

## **VIII- II -1. Project Research**

### **Project 5**

## PR5 Project Research on the Abnormal Aggregation of Proteins by Post-Translational Modifications, and Study of Repair Mechanism

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### Objectives and Allotted Research Subjects:

The aim of this project research is to elucidate the correlation between the change of the protein structure induced by various post-translational modifications with UV irradiation, gamma-irradiation, aging and protein function. We also investigate the repair mechanism for the damaged protein by irradiation. This research program has started in 2011. In this year, the 7 research subjects were carried out. The allotted research subjects (ARS) are as follows;

**ARS-1:** Protein deuteration for small-angle neutron Scattering (M. Sugiyama, N. Fujii and N. Fujii)

**ARS-2:** Comparison of properties of mammalian and aquatic animal D-aspartyl endopeptidases. (T. Kinouchi and N. Fujii)

**ARS-3:** Damage to biological molecules induced by ionizing radiation and biological defense mechanisms provided by radical scavengers II. (T. Saito and N. Fujii)

**ARS-4:** Effect of higher order structure of recombinant human  $\alpha$ A-crystallin on the rate of  $\beta$ -linkage isomerization of specific aspartyl residue.

(Y. Sadakane and N. Fujii)

**ARS-5:** Analysis of environmental stress-related imbalance in mice. (N. Ohgami and N. Fujii)

**ARS-6:** Factors permitting the combination of D-serine and pyridoxal 5'-phosphate at the active site of tryptophanase. (A. Shimada, N. Fujii and T. Saito)

**ARS-7:** A rapid survey of Asp isomers in lens proteins from the cataract. (N. Fujii, N. Fujii, H. Sakaue and H. Sasaki)

### Main Results and Contents of This Project

ARS-1: Sugiyama et al. investigated a structure of  $\alpha$ -crystallin complex by subunit deuteration and SANS.

As a result, it was revealed that there exists subunit exchange between  $\alpha$ A-crystallin and  $\alpha$ B-crystallin and also between  $\alpha$ B-crystallins.

ARS-2: Kinouchi et al. searched for and attempted to purify D-aspartyl-endopeptidase (DAEP) in aquatic animals. As a result, high DAEP activity was detected in gonads of African clawed frogs (*Xenopus laevis*) and

Japanese green sea urchins (*Hemicentrotus pulcherrimus*). Purified DAEP from *Xenopus* oocytes indicated common specific features with mouse DAEP: mitochondrial localization, high molecular weight, and sensitivity to a synthesized DAEP inhibitor. We are characterizing its fundamental functions of frog DAEP.

ARS-3: Saito et al. showed that astaxanthin inhibits or promotes gamma radiation induced oxidative degradation of  $\alpha$ -linolenic acid depending on the conditions, suggesting that carotenoids are involved in the protection against damage to lipid structures induced by gamma irradiation in vivo.

ARS-4: Sadakane et al. determined the rate of  $\beta$ -linkage isomerization of a specific Asp residue both in the recombinant human  $\alpha$ A-crystallin and fragmentary peptide at 50°C and 90°C, and revealed that higher order structure of protein suppressed the  $\beta$ -linkage isomerization of Asp58 residue of  $\alpha$ A-crystallin.

ARS-5: Ohgami et al. showed that chronic exposure to low frequency noise (below 0.5 kHz) at moderate levels causes impaired balance involving morphological impairments of the vestibule with enhanced levels of oxidative stress. We are investigating whether imbalance involves aggregation of a specific protein in the vestibule in inner ears.

ARS-6: Shimada et al. analyzed the kinetics of tryptophanase against various tryptophan-analogous inhibitors in terms of double reciprocal plots. The results indicated that small stereo-structural change was necessary to form an aldimine bond between D-serine and pyridoxal 5'-phosphate.

ARS-7: Fujii et al. showed that a convenient and robust biochemical method for identifying the isomeric Asp sites in crystallins using LC-MS systems. There are many advantages to this new method: 1) No requirement for large amounts of sample proteins, 2) No requirement for the purification of proteins 3) No requirement for complicated analytical steps. This new method is able to search comprehensively for the Asp isomers in damaged or aged proteins from all living tissues and cells.

M. Sugiyama, N. Fujii and N. Fujii

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**INTRODUCTION:** Small-Angle Scattering (SAS) is one of promising methods to analyze protein structure in an aqueous solution. The probes of SAS are X-ray and neutron: they are called Small-Angle X-ray and Neutron Scatterings (SAXS) and (SANS). A merit of use of X-ray as probe is strong intensity of source by using a synchrotron. On the other hand, typical neutron sources are weaker than that of X-ray but neutron is very sensitive for isotope effect. An interesting point of neutron probe for biological samples is the large difference in scattering length between proton and deuteron: the scattering length of proton is 3.74 fm but that of deuteron is 6.64 fm. Because hydrogen is one of main components of protein, we can make the scattering length density of the protein and/or a subunit changed by replacing the proton by deuteron. In other word, deuteration can be for used a labeling method with less modification of the protein itself.

Recently, Sugiyama et al. applied the combination technique of subunit deuteration and SANS for measurement of large scale kinetics in protein, such as subunit exchange. This technique has possibility to analyze the similar large scale kinetics in the other proteins. So, we begin to apply this technique to analyze the formation and time evolution of  $\alpha$ -Crystallin. It is well-known that  $\alpha$ -Crystallin consists of two kinds of subunits, named as  $\alpha$ A-Crystallin and  $\alpha$ B-Crystallin. Moreover, the ratio of two subunits in  $\alpha$ -Crystallin could be change by aging. However, the mechanism has been unknown but the subunit exchange could be one of candidates of the mechanism. Therefore, our first goal is to prove the existence of subunit exchange in  $\alpha$ -Crystallin system. To perform the combination technique of subunit deuteration and SANS, the key technique is protein deuteration. Here, we report the results of our preparation of deuterated protein ( $\alpha$ -Crystallin) and SANS measurements with them.

**EXPERIMENTS:** We prepared for fully deuterated  $\alpha$ B-Crystallin and not deuterated (normal)  $\alpha$ A-Crystallin and  $\alpha$ B-Crystallin in our laboratory. Then, we made two mixtures of deuterated and not deuterated  $\alpha$ B-Crystalins and of deuterated  $\alpha$ B-Crystallin and not deuterated  $\alpha$ A-Crystallin, respectively. The ratio of D<sub>2</sub>O in solvent was tuned to be 81%. The SANS measurements were performed with Small and Wide Angle Scattering Spectrometer (TAIKAN) installed at Material and Life Laboratory in J-PARC, Tokai, Ibaraki.

**RESULTS:** Figure 1 shows SANS profiles of mixture of deuterated  $\alpha$ B-Crystallin and not deuterated (normal)  $\alpha$ A-Crystallin: closed and open circles denote just

mixture and 12 hours later, respectively. SANS intensity clearly decreased 12 hours later than that just after mixture. This means that subunit exchange occurs between  $\alpha$ A-Crystallin and  $\alpha$ B-Crystallin.

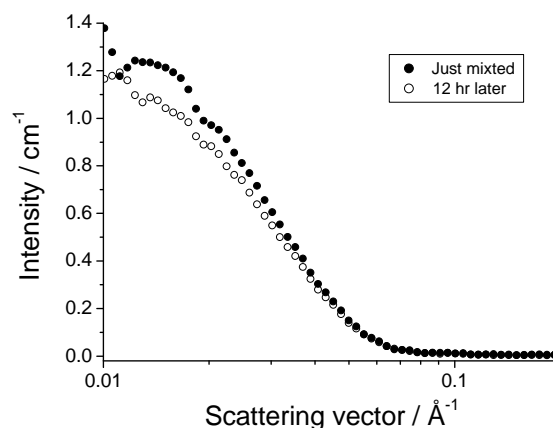


Fig. 1. SANS profiles of mixture of deuterated  $\alpha$ B-Crystallin and not deuterated (normal)  $\alpha$ A-Crystallin: closed and open circles denote just mixture and 12 hours later, respectively.

Figure 2 shows SANS profiles of mixture of deuterated and not deuterated (normal)  $\alpha$ B-Crystalins: closed and open circles denote just mixture and 12 hours later, respectively. SANS intensity clearly decreased 12 hours later than that just after mixture. This means that subunit exchange also occurs between  $\alpha$ B-Crystallin and  $\alpha$ B-Crystallin.

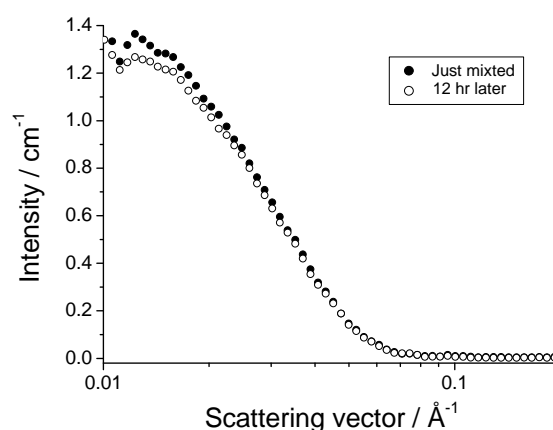


Fig. 2. SANS profiles of mixture of deuterated and not deuterated (normal)  $\alpha$ B-Crystallin: closed and open circles denote just mixture and 12 hours later, respectively.

T. Kinouchi and N. Fujii

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**INTRODUCTION:** As can be seen from many reports, D-Aspartate (D-Asp) is detected in abnormally aggregated proteins causing age-related diseases (i.e., cataract, prion disease and Alzheimer's disease). Therefore it is strongly suggested that formation of D-Asp in human proteins is potentially noxious for metabolic turnover of the normal protein. The D-aspartyl endopeptidase (DAEP), we have discovered from a mammal and have been characterizing [1-3], seems to physiologically serve as a scavenger against the noxious D-Asp containing-protein, because DAEP stereoselectively recognizes and degrades its substrate at the internal D- $\alpha$ -aspartate (Asp) residue. In mammals, which have a long life span and high body-temperature, DAEP is considered a kind of quality-control system to maintain the protein homeostasis. On the other hand, a distribution of DAEP in living things is not confined to mammals: for example, in African clawed frog (*Xenopus laevis*), high DAEP activity was detectable in its testes, ovaries and unfertilized eggs, though the frog naturally thrives around 20°C at the most. In order to elucidate physiological functions of DAEP, we therefore examined comparison of properties of DAEPs, purified from aquatic animals: African clawed frogs and Japanese green sea urchins, with mammalian DAEP.

**EXPERIMENTS:** Materials> African clawed frogs (*Xenopus laevis*) were purchased from Kato-S-Science (Chiba, Japan). Japanese green sea urchins (*Hemicentrotus pulcherrimus*) were purchased from a local vendor. Measurement of DAEP activity> We developed an assay system for DAEP activity using the synthetic D-Asp containing substrate, Succinyl-D-Aspartic acid  $\alpha$ -(4-methyl-coumaryl-7-amide) (Suc-D-Asp-MCA) [1]. Homogenate of the above biological materials or purified DAEP was mixed and incubated with 0.1 mM Suc-D-Asp-MCA and the assay buffer (10 mM Tris-HCl (pH 8.5), 200 mM NaCl, 3 mM MgCl<sub>2</sub>) at optimum temperature for each sample. The fluorescence of aminomethylcoumarin liberated from Suc-D-Asp-MCA by DAEP was measured at  $\lambda_{ex} = 380$  nm and  $\lambda_{em} = 460$  nm. Purification of DAEP> The purification of DAEP from mouse liver was previously described [1]. The purification of DAEP from frogs and sea urchins was as follows: The mitochondrial fraction separated from the unfertilized egg from frogs or sea urchins was suspended in extracting buffer (20 mM Tris-HCl (pH 8.5), 250 mM sucrose, 1 mM EDTA, 2% CHAPS) and stirred on a tube rotator for 12 h at 4°C. The extract was loaded onto HiTrap Q HP column (GE Healthcare, US). The eluted

DAEP-active fractions were loaded onto centrifugal filter devices: Amicon Ultra<sup>®</sup> (molecular cut-off limit: 100 kDa, Merckmillipore, Germany) to concentrate the extract and to remove smaller molecules included in the extract. Finally purification factor of the purified DAEP used in this study was above 100.

**RESULTS & DISCUSSION:** As we reported, mouse DAEP has some specific features [1]: the molecular weight is up to 600 kDa, and the activity is strongly inhibited by Zn<sup>2+</sup> and a synthesized DAEP inhibitor (benzyl-L-Arg-L-His-D-Asp-CH<sub>2</sub>Cl), which we developed. Those features were well conserved in frog and sea urchin DAEPs (Table). On the other hand, the tissue distribution of DAEP was quite different. Although mouse DAEP was detected in its liver as the highest activity, frog DAEP was detected in a gonad and not detectable in its liver. Therefore original and primitive physiological functions of DAEP are considered to be associated with fertilization and/or oocyte maturation. Further analyses of DAEP might elucidate its essential function.

Table. Summary of Properties of Mouse and frog DAEPs

Species	Mouse	African clawed frog
Tissue localization	Liver, Kidney...	Gonad, Oocyte
Subcellular localization	Mitochondria	Mitochondria
Optimum temperature	37°C	25°C
Molecular weight	600 kDa	300 kDa~
Sensitivity to DAEP inhibitor	Positive	Positive

### REFERENCES:

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- [2] T. Kinouchi *et al.*, *Chem. Biodivers.*, **7** (2010) 1403-1407.
- [3] T. Kinouchi *et al.*, *J. Chromat. B.*, **879** (2011) 3349-3352.

Kinouchi and Fujii searched for and attempted to purify D-aspartyl-endopeptidase (DAEP) in aquatic animals. As a result, high DAEP activity was detected in gonads of African clawed frogs (*Xenopus laevis*) and Japanese green sea urchins (*Hemicentrotus pulcherrimus*). Purified DAEP from *Xenopus* oocytes indicated common specific features with mouse DAEP: mitochondrial localization, high molecular weight, and sensitivity to a synthesized DAEP inhibitor. We are characterizing its fundamental functions of frog DAEP.

T. Saito and N. Fujii

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**INTRODUCTION:** Some bacteria exhibit extreme resistance to ionizing radiation [1]. A common feature of these bacteria is that they contain red carotenoid pigments [1, 2, 3]. Colorless mutants of these radioresistant bacteria are more sensitive to gamma irradiation than wild types [1]. Therefore, carotenoids are thought to be involved in the bacterial defense mechanisms against ionizing radiation [1]. Biological effects induced by low-linear energy transfer ionizing radiation are mainly attributed to radicals generated by radiolysis. Carotenoids have high radical scavenging activity, and they are localized in cell surface lipids in prokaryotes. These facts indicate that carotenoids are likely to defend the cell surface lipids of radioresistant bacteria against ionizing radiation.

When considering the biological defense mechanism of these radioresistant bacteria against ionizing radiation, it is important to elucidate the effects of carotenoids on damage to biological molecules, especially biological lipids. In this study, we analyzed the effect of astaxanthin, a typical carotenoid, on gamma radiation induced oxidative degradation of  $\alpha$ -linolenic acid, a type of fatty acid.

**EXPERIMENTS:** **Sample Preparation:** Linolenic acid was dissolved in benzene at a final concentration of  $5.0 \times 10^{-1}$  M, and astaxanthin was added at a final concentration of  $5.0 \times 10^{-8}$  to  $5.0 \times 10^{-4}$  M. **Gamma Irradiation:** The prepared solutions were irradiated with  $^{60}\text{Co}$  gamma rays at a dose of 30 kGy and a dose rate of 400 Gy/min. **Analysis of Oxidative Degradation of  $\alpha$ -Linolenic Acid:** The method described by Buege and Aust was used with some modifications [4]. TCA-TBA-BHT-HCl reagent (15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.04% butylated hydroxytoluene, and 0.25N hydrochloric acid) was prepared. The gamma irradiated sample was diluted 50-fold with benzene. The diluted solution (3.0 mL) was evaporated in vacuo. The residue was dispersed in 9.0 mL of PBS(-) with a sonicator. The dispersed solution (1.0 mL) was combined with 2.0 mL of TCA-TBA-BHT-HCl reagent and thoroughly mixed. The mixed solution was heated in a boiling water bath for 15 min, and absorption at 535 nm was measured. The amount of malondialdehyde (MDA) formed was calculated using the molar absorption coefficient ( $\epsilon$ ) of the

color substance formed by the reaction (i.e.,  $1.56 \times 10^5$  at 535 nm). Thus, oxidative degradation of  $\alpha$ -linolenic acid was evaluated by measuring the amount of MDA.

**RESULTS:** Under the experimental conditions used,  $5.0 \times 10^{-4}$  M astaxanthin inhibited the oxidative degradation of  $\alpha$ -linolenic acid induced by gamma irradiation, although it was not statistically significant (Fig. 1). In contrast, treatment with  $5.0 \times 10^{-7}$  and  $5.0 \times 10^{-8}$  M astaxanthin significantly promoted the oxidative degradation of  $\alpha$ -linolenic acid (Fig. 1). We have previously reported that  $\beta$ -carotene, a type of carotenoid, shows effects similar to those of astaxanthin [5]. We found that  $8.5 \times 10^{-3}$  M  $\beta$ -carotene significantly inhibited oxidative degradation of  $\alpha$ -linolenic acid induced by gamma irradiation. These results suggest that carotenoids play a role in the protection against damage to the lipid structure induced by gamma irradiation in radioresistant bacteria and that the intracellular concentrations of carotenoids are strictly regulated.

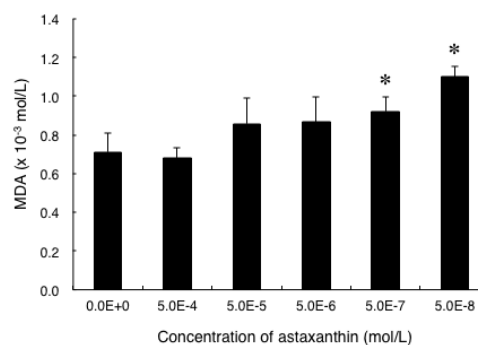


Fig. 1. Effects of astaxanthin on gamma radiation-induced oxidative degradation of  $\alpha$ -linolenic acid. The horizontal axis shows the concentration of astaxanthin and the vertical axis shows the amount of MDA formed. Each data set is presented as the mean  $\pm$  SD of six samples from three independent experiments. An asterisk (\*) indicates a sample with a significant difference from the control sample with no astaxanthin added ( $P < 0.05$ ).

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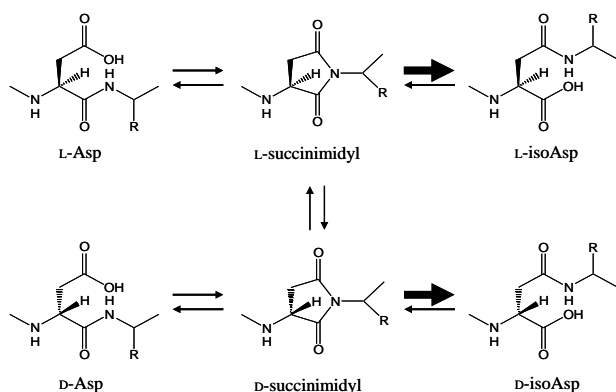
## PR5-4 Effect of Higher Order Structure of Recombinant Human $\alpha$ A-crystallin on the Rate of $\beta$ -Linkage Isomerization of Specific Aspartyl Residue

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**INTRODUCTION:** The stereoconversion of aspartyl (Asp) residue arise through intramolecular rearrangement, such as *via* a succinimide intermediate (Fig. 1). The native L-Asp residue in the protein was converted to the L-succinimide intermediate, and it is quickly hydrolyzed and produced the mixture containing L-Asp and L-isoAsp residue in a ratio of approximately 1:3. A small amount of L-succinimide intermediate undergoes reversible stereoconversion, and also produced the mixture of D-Asp and D-isoAsp residue in a same ratio above.



**Fig. 1** The beta-linkage isomerization and stereoinversion to D-form of aspartyl residue

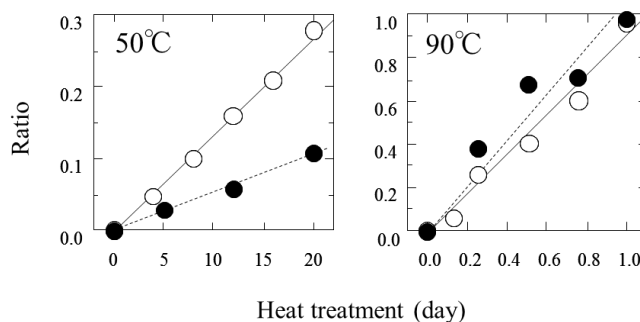
Fujii et al. reported that the stereoinversion of amino acids occurred at specific L-Asp residues of  $\alpha$ -crystallin, and also found that the D/L ratios of the Asp residues was higher than 1.0 in the  $\alpha$ A-crystallin obtained from aged human eye lens. We also determined the rate constants for  $\beta$ -linkage isomerization of three specific Asp residues in recombinant human  $\alpha$ A-crystallin protein by reversed-phase HPLC [1].

In this study, we examined effect of higher order structure of protein on the rates of  $\beta$ -linkage isomerization of a specific Asp using a recombinant human  $\alpha$ A-crystallin protein and fragmentary peptide.

**EXPERIMENTS:** The peptide fragments and recombinant protein of  $\alpha$ A-crystallin were prepared by the methods described in [1]. Both protein and peptide were incubated at 50°C or 90°C in the phosphate buffer (pH 7.4). The rate of  $\beta$ -linkage isomerization of Asp residue both in the protein and peptide was determined by re-

versed-phase HPLC.

**RESULTS:** The  $\alpha$ A-crystallin protein and fragmentary peptide containing Asp58 were incubated at 50°C for 20 days and at 90°C for 1 day. The  $\beta$ -linkage isomerized Asp58 residue was determined by reversed-phase HPLC, and the ratios of the amount of L-isoAsp residue to that of L-Asp residue were plotted (Fig. 2).



**Fig. 2** Rates of  $\beta$ -linkage isomerization of Asp58 residue located in T6 peptide of synthetic fragmentary peptides (open circles) and recombinant  $\alpha$ A-crystallin protein (solid circles) at 50 or 90 °C.

The  $\beta$ -linkage isomerization of Asp58 residue of the  $\alpha$ A-crystallin protein were significantly suppressed in comparison with that in the synthetic fragmentary peptide at 50°C, however rates of  $\beta$ -linkage isomerization of Asp58 residue was not different between the protein and peptide at 90 °C. Since  $\alpha$ A-crystallin is relatively heat stable protein, higher order structure of the protein seems not to be affected at 50°C. The experiment of CD spectrum of  $\alpha$ A-crystallin protein revealed that the major transition by a secondary structural change took place between 47 and 60°C [1]. These results suggest that higher order structure of protein affects the rate of  $\beta$ -linkage isomerization of Asp58.

**DISCUSSION:** We show here that higher order structure affected the isomerization of Asp in the  $\alpha$ A-crystallin protein. The isomerization and stereo-conversion of Asp residues are post translational modification, and such modification of  $\alpha$ -crystallin would affect the three-dimensional packing of the lens protein and may affect the transparency of the lens. Thus, the higher order structure of protein may protect such alterations.

### REFERENCE:

[1] Y. Sadakane, N. Fujii, K. Nakagomi, J Chromatogr B **879** (2011) 3240-3246.

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**INTRODUCTION:** Exposure to noise generated in occupational and daily environments is one of the community hazards [1,2]. Noise contains sound with broad frequencies, but there is very limited information regarding the frequency-dependent influence of noise on health. Low frequency noise (LFN) is generated from natural and artificial sources at all times. The frequency range of LFN is defined as being below 100 Hz [3]. LFN is detected in various situations in our modern society and is generated from many occupational and daily sources including transportation systems, industrial devices, air movement devices and household appliances. Thus, we are constantly exposed to LFN generated from various devices in the daily environment. Inner ears have the vestibule in the vicinity of the organ of Corti. Vestibular hair cells covered with otoconia play an important role in mechanotransduction, by which gravity stimulus are converted into neural impulses. Dysfunctions of vestibular hair cells have been shown to cause abnormal behaviors including balance [4]. Thus, the vestibule plays an important role for balance. On the other hand, exposure to audible noise (at 1-20 kHz) has been shown to induce damage of hair cells with increased oxidative stress in the organ of Corti in the inner ear, leading to noise-induced hearing loss in mice and humans. Thus, exposure to noise can cause damage of hair cells with increased oxidative stress in inner ears, although most of the previous studies used broadband noise with no consideration of specific frequencies. However, there is no information regarding whether exposure to LFN induces oxidative stress in vestibular hair cells, which play a critical role in regulation of balance. In this study, therefore, we used LFN (0.1 kHz) at a moderate level of 70 dB SPL for exposure of mice to noise in order to analyze the pathogenesis of imbalance caused by LFN stress.

**EXPERIMENTS:** Randomly bred wild-type female mice (ICR) at 6 weeks of age were used for exposure experiments. All experiments were authorized by the Institutional Animal Care and Use Committee in Chubu University (approval number: 2410030) and followed the Japanese Government Regulations for Animal Experiments. Mice were continuously exposed for 1 month to LFN at 70 dB SPL from a speaker as previously reported [6]. Measurement of balance and morphological analyses were performed according to previous studies [5, 6].

**RESULTS:** After exposure for one month to LFN at 70 dB SPL, behavior analyses including rotarod, beam-crossing and footprint analysis showed impaired balance in LFN-exposed mice but not in non-exposed mice. Morphological analyses including immunohistochemistry showed a decreased number of vestibular hair cells and an increased number of D-beta-Asp-positive cells in LFN-exposed mice compared to those in non-exposed mice. Our results suggest that chronic exposure to LFN at moderate levels causes imbalance involving morphological impairments of the vestibule with enhanced levels of impaired proteins caused by oxidative stress. Thus, the results of this study suggest the importance of considering the risk of chronic exposure to LFN at a moderate level for imbalance. Further studies are needed to identify the impaired proteins in vestibule caused by LFN stress.

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- [4] X. Zhao *et al.*, *Neuroscience* **153** (2008) 289-299.
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- [6] H. Tamura, N. Ohgami *et al.*, *PLoS ONE*, **7**(6) (2012) e39807

## PR5-6 Factors Permitting the Combination of D-serine and Pyridoxal 5'-phosphate Pyridoxal 5'-phosphate at the Active Site of Tryptophanase

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**INTRODUCTION:** A stable supply of homochiral molecules, which is necessary for synthesizing biological macromolecules with highly organized stereostructures such as proteins or polysaccharides, is achieved by enzyme enantioselectivity that functions to select the right enantiomer; and therefore the stability of this enantioselectivity is essential for sustaining vital activity. We have so far studied the enantioselectivity of tryptophanase, with the aim of acquiring a better understanding of the enantioselectivity. Tryptophanase is usually known as an enzyme that has a very wide substrate specificity for L-tryptophan derivatives and various  $\beta$ -substituted L-amino acids, but has an extremely tight enantioselectivity. Because of this absolute enantioselectivity, tryptophanase has no activity on D-tryptophan and D-serine at all. However, previous studies have shown that tryptophanase becomes active towards the D-enantiomers in highly concentrated diammoniumhydrogen phosphate ( $(\text{NH}_4)_2\text{HPO}_4$ ), DAP for short) solution. Tryptophanase enantioselectivity is very flexible in the presence of DAP, contrary to conventional knowledge about enantioselectivity. D-tryptophan or D-serine, respectively, was degraded or synthesized through  $\beta$ -elimination or  $\beta$ -replacement reactions after D-tryptophan or D-serine formed an aldimine bond with pyridoxal 5'-phosphate. The formation of the external aldimine bond between the D-enantiomers and pyridoxal 5'-phosphate was the first step for D-enantiomers to function as active substrates. However, it is not enough for the flexible tryptophanase enantioselectivity to convert D-enantiomers into active substrates. Perhaps other factors such as conformational change are required as well. Previous reports showed that tryptophanase underwent a small reversible conformational change in the presence of DAP. We think this small conformational change possibly modifies the enantioselectivity of tryptophanase so that D-enantiomers can be activated *via* the external aldimine bond formation. Inhibitors, which structurally resemble their enzymes' substrates but either do not react or react only very slowly compared to the substrate, are commonly used to probe the conformational nature of a substrate-binding site to elucidate the enzymes' catalytic mechanisms.

Therefore, a tryptophan-analogous inhibitor was used for kinetic analysis in this study because it was powerful enough to understand what happens at the active site of the tryptophanase with a conformational change.

**EXPERIMENTAL:** When tryptophanase degraded L-tryptophan, all reaction mixtures included 0.2 mM of pyridoxal 5'-phosphate and 0.23  $\mu\text{M}$  of tryptophanase in 100 mM potassium phosphate buffer of pH 8.3. DAP concentrations were prepared to the required concentrations of 0, 0.6, 1.2, 1.9 or 3.1 M L-tryptophan. The reaction mixture was prepared to a concentration of 98, 108, 128, 137, 147, 167, 196, 225, 245, 294, 343, 392, 490, 680 or 980  $\mu\text{M}$  for kinetic analyses. Reactions were performed at 37 °C for 30 min in a Dry Thermo Unit DTU-1B (Taitec, Tokyo, Japan). On the other hand, the reaction condition of D-tryptophan degradation differed in several ways from that of L-tryptophan degradation. Each concentration of pyridoxal 5'-phosphate and tryptophanase was 1.2 mM and 0.92  $\mu\text{M}$ , and additionally reaction temperature and time was 55 °C and 2 h, respectively. All the other reaction conditions were the same as in the L-tryptophan degradation. Tryptophanase activity was calculated as described elsewhere.

**RESULTS AND DISCUSSION:** Tryptophanase is one of the enzymes with absolute enantioselectivity, but it reversibly changes in concentrated DAP solution. This flexible enantioselectivity is caused by a small conformational change in the presence of DAP. When D-tryptophan, pyruvate, indole pyruvate and D-histidine were used as inhibitors, their inhibition patterns were examined in terms of kinetics to see how the conformation affected the activity of tryptophanase on L-tryptophan degradation. The inhibition of D-tryptophan shifted from competitive to noncompetitive *via* mixed type inhibition with increasing DAP concentrations. This inhibition pattern was also obtained from experiments with indole pyruvate. We indicated that a heterocyclic benzene ring of D-tryptophan was responsible for the inhibition pattern on the basis of experimental results using D-histidine. Additionally, we suggested that tryptophanase had two different conformations (L-conformation and D-conformation) between which it switched depending on the saline environment in aqueous solution. Our results shed light on the origin of homochirality; that is to say they provide the possibility that today's exclusive use of L-amino acids in the biological world might be associated with the saline environment *in vivo*. This study provides an attractive mechanism to explain the origin of homochirality.



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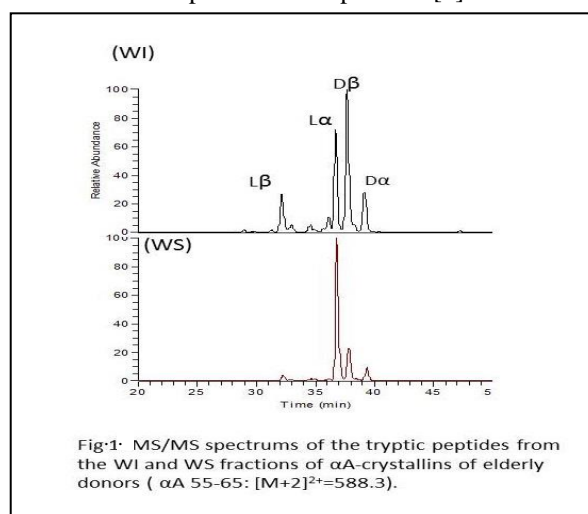
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**INTRODUCTION:** A cataract, which is the most common age-related disease, is caused by clouding of the eye lens that may lead to a partial or total loss of vision. The mechanism of cataract development is not well understood. However, it is thought that eye lens proteins of a cataract are abnormally aggregated, resulting in clumping that scatters the light and interferes with focusing on the retina. Human lens proteins are mainly composed from the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -crystallin superfamily of proteins. The overall structure, stability and short-range interactions of these proteins are thought to contribute to the transparent properties of the lens [1]. Because the lens crystallins are long-lived proteins, they undergo various posttranslational modifications including isomerization, inversion, deamidation, oxidation, glycation and truncation. These posttranslational modifications may lead to age-related cataract. Therefore, analysis of posttranslational modifications of individual amino acid residues in proteins is important. However, detection of the optical isomers of amino acids formed in these proteins is difficult because optical resolution is only achieved using complex methodology. In this study, we describe a new method for the analysis of isomerization of individual Asp residues in proteins using LC-MS and the corresponding synthetic peptides containing the Asp isomers. This makes it possible to analyze isomers of Asp residues in proteins precisely and quickly.

**EXPERIMENTS:** Lens samples from elderly individuals were homogenized in 20 mM Tris/HCl, pH 7.8, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA) by ultrasonication and fractionated into water-insoluble (WI) and water-soluble (WS) fractions by centrifugation at 16,000 g for 20 min at 4°C. The WI proteins were dissolved in 8 M urea, 50 mM Tris/HCl, pH 7.8, 1 mM CaCl<sub>2</sub>, and then the urea concentration diluted to less than 1 M in 50 mM Tris/HCl pH 7.8, 1 mM CaCl<sub>2</sub> buffer before enzymatic digestion. The WI and WS proteins were digested with trypsin and the resulting peptides were analyzed by LC-MS using a nano flow HPLC system (Paradigm MS4, Michrom BioResources, USA). Mass spectrometry (MS) was performed on an Ion Trap (IT) system (LCQ Fleet,

Thermo, USA).

**RESULTS:** The detection of the isomeric Asp residues in the protein was achieved by the combination of finding peptides with the same mass which are separated into multiple peaks in the LC-MS followed by their MS/MS analysis. Fig. 1 shows the 4 different isomers Asp 58 of  $\alpha$ A-crystallin. We found that Asp 58, 76, 84 and 151 of  $\alpha$ A-crystallin, and Asp 62 and 96 of  $\alpha$ B-crystallin are highly converted to L $\beta$ -, D $\beta$ - and D $\alpha$ -isomers. The amount of isomerization of Asp is greater in the insoluble fraction at all Asp sites in lens proteins [2].



**DISCUSSION:** Here, we describe a convenient and robust biochemical method for identifying the isomeric Asp sites in crystallins using LC-MS systems. There are many advantages to this new method: 1) No requirement for large amounts of sample proteins, 2) No requirement for the purification of lens proteins from WI and WS fractions, 3) No requirement for complicated analytical steps which usually include the hydrolysis of the peptides followed by derivatization to the diastereoisomers of amino acids. This new method is able to search comprehensively for the Asp isomers in damaged or aged proteins from all living tissues and cells.

#### REFERENCES:

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