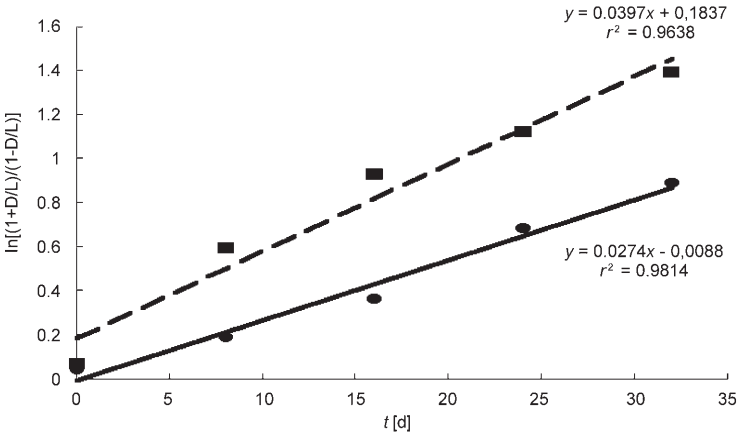

Figure 1. Racemization of Asp residues in (Leu-Asp-Asp-Leu)$_8$-Asp (■) and (Asp-Leu)$_{15}$ (●) peptides at 80 °C.

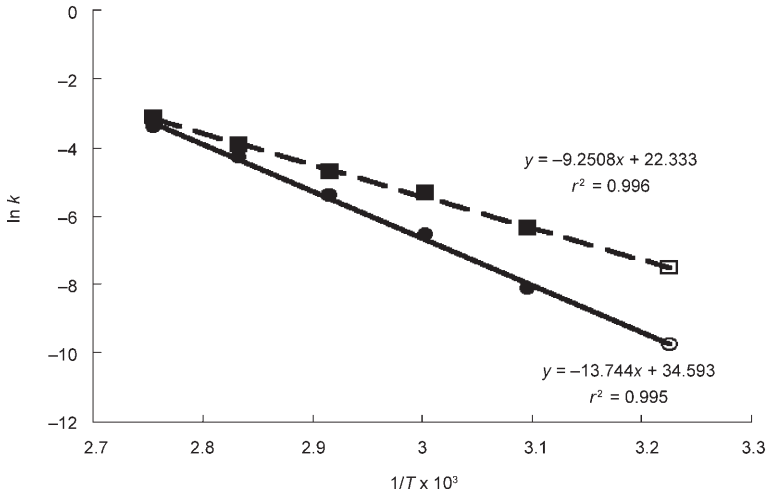

Figure 2. Arrhenius plot of the rate constants derived from incubations of (Leu-Asp-Asp-Leu)$_8$-Asp (■) and (Asp-Leu)$_{15}$ (●) peptides at 50–90 °C. Racemization rates of Asp in the two peptides at 37 °C were estimated by the Arrhenius equation.

Table 2. Racemization of the Asp residues in two peptides.

Sequence	E [kcal mol^{-1}]	$k_{37}\times10^2$ per year	Year$_{37}$
(Asp-Leu)$_{15}$	27.3	2.14	122.6
(Leu-Asp-Asp-Leu)$_8$-Asp	18.4	20.02	13.1

we estimated the rate constants (k_{37}) and the time (t) required to reach an Asp D/L ratio of 0.99 at body temperature (37 °C) by using Equation (2). The results were for Asp residues in (Leu-Asp-Asp-Leu)$_8$-Asp: $k_{37}=20.02\times10^{-2}$ per year and $t=13.1$ years, and for Asp residues in (Asp-Leu)$_{15}$: $k_{37}=2.14\times10^{-2}$ per year and $t=122.6$ years. The results are also summarized in Table 2 and consistently indicate that Asp residues in (Leu-Asp-Asp-Leu)$_8$-Asp are extremely susceptible to racemization compared with those in (Asp-Leu)$_{15}$.

Discussion

In this study, we report on the kinetic analysis of racemization rates of Asp residues in (Leu-Asp-Asp-Leu)$_8$-Asp and in (Asp-Leu)$_{15}$ to investigate the influence of secondary structures on the racemization of Asp residues. The two peptides have almost the same number of Asp residues but adopt different chain conformations. Bertrand and Brack[19] reported that, as a general rule, strict alternation of hydrophilic (hi) and hydrophobic (ho) amino acids induces a β-sheet structure, whereas a tetrapeptide periodicity (-hi-hi-ho-ho-) induces an α-helical conformation. Sequential Asp and Leu peptides adopt a random coil conformation in pure water due to charge repulsion but addition of low concentrations of Zn^{2+} cations, specifically induces an α-helical structure for (Leu-Asp-Asp-Leu)$_8$-Asp and a β-sheet structure for (Asp-Leu)$_{15}$.[20] The present data clearly show that the activation energy of racemization of Asp residues in α-helical (Leu-Asp-Asp-Leu)$_8$-Asp is higher than that in β-sheets of (Asp-Leu)$_{15}$. Furthermore, at 37 °C, the time required for the D/L ratios of Asp residues in the two peptides to approximate 1.0 were estimated at 13 and 123 years, respectively. These results indicate that the Asp residues in α-helix structures are more susceptible to racemize than Asp residues in β-sheet structures. Peptides with β-sheet structures are expected to protect the peptides from racemization because the β-sheet cannot accommodate heterochiral chains containing both L- and D-amino acids. Figure 3 represents a β-sheet structure obtained with a homochiral alternating sequence of acidic and hydrophobic L-amino acids. In such a structure, the side chains point perpendicular to the plane of the sheet and the hydrogen atoms lie almost in the plane. Stereoinversion, that is, the replacement of L enantiomers by D enantiomers, would force the side chains of the D residues to lie in the plane of the sheet, an orientation which has been shown to be impossible.[21] Indeed, when increasing amounts of D enantiomers are introduced in a β-sheet forming all-L peptide, only those segments having seven adjacent residues of identical chirality

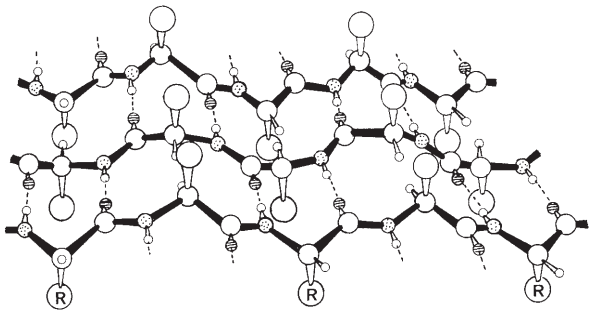

Figure 3. β-Sheet structure with a homochiral sequence of alternating acidic and hydrophobic L-amino acids showing side chains pointing perpendicular to the plane of the sheet, where R stands for aspartic acid side chain ($-CH_2-COO^-$).

can associate to form β-sheet structures surrounded by random coil heterochiral segments.[21] By contrast, it has been demonstrated that peptides with α-helical structures are capable to accommodate both L and D-amino acids.[22]

The present data explain the peculiar behavior of Asp36 in alpha B-crystallin obtained from lens tissue from an 80 year-old human.[11] This amino acid would be highly racemized if it was followed by a small residue such as Gly, Ser or Ala. Actually, it is followed by a bulky amino acid, Leu37, a situation which normally should impede the racemization reaction.[11] In this particular case, Asp36 is inserted in a "racemizing" α-helical sequence.

Conclusion

We examined the kinetics of the Asp racemization in two model peptides, that is, $(Asp\text{-}Leu)_{15}$ and $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp, which form a β-sheet structure and an α-helix structure, respectively. The Asp residues inserted in α-helical peptides were more sensitive to racemization than Asp residues inserted in peptides adopting a β-sheet structure. These results clearly indicate that the racemization rate of Asp residues in peptides depends on the secondary structure of the peptide. Since β-sheet structures are thermostable and stereoselective,[23] the data suggest that these structures might have been a means of collecting and storing homochiral prebiotic peptidic sequences at the same time as protecting them against racemization.

Experimental Section

Peptides and heating experiments: Model peptides $(Asp\text{-}Leu)_{15}$ for β-sheet structure and $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp for α-helix structure obtained as previously published.[15] To solubilize peptides in deionized, distilled water, a stoichiometric amount of NaOH (0.1 M) was added to a suspension of the polypeptide in deionized, distilled water and stirred overnight. These dissolved peptides were neutralized with HCl to pH 7.0 and 0.5 equivalent per Asp residue of zinc chloride (0.01 M) was added to form the secondary structure. The peptide solution was diluted 20 times with deionized, distilled water. To determine the racemization rates of Asp residues in these model peptides, the peptide solution was incubated at 50, 60, 70, 80, 90 °C for between 8 and 32 d.

Determination of the D/L ratio of amino acid in the peptide: Amino acid contamination was prevented by heating all glassware at 500 °C for 4 h. The peptides were dried and then hydrolyzed with gas-phase 6 N HCl in vacuo 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*-phthalaldehyde (OPA) and *N-tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys-OH) 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 acetate buffer (pH 6.0) in 120 min at a flow rate of 0.8 mL min^{-1}, at 30 °C. The D/L values were determined from the mean of duplicate measurements.

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

$$-\mathrm{d[L]}/\mathrm{d}t = k[\mathrm{L}] - k[\mathrm{D}] \quad (1)$$

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 Equation (1) gives:

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

where t is the time of the reaction and t_0 stands for $t = 0$. The t_0 term in Equation (2) is due to racemization induced by acid hydrolysis. We determined the rate constants of racemization of Asp residues in the two peptides at five temperatures (50, 60, 70, 80 and 90 °C) by using Equation (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 Equation (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).

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