D-Amino Acid Biosystem

D-Amino Acid in Elderly Tissues

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All living organisms on earth are composed of L-amino acids and D-sugars. Therefore the presence and function of D-amino acids in living organisms have not been studied. Recently, however, D-aspartic acid (D-Asp) which is a D-amino acid, has been detected in various proteins from human tissue samples such as eye lens, brain, skin, bone, aorta from elderly individuals. It has been explained that the presence of D-Asp is the result of racemization of Asp residues in the protein during the life span, inasmuch as the proteins in these tissues are metabolically inert. The Asp-151 and Asp-58 residues in alpha A-crystallin from elderly human lens are especially stereochemically labile and the D/L ratios of these residues were greater than 1.0. A D/L ratio greater than 1.0 is not defined as racemization, but as the inversion of configuration. This was the first observation that inversion occurred in the configuration of amino acids in vivo during the natural aging process. In this review, we summarize the D-Asp in the various tissues reported by many researchers and describe the mechanism of D-Asp formation in protein. We suggest that a chiral reaction field exists in the native higher order structure of protein which induces the inversion of L-Asp to D-Asp residue.

Key words aging; D-amino acid; homochirality; isomerization; racemization; stress

INTRODUCTION

It has been believed that all living organisms are composed exclusively of L-amino acids, and that D-amino acids, which are the enantiomers of L-amino acids, were eliminated before the emergence of life. Therefore the presence and function of D-amino acids in living organisms except in the cell wall of microorganisms have not been studied. Recently, however, D-aspartic acids (D-Asp) have been detected in various human tissues such as eye lens, brain, skin, bone, aorta, erythrocytes, lung, and ligaments, from elderly individuals. D-Serine (D-Ser) was found in beta-amyloid protein of Alzheimer’s disease (Table 1).5,13) The Asp residue is the most racemizable amino acid among the 20 different amino acids that compose protein. Therefore the presence of D-Asp in aged tissues has been explained as a result of racemization of Asp residues in the protein over time inasmuch as the proteins in such tissues are metabolically inert. The accumulation of D-amino acid in protein will change the higher-order structure of the protein and subsequently will decrease the protein. D-Asp residues are observed in cataract lens, in the brain in Alzheimer disease, and in the aorta in arteriosclerosis from elderly donors. The phenomena suggest a relationship among D-amino acids, stress, and aging. Most studies have documented exclusively the presence of D-Asp in whole tissues. However, the specific sites where D-Asp was identified were in the human lens alpha A-crystallin,2 human lens alpha B-crystallin,3 and a beta-amyloid protein in the brain.5 We have also studied the mechanism of formation of D-Asp in a specific lens protein.14,15)

D-ASP RESIDUES ARE ABUNDANTLY ACCUMULATED IN THE SPECIFIC SITES OF ALPHA A- AND ALPHA B-CRYSTALLINS IN ELDERLY HUMAN THE LENSES

Earlier studies showed that D-Asp accumulated in the proteins of the human lens with age.17) However, because D-Asp was detected in homogenates of lenses, it could not be determined whether all of the aspartic acid in the lens protein was racemized uniformly, or whether particular aspartic acid residues with a greater tendency to racemization exist in specific lens proteins. We predicted that D-Asp residues might be present at some specific sites in some specific lens proteins. First, human lenses were homogenized and the sample was subjected to DEAE chromatography and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, all proteins of the bands observed on SDS-PAGE were obtained by electronic elution and were hydrolyzed and analyzed for the D/L ratio of Asp. It was found that alpha A- and alpha B-crystallin contained D-Asp isomer.16) Alpha A- and alpha B-crystallins are subunits of alpha-crystallin aggregate with a molecular mass of 600—1000 kDa. Human alpha A- and alpha B-crystallin are composed of 173 and 175 amino acids, and have molecular mass of approximately 20 kDa, respectively. The alpha A-crystallin has 15 Asp and 2 Asn residues (Fig. 1) and alpha B- crystallin has 11 Asp and

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Protein</th>
<th>Amino acid</th>
<th>Related disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tooth</td>
<td>Phosphoryn</td>
<td>D-Asp</td>
<td>?</td>
</tr>
<tr>
<td>Bone</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Aorta</td>
<td>Elastin</td>
<td>D-Asp</td>
<td>Arteriosclerosis</td>
</tr>
<tr>
<td>Brain</td>
<td>?</td>
<td>D-Asp</td>
<td>?</td>
</tr>
<tr>
<td>Brain</td>
<td>β-Amyloid</td>
<td>D-Asp, D-Ser</td>
<td>Alzheimers</td>
</tr>
<tr>
<td>Lens</td>
<td>αA-Crystallin</td>
<td>D-Asp</td>
<td>Cataract</td>
</tr>
<tr>
<td>Lens</td>
<td>αB-Crystallin</td>
<td>D-Asp</td>
<td>Cataract</td>
</tr>
<tr>
<td>Skin</td>
<td>?</td>
<td>D-Asp</td>
<td>Elastosis</td>
</tr>
<tr>
<td>Lung</td>
<td>Elastin</td>
<td>D-Asp</td>
<td>?</td>
</tr>
<tr>
<td>Bone</td>
<td>Osteocalcin</td>
<td>D-Asp</td>
<td>?</td>
</tr>
</tbody>
</table>

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Table 1. The Presence of D-Aspartic Acid in Protein from Various Tissues

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2 Asn residues (Fig 2). To determine the D/L ratio of individual Asp/Asn residues in alpha A- and alpha B-crystallins were digested with trypsin and the resulting peptides were separated by reverse-phase HPLC. After the resulting peptides were identified by mass and sequence analysis, the peptides were hydrolyzed and derivatized with o-phthaldialdehyde (OPA) and N-tert-butyloxycarbonyl-L-cysteine (Boc-L-Cys). The D/L ratio of amino acids was determined by reverse-phase HPLC. We found that Asp-58 and Asp-151 residues in aged human alpha A-crystallin were highly inverted to D-isomers (D/L ratio of Asp-58, 3.1; Asp-151, 5.7) and Asp-36 and Asp-62 were highly racemized (D/L ratio of Asp-36, 0.92; Asp-62, 0.57) (Table 2). Other Asp residues in the proteins were not racemized. As shown in Table 3, site-specific racemization of the Asp-151 residue of alpha A-crystallin was observed in cattle, mice, and rats despite the difference in species. The results show that Asp-151 residue in alpha A-crystallin is stereochemically labile. Especially in the case of human alpha A-crystallin, the D/L ratio of Asp-151 residue is greater than 1.0. Since racemization is defined as a reversible first-order reaction, when the D/L ratio reaches 1.0, the racemization is in equilibrium. Therefore, Asp-151 in human alpha A-crystallin was not simply racemized but stereoinverted to the D-isomer. This is the first observation of the inversion of amino acid residues in protein. Why is D-Asp predominantly formed at the 58th and 151th specific sites in alpha A-crystallin? We observed that the D-Asp formation was accompanied with isomerization of the normal alpha-linkage to an abnormal beta-linkage of the peptide bond in alpha A- and alpha B-crystallins. The next describes the mechanism of D-Asp and beta-Asp formation at specific sites in proteins.

**MECHANISM OF D-ASP AND BETA-ASP FORMATION IN PROTEIN**

In alpha A- and alpha B-crystallin, D-Asp and beta-Asp formation occur simultaneously. This result indicates that D-Asp formation in protein occurs via a succinimide intermediate. As shown in Fig. 3, the simultaneous formation of beta- and D-Asp residues in the protein could be explained as follows: i) When the carbonyl group of the side chain of the L-alpha-aspartyl residue is attacked by the nitrogen of the amino acid residue following the Asp residue, L-succinimide is formed by intramolecular cyclization. ii) L-succinimide may be converted to D-succinimide through an intermediate [I] that has the prochiral alpha-carbon in the plane of the ring. iii) Protonation of the intermediate [I] would occur with equal probability from the upper or lower side of the plane in an ordinary peptide or protein (racemization). iv) D- and L-succinimide are hydrolyzed at either side of their two carbonyl groups, yielding both beta- and alpha-Asp residues, respectively. The rate of succinimide formation is expected to depend on the neighboring residue of the Asp residue. When the neighboring amino acid of the Asp residue has a small side chain, such as glycine, alanine, or serine, the formation of succinimide occurs easily because there is no steric hindrance. We synthesized three peptides corresponding to fragments of alpha A-crystallin (T18, T6, and T10 in Fig. 1) and carried out kinetic studies of the racemization of Asp in these peptides. The results indicated that the Asp residue in peptide T18 (Asp-151) was the most susceptible to racemization, while the Asp residue in peptide T10 (Asp-84) was the least susceptible. The racemization rate of Asp decreases according to the level of steric hindrance of the carboxyl side chain of the Asp residue. This order of susceptibility is consistent with that of native human alpha A-crystallin.

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**Table 2. Stereoinversion and Isomerization of αA, αB-Crystallin from Aged Human Lens**

<table>
<thead>
<tr>
<th>Crystallin</th>
<th>Asp</th>
<th>D/L of Asp</th>
<th>Next amino acid</th>
<th>Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>αA</td>
<td>Asp-58</td>
<td>3.10</td>
<td>Ser-59</td>
<td>Beta</td>
</tr>
<tr>
<td>αA</td>
<td>Asp-151</td>
<td>5.70</td>
<td>Ala-152</td>
<td>Beta</td>
</tr>
<tr>
<td>αB</td>
<td>Asp-36</td>
<td>0.92</td>
<td>Leu-37</td>
<td>Beta</td>
</tr>
<tr>
<td>αB</td>
<td>Asp-62</td>
<td>0.57</td>
<td>Thr-63</td>
<td>Beta</td>
</tr>
<tr>
<td>αB</td>
<td>Asp-140</td>
<td>0.01</td>
<td>Gly-141</td>
<td>Alpha</td>
</tr>
<tr>
<td>αB</td>
<td>Asn-143</td>
<td>0.02</td>
<td>Gly-146</td>
<td>Alpha</td>
</tr>
</tbody>
</table>

**Table 3. A Part of Amino Acid Sequences of around Asp-151 in the αA-Crystallins Obtained from Various Species**

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequences</th>
<th>D/L of Asp151</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>IQTGLD151ATHAER</td>
<td>5.7</td>
</tr>
<tr>
<td>Cattle</td>
<td>IPSGVD151AGHSER</td>
<td>0.5</td>
</tr>
<tr>
<td>Rat</td>
<td>VQSGLD151AGHSER</td>
<td>0.2</td>
</tr>
</tbody>
</table>

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Fig. 1. The Primary Structure of Human αA-Crystallin

αA-Crystallin was digested with trypsin.

Fig. 2. The Primary Structure of Human αB-Crystallin

αB-Crystallin was digested with trypsin.
However, a very important difference concerning D-Asp formation in the model peptide and in the native protein is that inversion (D/L ratio) of the L-Asp residues occurred in the native protein but not in the short model peptide. In the native protein, we found that the D/L ratios of the Asp-151 and Asp-58 residues in the 80-year-old human alpha A-crystallin were much higher than 1.0 (D/L ratio: Asp-151, 5.7; Asp-58, 3.1) (Table 2). Since racemization is defined as a reversible first-order reaction, when the D/L ratio reaches 1.0, the racemization is at equilibrium. Thus the D/L ratios that were greater than 1.0 would not be defined as racemization, but as the inversion of L-Asp to its D-isomer. In the short model peptides, the racemization of the Asp residue proceeded normally but the stereoinversion of the Asp residue did not occur. This result suggests that the area surrounding the Asp-151 and Asp-58 residues might form a chiral environment that allows the inversion of L-Asp residues to D-Asp residues in alpha A-crystallin.

POSSIBLE CHIRAL FIELD INDUCING THE STEREOINVERSION IS PRESENT IN ALPHA A-CRYSTALLIN ITSELF

As described in the previous section, the D/L ratios of the Asp residues in the model peptide did not exceed 1.0. In other words, D-Asp formation in unstructured peptides proceeded according to the theory of racemization. This suggests that a chiral reaction field exists in the native higher-order structure of human alpha A-crystallin which induces the inversion of L-Asp to D-Asp residues. If such a chiral reaction field exists in the native structure of the protein, we speculated that the rate of inversion of Asp-151 should decrease in unfolded alpha A-crystallin due to the disappearance of the chiral environment. Conversely, we reasoned that upon the unfolding of alpha A-crystallin from an aged human lens, which is enriched for the D-form at Asp-151, the D-form might be converted to the L-form, implying that a chiral field exists in the folded protein which initially induces the specific stereoinversion. Therefore we unfolded the alpha A-crystallin obtained from the aged human lens to a random coil structure by exposure to 6M urea or heating and measured the D/L ratio of the Asp-151 residue. In the native alpha A-crystallin, the D/L ratio of the beta-linked Asp-151 residue was 5.7, while the D/L ratio of the beta-linked Asp-151 residues in unfolded alpha A-crystallin approached approximately 1.0 (Table 4). These results strongly indicated that a chiral reaction field exists in the native higher-order structure of human alpha A-crystallin which induces the inversion of L-Asp to D-Asp residues. As shown in Fig. 3, the protonation of the intermediate [I] does not occur with equal probability from the upper or lower side of the plane. Rather, a sterically hindering structure composed of the native higher-order structure of alpha A-crystallin which induces the protonation of the intermediate [I] (Fig. 3, shaded parts), resulting in the protonation of the intermediate [I] from the upper side of the plane. This, in turn, causes the configuration to be inverted to the D-form (Fig. 3). Conversely, when the native higher-order structure is destroyed...
by urea or heating, protonation to the intermediate [I] could occur with an equal probability from either side of the plane, showing the d/l ratio of Asp = 1.0. This result indicates that the structural field that surrounds the Asp-151 residue induces the formation of d-beta-Asp, and that this field is composed of the higher-order conformation of alpha A-crystallin. Where is the chiral field that predominantly induces d-Asp in the part of alpha A-crystallin?

Recently, we have identified truncated peptides formed by a posttranslational cleavage event between His-154 and Ala-155 residues in aged alpha A-crystallin protein. The d/l ratio of Asp-151 in the truncated alpha A-crystallin 1—154 was 0.3 (Table 4). Unlike the native full-length (1—173) alpha A-crystallin, the stereoinversion of Asp-151 was not observed in the cleaved 1—154 polypeptide from alpha A-crystallin. Considered together with the above results, the chiral reaction field of native human alpha A-crystallin might consist of the region from Ala-155 to the C-terminus residue along with other residues close to the C-terminus.

The stereoinversion and isomerization of the Asp residues occur simultaneously. Therefore four isomers, which are normal L-alpha-Asp, biologically uncommon L-beta-Asp, D-alpha-Asp, and D-beta-Asp, are formed in alpha A-crystallins. Recently, we have measured the ratio of the four isomers of Asp-151 in alpha A-crystallins obtained from human lenses of the newborn, 30-, 60-, and 80-year-old range lenses. The isomers increased with age, and the total amount of the three isomers was greater than that of normal L-alpha-Asp in the alpha A-crystallin in the human lenses of the 80-year-old range. The drastic changes started at birth, with about 45% of normal L-alpha-Asp-151 lost by the 30-year-old.21)

D-ASPARTIC ACID IN THE BRAIN

As shown in Table 1, the presence of d-Asp residues in various proteins has been reported; however, it is not clear which protein in tooth, bone, aorta, skin, and lung contains d-Asp. As described in the previous sections, the localization of d-Asp in the lens and the mechanism of d-Asp formation have been investigated in detail. Localization of d-amino acids in tissues other than lens was reported in the beta-amylloid protein of brains obtained from patients with Alzheimer’s disease. Beta-amylloid protein is composed of 42 amino acids. Asp-1, Asp-7,5) and Ser-2613) residues were specifically racemized. However, the amount of d-Asp in beta-amylloid protein was lower than that in lens protein. As described in the previous section, 50% of the Asp-151 of alpha A-crystallin comprised beta-Asp and the d/l ratio of beta-Asp was 5.7; 40% of Asp-58 was beta-Asp of alpha A-crystallin and the d/l ratio of beta-Asp-58 was 3.1, while in beta-amylloid protein, 18% of Asp-7 was beta-Asp and the d/l ratio of beta-Asp was about 1.0. The major modification of Asp-7 in beta-amylloid protein was isomerization from alpha-Asp to beta-Asp (60%) and the beta-Asp-7 did not racemize (d/l ratio, 0.1). The modification of Asp-1 is also a situation similar to Asp-7, that is, a major component was l-beta-Asp, although the exact amount could not be calculated accurately because of incomplete separation of Asp-7-containing peptide by reverse-phase HPLC.

It is well known that the deposition of beta-amylloid protein in the brain causes Alzheimer’s disease. Roher et al. suggested that the beta-amylloid protein is aggregated by the racemization of these amino acids and accumulated in the brain.5) To elucidate the effect of racemization on the aggregation properties of beta-amylloid protein, Tomiyama et al. synthesized four beta-amylloid protein analogues in which d-Asp was substituted for L-Asp residues: normal beta-amylloid protein 1-35; [D-Asp-7] beta-amylloid protein 1-35; [D-Asp-23] beta-amylloid protein 1-35; and [D-Asp-7,23] beta-amylloid protein 1-35. The results indicated that racemization of Asp-23 accelerated peptide aggregation and fibril formation, while racemization of Asp-7 slowed down the aggregation.22)

D-ASPARTIC ACID IN SKIN

Recently, we have found d-beta-Asp-containing protein in the elastic fibers of skin from elderly donors.7) The formation of d-beta-Asp in protein is related not only to aging but also to UV irradiation. Figure 4 clearly indicates that d-beta-Asp-containing protein was observed only in the facial skin of elderly donors (Figs. 4b, c), but was not detected in the facial skin of young donors (Fig. 4a), nor in sun-protected skin (buttoks) of elderly donors (Fig. 4d). These results suggest that d-beta-Asp-containing protein accumulation is accelerated by exposure to sunlight and that the exceptional protein accumulates in aged skin during UV-related aging.

D-ASPARTIC ACID IN OTHER TISSUES

In the 1970s, Helfman and Bada reported that d-aspartic acid accumulated in tooth enamel23) and dentine,24) and that the amount increased with aging. The rate constant for the racemization reaction of aspartic acid in human tooth enamel was found to be 8.29×10^{-4} yr^{-1}. This rate constant suggests that in any protein with a long in vivo lifetime, d-aspartic acid will accumulate with age (about 8% of total aspartic acid in enamel will be the d-enantiomer after 60 years).23) Subsequently, Ohtani have reported on Asp racemization in teeth in detail.25) Most recently, Masuda et al. have suggested that d-Asp containing protein in teeth may be phosphophoryn.26)

In other tissues, d-Asp-containing proteins were observed in osteocalcin of bone,27) in elastin of ligament,28) and aorta.9) Recently, Cloos and Fledelius29) have shown that Asp-1211 of the alpha 1 C-terminal telopeptide of type I collagen (AHDDGGR) in urine from bone resorption was racemized and isomerized.28)

CONCLUSIONS

Most researchers have considered that l-amino acids in
proteins could not change to D-isomers under the physical conditions in the living body. However, as shown in Table 1, the number of reports concerning the presence of D-Asp residues in various proteins has rapidly increased. The localization of D-Asp in these proteins will undoubtedly be clarified by further research. The present study indicates that D-beta-Asp formation occurs much more easily in proteins than was thought. Recent improvements in analytical techniques now enable accurate analysis of amino acid enantiomers at the picomole level. Therefore we are able to identify a very small quantity of D-aspartic acid at specific sites in lens proteins consisting almost entirely of L-amino acids, a task similar to looking for a needle in a haystack. D-amino acids in proteins can be interpreted as molecular markers of aging.

REFERENCES