CMLS, Cell. Mol. Life Sci. 62 (2005) 199–205
1420-682X/05/020199-7
DOI 10.1007/s00018-004-4412-5

CMLS Cellular and Molecular Life Sciences

Research Article

Evidence for a novel racemization process of an asparaginyl residue in mouse lysozyme under physiological conditions

K. Ueno [a], T. Ueda [b,*], K. Sakai [b], Y. Abe [b,c], N. Hamasaki [c], M. Okamoto [a] and T. Imoto [d]

[a] Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8581 (Japan)
[b] Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582 (Japan), Fax: +81 92 642 6667, e-mail: ueda@phar.kyushu-u.ac.jp
[c] Department of Clinical Chemistry and Laboratory Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582 (Japan)
[d] Department of Applied Microbial Technology, Faculty of Engineering, Sojo University, Kumamoto 860-0082 (Japan)

Received 16 September 2004; received aftrer revision 26 October 2004; accepted 12 November 2004

Abstract. We examined chemical reactions in mouse lysozyme after incubation under physiological conditions (pH 7 and 37 °C). After incubation for 8 weeks, racemization was observed specifically at Asn127 among the 19 Asp/Asn residues in mouse lysozyme. Furthermore, analysis of the primary structure showed that the racemized residue was not Asp, but Asn, which demonstrates that deamidation and isomerization did not occur. These results mean that this racemization occurs without forming a succinimide intermediate. This is the first example of D-asparaginyl formation in a protein occurring during the racemization process under physiological conditions.

Key words. Deterioration; mouse lysozyme; protein aging; racemization.

Many protein and peptide pharmaceuticals have been developed and are utilized in medical practice, including erythropoietin, interferon and granulocyte colony-stimulating factor. Because deterioration during the preservation of protein and peptide pharmaceuticals can lead to autoimmune responses [1] as well as functional disorders, we must elucidate the mechanisms of protein deterioration and propose methods to prevent it [2–4]. Protein deterioration is reportedly caused by spontaneous reactions, enzymatic reactions or environmental stresses such as high temperature, salt and ultraviolet irradiation [5]. Notable examples of spontaneous modification are pyroglutamylation of Glu/Gln residues, deamidation of Asn/Gln residues, isomerization of Asp/Asn residues cleavage of the peptide bond in Asp/Asn-Pro sequences [6] and racemization of other amino acids.

Proteins in living tissues are comprised exclusively of L-amino acids; however, Asp/Asn residues in peptides and proteins are prone to racemize to D-Asp residues through deamidation and isomerization [7–13]. There are numerous examples of proteins that exhibit racemization in aged human tissues: dentin in teeth, membrane proteins in erythrocytes, elastin in aortic tissue, myelin basic protein in the brain, beta amyloid peptides in Alzheimer's disease plaques and αA-crystallin in the eye lens [7–15]. Racemization of αA-crystallin in the eye lens has also been reported to be site specific [16]. Considering the relationship between racemization and protein structure, we decided to investigate site-specific racemization of Asp/Asn residues.

Mouse lysozyme (ML) is secreted by macrophages, monocytes and polymorphonuclear leukocytes, and is widely distributed in body fluids and tissues [17, 18]. ML, a globular protein, is composed of 130 amino acid residues and contains all the usual 20 amino acids. The

* Corresponding author.

three-dimensional structure has already been analyzed using nuclear magnetic resonance (NMR) spectroscopy [19]. Therefore, it is a suitable model to study protein deterioration under physiological conditions. Moreover, ML is a self protein for mice, and the deteriorated ML is an ideal protein for immunological studies. Here, we examined the deterioration of ML and found that Asn127 in ML was preferentially racemized after 8 weeks of incubation under physiological conditions.

Materials and methods

Materials

Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden). CM-Toyopearl 650S was obtained from Tohsoh (Tokyo, Japan). All other reagents were of analytical grade for biochemical use. Amino acid analyses were performed on a Hitachi L-8500 amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis of protein peptide samples in 6 N HCl under a vacuum at 110 °C for 20 h. Mass analyses were performed using a matrix-assisted laser desorption-time-of-flight mass spectrometer (MALDI-TOF/MS) Voyager (Applied Biosystems, Framingham, Mass.).

Preparation of ML

ML was obtained using a *Pichia pastoris* transformant according to our previous method [20]. The protein was purified by cation exchange (CM-Toyopearl 650S) chromatography.

Preparation of peptides

ML was reduced with 2-mercaptoethanol (2-ME) and S-alkylated with 3-bromopropyl trimethylammonium bromide (TAP-Br) according to a previous method [21]. Reduced S-alkylated ML was dissolved in 5 ml 0.1 M phosphate buffer (pH 8). To this solution, N-tosyl-L-phenylalanine chloromethylketone (TPCK)-trypsin (1% lysozyme by weight) was added and the solution was incubated for 2 h at 40 °C. The digested sample was separated by RP-HPLC using a C18 column (Mightysil RP-18 GP, 250 × 4.6 mm; Kanto Chemical, Osaka, Japan) with a linear gradient of 0–50% acetonitrile containing 0.1% HCl, at a flow rate 0.4 ml/min. Peptides were detected based on absorbance at 210 nm.

Analysis of racemization

Analysis of racemization at Asp and Asn residues in incubated ML was carried out using the method of Aswad [22] with slight modification. In brief, 4 mg o-phthaldialdyde (OPA) was dissolved in 300 µl methanol. To this solution was then added 250 µl 0.4 M sodium borate (pH 9.4), 390 µl distilled water and 60 µl 1.0 M N-acetyl-L-cysteine (NAC) solution at pH 5.5 (OPA-NAC solution). The OPA-NAC solution was stored at 4 °C until use. ML or peptides were hydrolyzed in 6 N HCl under a vacuum at 110 °C for 4 h. The hydrolysate was freeze-dried, and distilled water was added to the residue (Asp solution). Twenty microliters of the Asp solution was mixed with 10 µl OPA-NAC solution and this was left to stand for 3 min before 470 µl 0.05 M sodium acetate (pH 5.2) was added to the solution. The sample solution was centrifuged at 15,000 g for 1 min, and 20 µl of supernatant was subjected to RP-HPLC on a Vydac Protein & Peptide C18 (250 × 4.6 mm; Vydac, Columbia, Md.). The column was isocratically eluted with 0.05 M sodium acetate (pH 5.8) containing 4% acetonitrile at a flow rate of 0.4 ml/min. Column effluents were monitored based on absorbance at 350 nm.

Identification of N-terminal residue in T18 tryptic peptide

After reduced S-alkylated ML had been digested by TPCK-trypsin, the digested sample was separated by RP-HPLC as described above. The obtained tryptic peptide T18 was subjected to gas-phase protein sequencer N-terminal sequence analyses performed using an ABI 473A peptide sequencer (Applied Biosystems, Foster City, Calif.).

Preparation and separation of epimers in T18 tryptic peptides

Three types of peptide, in which the Asn127 residues were L-Asn, D-Asn and L-Asp, were synthesized as 11-mer peptides (D-L-S-Q-Y-I-R-N-C-G-V; N: one of the studied residues) using a peptide synthesizer (Pioneer peptide synthesizer; Applied Biosystems) [23–25]. Reduced and S-carboxymethylated peptides were digested with TPCK-trypsin as describe above, resulting in authentic tetramer peptides (L-N-carboxymethyl C-G-V, D-N-carboxymethyl C-G-V and L-D-carboxymethyl C-G-V). Confirmation of the purities of authentic peptides was made by amino acid analysis. Separation of each of the T18 tryptic peptide epimers was performed by RP-HPLC using a C18 column (Shiseido Capcellpack C18, 250 × 4.6 mm; Shiseido, Tokyo, Japan). The column was isocratically eluted with 3% acetonitrile containing 0.1% HCl at a flow rate 0.5 ml/min.

Results

Analyses of racemization of Asp/Asn residues in ML

In this experiment, we used ML obtained from *P. pastoris* transformants as described previously by our group [20]. We investigated racemization of Asp/Asn residues in ML after incubating at pH 7 and 37 °C in a glass tube. After incubating for 5 or 8 weeks, ML was partially hydrolyzed for 4 h to produce free aspartic acid, and we analyzed the racemization of the resulting aspartic acid. The D-Asp ratio in ML increased slightly but significantly with incu-

Table 1. Formation of D-Asp/Asn in ML after incubation in 0.05 M PBS at pH 7 and 37 °C.

	Incubation time		
	0 h	5 weeks	8 weeks
D-Asp (%)	1.95	3.75	4.35
SD	± 0.07	± 0.35	± 0.07

Data include all 19 Asp/Asn residues. Values represent the average and SD (n = 2).

bation when compared with that in ML without incubation (table 1). The D-Asp ratio in ML after incubation for 8 weeks was 2.4% after a correction of 1.95% for the racemization occurring during partial hydrolysis.

Specific racemization occurred at Asn127 in ML

Figure 1 shows the primary structure of ML. ML has 11 Asn and 8 Asp residues. To analyze the racemized Asp/Asn residues after incubating for 8 weeks, we performed tryptic digestion of reduced S-alkylated ML. Individual tryptic peptides were identified based on amino acid composition and mass analysis. The RP-HPLC elution patterns of tryptic peptides from ML incubated for 8 weeks and from ML without incubation are shown in figure 2A and B, respectively. Similar patterns could be seen irrespective of incubation. We examined the racemization at Asp/Asn residues in individual tryptic peptides. As shown in the left column of table 2, in ML incubated for 8 weeks, the D-Asp ratio of tryptic peptide T18 containing an Asn127 residue was extremely high (39.6%), whereas those of other peptides were about 2%. In ML without incubation, the D-Asp ratio of tryptic peptide T18 was 1.85%. These results indicated that, among all the Asp/Asn residues of ML, Asn127 was the only residue that racemized after 8 weeks of incubation under physiological conditions.

Confirmation of racemization at Asn127 in ML

To confirm the racemization at Asn127, we established a separation system of tryptic peptide T18 depending on

T1+2 :K^{1}-V-Y-E-R^{5}
T3 :C^{6}-E-F-A-R^{10}
T4+5 :T^{11}-L-K-R^{14}
T6 :N^{15}-G-M-A-G-Y-Y-G-V-S-L-A-**D**^{27}-W-V-C-L-A-Q-H-E-S-N^{37}-Y-**N**^{39} -T-R^{41}
T7 :A^{42}-T-**N**^{44}-Y-**N**^{46}-R^{47}
T8 :G^{48}-**D**^{49}-Q-S-T-**D**^{53}-Y-G-I-F-Q-I-**N**^{60}-S-R^{62}
T9 :Y^{63}-W-C-**N**^{66}-**D**^{67}-G-K^{69}
T10 :T^{70}-P-R^{72}
T11+12 :A^{73}-V-**N**^{75}-A-C-G-I-**N**^{80}-C-S-A-L-L-Q-**D**^{87}-**D**^{88}-I-T-A-A-I-Q-C-A-K-R^{98}
T13 :V^{99}-V-R^{101}
T14 :**D**^{102}-P-Q-G-I-R^{107}
T15 :A^{108}-W-V-A-W-R^{113}
T16 :A^{114}-H-C-Q-**N**^{118}-R^{119}
T17 :**D**^{120}-L-S-Q-Y-I-R^{126}
T18 :**N**^{127}-C-G-V^{130}

Figure 1. Primary structure of ML. T represents tryptic peptides, and peptide numbering is from the N-terminal peptide. Asparagine and aspartic acid residues are shown in bold.

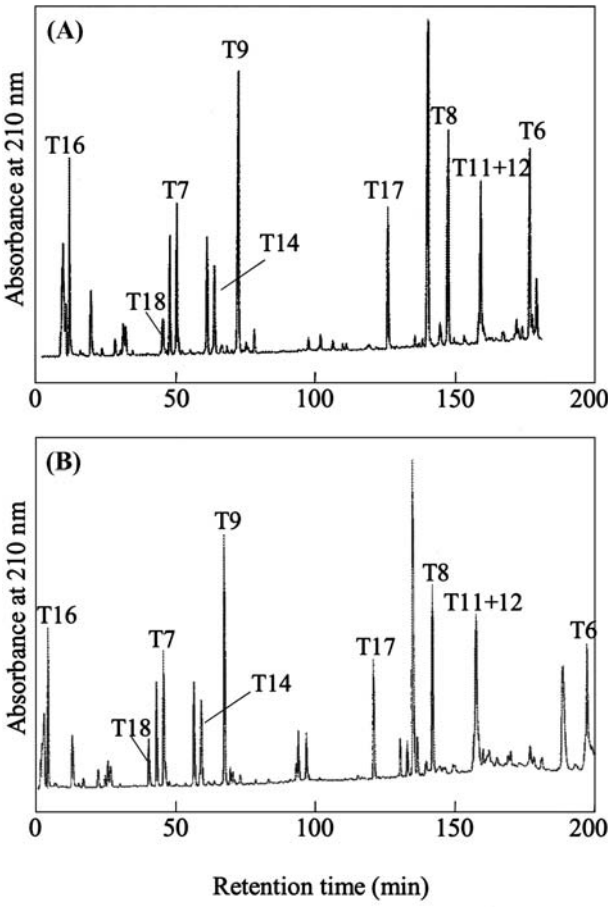

Figure 2. RP-HPLC patterns of tryptic peptides from reduced S-alkylated ML. ML was incubated for 8 weeks (*A*) or 0 h (*B*). Peptides were separated by RP-HPLC using a C18 column (RP-18 GP, 4.6 × 250 mm, Mightysil) with a linear gradient of 0–50% acetonitrile containing 0.1% HCl, at a flow rate of 0.4 ml/min. Peptides were detected by measuring their absorbance at 210 nm. Each peak was assigned based on amino acid composition and mass analysis. Tryptic peptides containing Asp/Asn residues are denoted.

the epimerism at Asn127. S-alkylated T18 peptides [Asn-carboxymethyl (CM)Cys-Gly-Val] were analyzed by RP-HPLC (see Materials and methods). Figure 3A shows that the epimers of the synthesized peptide could be clearly separated using RP-HPLC. When tryptic peptide T18 from ML after 8 weeks of incubation was analyzed using this system, we found two peptide peaks (fig. 3B). The results of co-chromatography of the tryptic peptide T18 and the synthesized peptides (fig. 3C–E) suggested that the tryptic peptide T18 from ML after 8 weeks of incubation contained D-Asn-(CM)Cys-Gly-Val. The former peak intensity in figure 3B indicated that 30–40% of the tryptic peptide was D-Asn-(CM)Cys-Gly-Val, which was consistent with the result in table 2.

Furthermore, we examined the N-terminal amino acids of the tryptic peptide T18 using an amino acid sequencer. As shown in figure 4, we detected mainly PTH-Asn. From the above results, we concluded that racemization oc-

Table 2. Formation of D-Asp/Asn in tryptic peptides containing Asp/Asn residues derived from ML after incubation for 8 weeks at pH 7 and 37 °C.

Peptide	D-Asp (%)	Number of Asp/Asn residues	Total amount of D-Asp [a]
T6	2.9	4	3.8
T7	2.2	2	0.5
T8	2.3	3	1.1
T9	2.4	2	1.0
T11 + 12	2.5	4	2.2
T14	1.9	1	– 0.1
T16	2.4	1	0.5
T17	2.0	1	0.1
T18	39.6	1	37.7
0 h [b] (ML)	1.95	19	

[a] Total amount of D-Asp (%) = [(D-Asp) – 1.95] × corresponding number of Asp/Asn residues.
[b] See table 1.

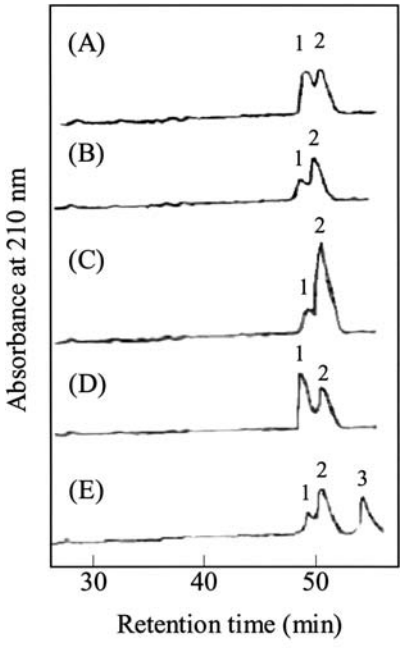

Figure 3. RP-HPLC patterns of tryptic peptides [Asn-(CM)Cys-Gly-Val]. Peptides were separated by RP-HPLC using a C18 column (Shiseido Capcellpack C18, 250 × 4.6 mm) with isocratic elution buffer (3% acetonitrile and 0.1% HCl) at a flow rate of 0.5 ml/min: mixture of the synthetic peptide [L-Asn-(CM)Cys-Gly-Val] and the synthetic peptide [D-Asn-(CM)Cys-Gly-Val] (*A*); tryptic peptide T18 derived from ML after incubation for 8 weeks (*B*); mixture of the tryptic peptide T18 derived from ML after incubation for 8 weeks and the synthetic peptide [L-Asn-(CM)Cys-Gly-Val] (*C*); mixture of the tryptic peptide T18 derived from ML after incubation for 8 weeks and the synthetic peptide [D-Asn-(CM)Cys-Gly-Val] (*D*); mixture of the tryptic peptide T18 derived from ML after incubation for 8 weeks and the synthetic peptide [L-Asp-(CM)Cys-Gly-Val] (*E*). Peaks 1, 2 and 3 correspond to the peptides D-Asn-(CM)Cys-Gly-Val, L-Asn-(CM)Cys-Gly-Val and L-Asp-(CM)Cys-Gly-Val, respectively.

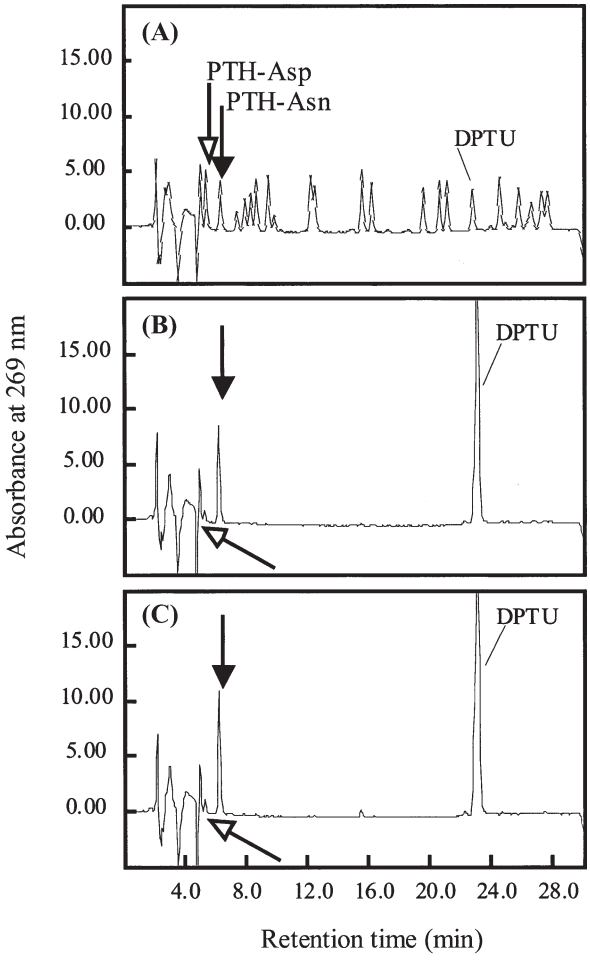

Figure 4. N-terminal analyses of the tryptic peptide T18 by a gas-phase protein sequencer. Closed arrows and open arrows indicate the retention time of phenyl thio hydantoin (PTH)-Asn and PTH-Asp, respectively; standard cycle (*A*); first cycle for mouse lysozyme incubated for 8 weeks (*B*) and 0 h (*C*).

curred at Asn127 in ML after 8 weeks of incubation under physiological conditions.

Discussion

As shown in table 1, 2.4 (= 4.35 – 1.95)% of Asp/Asn residues in ML were racemized after incubation for 8 weeks under physiological conditions, which means that 2.4% of D-Asp/Asn residues formed after 8 weeks of incubation. To determine the racemization site, we examined the formation of D-Asp/Asn in tryptic peptides derived from ML after 8 weeks of incubation. As shown in table 2, we found that D-Asn in the tryptic peptide T18 was preferentially racemized. Based on the total amount of D-Asp (table 2), we can calculate the average D-Asp ratio in ML by adding up the values the right column in table 2 and dividing by 19 (total number of Asp/Asn residues in ML) to give 2.45%. This value was similar to Asp ratio (2.4%) derived from ML after 8 weeks of incubation, suggesting that specific racemization occurred at Asn127 in ML after 8 weeks of incubation. Moreover, from a comparison of the retention time of the tryptic peptide T18 derived from ML after 8 weeks of incubation with those of the authentic peptides on RP-HPLC, and analysis of the N-terminal amino acids of the tryptic peptide T18 derived from ML after 8 weeks of incubation, we concluded that L-Asn at position 127 in ML was preferentially converted to D-Asn after 8 weeks of incubation under physiological conditions.

The mechanism of racemization at Asn residues is considered to involve the formation of an aminosuccinimidyl (Asu) peptide intermediate which is an obligatory first step in these reactions (see fig. 5) [26–29]. General base catalysis by nucleophilic attack, in which the lone pairs of electrons on the nitrogen of the carboxyl-side backbone attack the side chain carbonyl group, causes the formation of intramolecular succinimide, and the subsequent hydrolyzation of succinimide leads to D/L-aspartyl and D/L-isoaspartyl residues. The formation of succinimide thus plays an important role in the racemization of Asp/Asn residues [30]. However, as shown above, after 8 weeks of incubation of ML under physiological conditions, we found that the racemization occurred at Asn127 in ML, indicating that the racemization did not form by way of the succimide intermediate. This is the first example of racemization at an Asn residue in a protein occurring without formation of the succinimide intermediate.

In the mechanism with the succinimide intermediate, specific racemization in a protein depends on the primary structure, the secondary structure, the higher order structure [27, 28, 31–34] and the steric disorder of the neighboring C terminal residue [16]. Therefore, since the C terminus residue of Asn127 is Cys128 in ML, which forms a disulfide bond with Cys6, the succinimide formation mechanism cannot explain the specific racemization at Asn127 in ML during incubation of ML under physiological conditions. The reactive C-terminal (Val130) carboxylate in ML is one of the candidates for involvement in the racemization at Asn127. However, the solution structure of ML using NMR [19] and molecular dynamic simulation (data not shown), suggested that the C-terminal carboxylate is unlikely to access Asn127. Recently, Li et al. [35] examined the conversion from L-Asn to D-Asn using the peptide containing Asn, and found that this conversion in the peptide partially occurred without deamidation, indicating that succinimide formation at the Asn residue did not occur. Conversion from L-Asn to D-Asn in the peptide was considered to proceed by way of the tetrahedral intermediate (see fig. 6) [35]. Thus, in the present case, the racemization at Asn127 in ML during incubation under physiological conditions may have occurred by way of the tetrahedral intermediate.

For alternative mechanisms, we focus on the C-alpha radical at Asn127. The C-alpha radical at an amino acid residue in a peptide was reported to be stable in oxidative

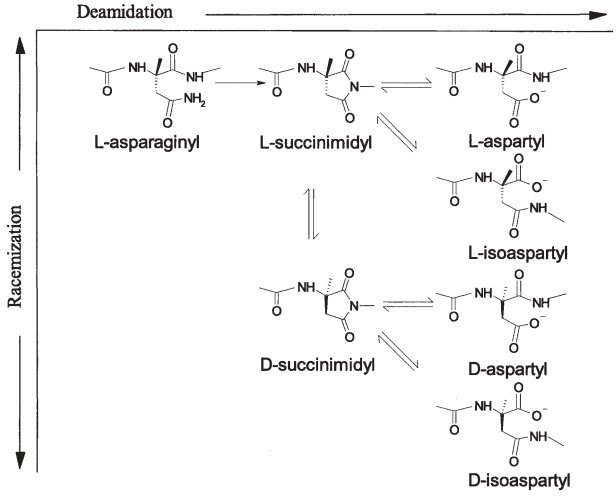

Figure 5. The conventional mechanism of the Asn racemization reaction at neutral or basic pH [16].

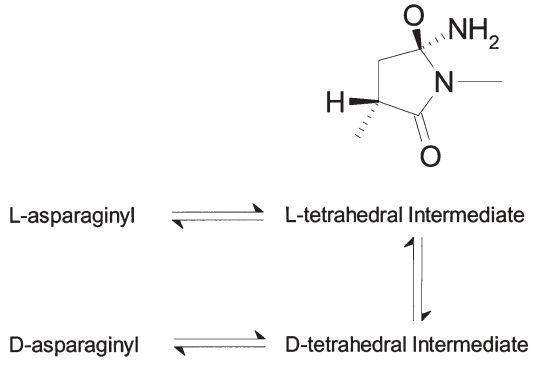

Figure 6. Possible conversion pathway from L-Asn to D-Asn. Cited from Li et al. [35].

circumstances [36–38], and the C-alpha-H bond dissociation energy at Asn is lower than that at the other amino acid residues [39]. Generally, the O-H bond dissociation energy of the water molecule is much higher than the C-alpha-H bond dissociation energy of Asn and the hydroxyl radical would react nonselectively with Asn; nevertheless, the hydroxyl radical would first react with the side chain Tyr or Trp, and the side chain radical could then selectively react with C-alpha-H in the main chain [40–42]. The disufide bond is not chemically inert and radical transfer possibly occurs from Tyr to Cys [43], thus Asn127 can be an intermediate with radical transfer from Tyr to Cys resulting in the racemization. In fact, Asn127 could interact with Tyr124 through the hydrogen bond network in the vicinity of Cys128-Cys6, and racemization of hydroxyproline in *Homo tirolensis* relates to Tyr oxidation and hydroxyl radical generation [44]. Additionally, from the solution structure of ML using NMR [19] and molecular dynamics simulation (data not shown here), Asn127 was suggested to locate at an exposed position of loose structure in which it contacts with bulk water. Thus, the loose structure allows the specific racemization at Asn127. Therefore, if the racemization in ML is induced by the oxidation described above, scavengers might prevent the racemization and prevent protein aging and conformational disease. In summary, as an alternative mechanism, we propose that the racemization at Asn127 is induced by an oxidation mechanism, and we are now verifying the validity of the alternative mechanism using theoretical calculations.

On the other hand, the racemization of Asn127 is also similar to that of other amino acid residues, rather than through the succinimide pathway, during the aging process [14, 44, 45]. Furthermore, amyloid beta peptides in Alzheimer disease plaques show racemization at Ser as well as Asp [14], and the amyloid beta peptide 1–42 generates hydroxyl radicals during incubation under physiological conditions [46]. Fujii et al. [5, 47] reported that site-specific racemization and isomerization at Asp151 in αA-crystallin of the lens protein in rat and bovine are related to UV-B irradiation or gamma-irradiation which might be relevant to the generation of hydroxyl and hydrogen radicals. Notably, in bovine αA-crystallin, the isomerization is reported to increase but the racemization decreases at Asp151 [47], which could suggest that racemization is independent of succinimide formation.

In this study, we experimentally elucidated that the specific racemization of Asn127 occurred without formation of succinimide, the typical obligatory intermediate for racemization, in ML after incubation for 8 weeks in phosphate buffer at pH 7 and 37 °C. To date, it has commonly been accepted that the racemization at Asn in a protein under physiological conditions was accompanied with the formation of Asp, through the deamidation of Asn. The present finding may lead us to pay more attention to the racemization at Asn in proteins. This may contribute to a better understanding of aging disorders at the atomic level. As for the biological significance of the racemization: it has been found in amyloid beta peptide in senile plaques of Alzheimer patients [14, 15] and in αA-crystallin of lens protein in rat and bovine [5, 47]. According to the study on amyloid beta peptide, racemization is related to the protein aging process and conformational disease, depending on the modified product and intermediate of the reaction [15, 42, 48]. Furthermore, modification of an amino acid residue in a protein has been suggested to cause an immunological response [1]. So far, there has been no report on the effect of the racemization in a protein on the immunological response. Since the present racemization occurred under physiological conditions, we are interested in the effect of the racemization on autoimmune disease. The investigation is in progress in our laboratory.

1 Mamula M. J., Gee R. J., Elliott J. I., Sette A., Southwood S., Jones P.-J. et al. (1999) Isoaspartyl post-translational modification triggers autoimmune responses to self-proteins. J. Biol. Chem. **274:** 22321–22327

2 Liu D. T. (1992) Deamidation: a source of microheterogeneity in pharmaceutical proteins. Trends Biotechnol. **10:** 364–369

3 Pearlman R. and Nguyen T. (1992) Pharmaceutics of protein drugs. J. Pharm. Pharmacol. **44:** 178–185

4 Bischoff R. and Kolbe H. V. J. (1994) Deamidation of asparagine and glutamine residues in proteins and peptides: structural determinants and analytical methodology. J. Chromatogr. B **662:** 261–278

5 Fujii N., Momose Y., Ishibashi Y., Uemura T., Takita M. and Takehana M. (1997) Specific racemization and isomerization of the aspartyl residue of alpha A-crystallin due to UV-B irradiation. Exp. Eye Res. **65:** 99–104

6 Geiger T. and Clarke S. (1987) Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides: succinimide-linked reactions that contribute to protein degradation. J. Biol. Chem. **262:** 785–794

7 Saleh N., Deutsch D. and Gil-Av E. (1993) Racemization of aspartic acid in the extracellular matrix proteins of primary and secondary dentin. Calcif. Tissue Int. **53:** 103–110

8 Ogino T. and Ogino H. J. (1988) Application to forensic odontology of aspartic acid racemization in unerupted and supernumerary teeth. J. Dent. Res. **67:** 1319–1322

9 Helfman P. M. and Bada J. L. (1976) Aspartic acid racemisation in dentine as a measure of ageing. Nature **262:** 279–281

10 Brunauer L. S. and Clarke S. (1986) Age-dependent accumulation of protein residues which can be hydrolyzed to D-aspartic acid in human erythrocytes. J. Biol. Chem. **261:** 12538–12543

11 Powell J. T., Vine N. and Crossman M. (1992) On the accumulation of D-aspartate in elastin and other proteins of the ageing aorta. Atherosclerosis **97:** 201–208

12 Shapira R., Wilkinson K. D. and Shapira G. (1988) Racemization of individual aspartate residues in human myelin basic protein. J. Neurochem. **50:** 649–654

13 Fujii N., Muraoka S., Satoh K., Hori H. and Harada K. (1991) Racemization of aspartic acid at specific site in alpha-A-crystallin from aged human lens. Biomed. Res. Tokyo **12:** 315–321

14 Roher A. E., Lowenson J. D., Clarke S., Wolkow C., Wang R., Cotter R. J. et al. (1993) Structural alterations in the peptide backbone of beta-amyloid core protein may account for its de-

position and stability in Alzheimer's disease. J. Biol. Chem. **268:** 3072–3083
15 Tomiyama T., Asano S., Furiya Y., Shirasawa T., Endo N. and Mori H. (1994) Racemization of Asp23 residue affects the aggregation properties of Alzheimer amyloid beta protein analogues. J. Biol. Chem. **269:** 10205–10208
16 Fujii N., Momose Y., Ishii N., Takita M., Akaboshi M. and Kodama M. (1999) The mechanisms of simultaneous stereoinversion, racemization, and isomerization at specific aspartyl residues of aged lens proteins. Mech. Ageing Dev. **107:** 347–358
17 Riblet R. J. and Herzenberg L. A. (1970) Mouse lysozyme production by a monocytoma: isolation and comparison with other lysozymes. Science **168:** 1595–1597
18 Cross M., Mangelsdorf I., Wedel A. and Renkawitz R. (1988) Mouse lysozyme M gene: isolation, characterization, and expression studies. Proc. Natl. Acad. Sci. USA **85:** 6232–6236
19 Obita T., Ueda T. and Imoto T. (2003) Solution structure and activity of mouse lysozyme M. Cell. Mol. Life Sci. **60:** 176–184
20 Mine S., Ueda T., Hashimoto Y., Tanaka Y. and Imoto T. (1999) High-level expression of uniformly 15N-labeled hen lysozyme in *Pichia pastoris* and identification of the site in hen lysozyme where phosphate ion binds using NMR measurements. FEBS Lett. **448:** 33–37
21 Okazaki K., Imoto T. and Yamada H. (1985) A convenient protein substrate for the determination of protease specificity: reduced and S-3-(trimethylated amino)propylated lysozyme. Anal. Biochem. **145:** 87–90
22 Aswad D. W. (1984) Determination of D- and L-aspartate in amino acid mixtures by high-performance liquid chromatography after derivatization with a chiral adduct of o-phthaldialdehyde. Anal. Biochem. **137:** 405–409
23 Salisbury S. A., Tremeer E. J., Davies J. W. and Deia O. (1990) Acylation monitoring in solid-phase peptide-synthesis by the equilibrium distribution of colored ions. J. Chem. Soc. Chem. Commun. **7:** 538–540
24 Young S. C., White P. D., Davies J. W., Owen D. E., Salisbury S. A. and Tremeer E. J. (1990) Counterion distribution monitoring: a novel method for acylation monitoring in solid-phase peptide synthesis. Biochem. Soc. Trans. **18:** 1311–1312
25 Rink H. (1987) Solid-phase synthesis of protected peptide-fragments using a trialkoxy-diphenyl-methylester resin. Tetrahedron Lett. **28:** 3787–3790
26 Meinwald Y. C., Stimson E. R. and Scheraga H. A. (1986) Deamidation of the asparaginyl-glycyl sequence. Int. J. Pept. Protein Res. **28:** 79–84
27 Patel K. and Borchardt R. T. (1990) Chemical pathways of peptide degradation. II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. Pharm. Res. **7:** 703–711
28 Stephenson R. C. and Clarke S. (1989) Succinimide formation from aspartyl and asparaginyl peptides as a model for the spontaneous degradation of proteins. J. Biol. Chem. **264:** 6164–6170
29 Aswad D. W. (ed.) (1995) Deamidation and Isoaspartate Formation in Peptides and Proteins. CRC Press, Boca Raton, Fla.
30 Radkiewicz J. L., Zipse H., Clarke S. and Houk K. N. (1996) Accelerated racemization of aspartic acid and asparagine residues via succinimide intermediates: an ab initio theoretical exploration of mechanism. J. Am. Chem. Soc. **118:** 9148–9155
31 Tyler-Cross R. and Schirch V. (1991) Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. J. Biol. Chem. **266:** 22549–22556
32 Paranandi M. V., Guzzetta A. W., Hancock W. S. and Aswad D. W. (1994) Deamidation and isoaspartate formation during in vitro aging of recombinant tissue plasminogen activator. J. Biol. Chem. **269:** 243–253
33 Fujii N., Harada K., Momose Y., Ishii N. and Akaboshi M. (1999) D-amino acid formation induced by a chiral field within a human lens protein during aging. Biochem. Biophys. Res. Commun. **263:** 322–326
34 Xie M., Aube J., Borchardt R. T., Morton M., Topp E. M. and Vander Velde D. et al. (2000) Reactivity toward deamidation of asparagine residues in beta-turn structures. J. Pept. Res. **56:** 165–171
35 Li B., Borchardt R. T., Topp E. M. VandeVelde D. and Schowen R. L. (2003) Racemization of an asparagine residue during peptide deamidation. J. Am. Chem. Soc. **125:** 11486–11487
36 Seviila M. D., D'Arcy J. B. and Morehouse K. M. (1979) An electron spin resonance study of γ-irradiated frozen aqueous solutions containing dipeptides: Mechanisms of radical reaction. J. Phys. Chem. **83:** 2887–2892
37 Sevllla M. D., D'Arcy J. B. and Morehouse K. M. (1979) An electron spin resonance study of γ-irradiated frozen aqueous solutions containing N-acetylamino acids. J. Phys. Chem. **83:** 2893–2897
38 Burlinska B., Michalik J. and Bobrowski K. (1994) An electron spin resonance study of gamma-irradiated polycrystalline methionine-containing peptides. Radiat. Phys. Chem. **43:** 425–432
39 Rauk A., Yu D., Taylor J., Shustov G. V., Block D. A. and Armstrong D. A. (1999) Effects of structure on αC-H bond enthalpies of amino acid residues: relevance to H transfers in enzyme mechanisms and in protein oxidation. Biochemistry **38:** 9089–9096
40 Nauser T., Pelling J. and Schöneich C. (2004) Thiyl radical reaction with amino acid side chains: rate constants for hydrogen transfer and relevance for posttranslational protein modification. Chem. Res. Toxicol. **17:** 1323–1328
41 Irwin J. A., Østdal H. and Davies M. J. (1999) Myoglobin-induced oxidative damage: evidence for radical transfer from oxidized myoglobin to other proteins and antioxidants. Arch. Biochem. Biophys. **362:** 94–104
42 Brunelle P. and Rauk A. (2002) The radical model of Alzheimer's disease: specific recognition of Gly29 and Gly33 by Met35 in a β-sheet model of Aβ: an ONIOM study. J. Alzheimer Dis. **4:** 283–289
43 Favaudon V., Tourbez H., Houee-Levin C. and Lhoste J. M. (1990) Carboxyl radical induced cleavage of disulfide bonds in proteins: a gamma-ray and pulse radiolysis mechanistic investigation. Biochemistry **29:** 10978–10989
44 Lubec G., Weninger M. and Anderson S. R. (1994) Racemization and oxidation studies of hair protein in the *Homo tirolensis*. FASEB J. **8:** 1166–1169
45 Bada J. L. (1984) In vivo racemization in mammalian proteins. Methods Enzymol. **106:** 98–115
46 Monji A., Utsumi H., Ueda T., Imoto T., Yoshida I. and Hashioka S. et al. (2001) The relationship between the aggregational state of the amyloid-beta peptides and free radical generation by the peptides. J. Neurochem. **77:** 1425–1432
47 Fujii N., Hiroki K., Matumoto S., Masuda K., Inoue M. and Tanaka Y. et al. (2001) Correlation between the loss of the chaperone-like activity and the oxidation, isomerization and racemization of gamma-irradiated alpha-crystallin. Photochem. Photobiol. **74:** 477–482
48 Orpiszewski J., Schormann N., Kluve-Beckerman B., Liepnieks J. J. and Benson M. D. (2000) Protein aging hypothesis of Alzheimer disease. FASEB J. **14:** 1255–1263