VIII-Ⅱ-1. Project Research

Project 11

PR11 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: Structural change of eye lens protein (crystallin) in solution. (M. Sugiyama, Y. Ueki, N. Fujii and N. Fujii)

ARS-2: Evolutinal Consideration of D-Aspartyl Endopeptidase. (T. Kinouchi and N. Fujii)

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

ARS-4: Effect of structural alteration of asparagine residue in the Prion peptide fragment (106-126) on the amyloid fiber formation. (Y. Sadakane and N. Fujii)

ARS-5:Analysis of environmental stress-related hearing loss in the mice. (N. Ohgami and N. Fujii)

ARS-6: Effect of gamma-ray irradiation on the activity of tyrptophanase and tryptophan synthase.
(A. Shimada, N. Fujii and T. Saito)

ARS-7: Simultaneous stereoinversion and isomerization at the Asp-4 residue in beta B2-crystallin from the cataractous lenses. (N. Fujii, T. Kawaguchi, H. Sasaki and N. Fujii)

Main Results and Contents of This Project

ARS-1: Sugiyama et.al. investigated a structure of βB_2-crystallin in an aqueous solution . As a result, it was revealed that the structure of βB_2-crystallin is different from that in crystal.

ARS-2: Kinouchi and Fujii examined the distribution of D-aspartyl-endopeptidase (DAEP) in animals and microorganisms. As a result DAEP activity was detected in fishes, amphibians and birds, but was not detectable in archaea, prokaryotes, yeast and nematode. Interestingly high DAEP activity in African clawed frog (Xenopus laevis) was remarkable in testis and unfertilized eggs. It is suggested that a primitive physiological function of DAEP is associated with fertilization and/or oocyte maturation.

ARS-3: Saito et al. showed that beta-carotene does not affect the linolenic acid peroxidation reaction induced by gamma irradiation, suggesting that carotenoid pigments affect radical reactions that proceed after the lipid peroxidation reaction in the biological defense mechanism in vivo.

ARS-4: Sadakane et al. analyzed the effect of structural alterations of asparagine residue on the amyloid fiber formation by measuring thioflavin fluorescence and observing the solution viscosity, and revealed that the viscosity of peptide solution containing D-form aspartic acid increased. types of damage to biological lipids in vivo.

ARS-5: Ohgami *et al.* showed that partial impairment of *c-Ret* causes age-related hearing loss involving neurodegeneration of spiral ganglion neurons in mice. Neurodegenerative disorders usually involve impairments of protein including aggregation and oxidative stress. We are investigating whether hearing loss involves aggregation of a specific protein in inner ears.

ARS-6**:** This research aims to investigate how the activity of tryptophanase (TPase) and tryptophan synthase (TSase) changes when a low dose of γ-rays is administered to them. We first investigate the influence of it about tryptophanase's activity.

ARS-7: Fujii et al. found that the simultaneous stereo inversion and isomerization at the Asp 4 residue occur in beta B2-crystallin from the cataractous lenses of elderly donors.

採択課題番号 23P11　放射線や紫外線照射によるタンパク質の異常凝集とその防御及び修復機構に関する研究　プロジェクト

（京大原子炉）藤井紀子

Structural Change of Eye Lens Protein (crystalline) in Solution

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INTRODUCTION: Crystallin is a main component of eye lens proteins. There are three kinds of crystallins in human eye lens, α-crystallin, β-crystallin and γ-crystallin. α-Crystallin, the largerst proten among three crystallins (Mw is *ca* 800kDa), belongs to a family of heat shock proteins and it therefore works as a chaperon to repair the other proteins. On the other hands, the functions of β- and γ-crystallins have not been clarified yet. To understand their roles and functions in eye lens, as the first step, we begin to clarify the structure of β-crystallin in an aqueous solution.

It is well-known that protein structure can be determined with high resolution by single crystal X-ray diffraction method. However, it is difficult to reveal the protein structure in the an aqueous solution with this method. Therefore, we employed a small-angle neutron scattering (SANS) method for this propose, the clarification of protein structure in an aqueous solution. Even though Small-Angle Scattering has lower spatial resolution than that of single crystal diffraction, it does not require "crystal" as a sample and moreover can observe the particle structure in a solution. In addition, neutron is a soft probe for the sample, not to make less radiation damage on the protein. However, it is difficult to reveal the detailed structure by ordinary SANS data analysis method, which gives us approximative size and/or shape of a particle. Therefore, we are developing a new analysis method utilizing a RMC technique.

EXPERIMENTS: Human βB_2-crystallin expressed by *E.Coli* was used as a sample. The concentrations of samples were tuned to be 3.0 mg/ml and the solvent was 20 mM Tris/HCl (pH 7.8) + 150 mM NaCl.The used SANS data were observed with SANS-U installed at JRR-3 in Tokai.

ANALYIS: The structure of βB_2-crystallin (1ytq) is shown in Fig.1. This protein has two domains with the size of 10Å apart from 24Å. Firstly, we have to develop the SANS simulation method; the simulation code makes the SANS profile from the single crystal structure data. By cubic-dividing method, we successfully obtain a SANS profile from PDB data.

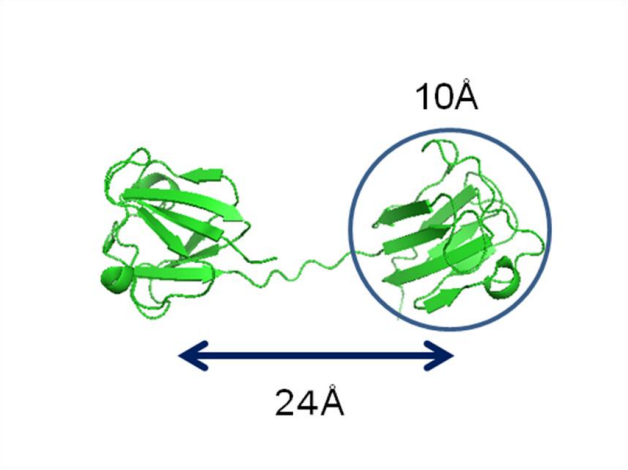

Fig. 1. Structure of βB_2-crystallin (PDB:1ytq) .

Figure 2 shows a SANS profile of βB_2-crystallin in an aqueous solution and the simulated one. The simulated SANS profile does not reproduce the experimental one. The gyration radius calculated from the experimental one is 20.8 Å but that from the simulated one is 23.5Å. This clearly suggested that the distance between two domains changes in an aqueous solution, compared with that in the crystal.

Now, we are trying to reveal the distance in aqueous solution by RMC-coupled analysis.

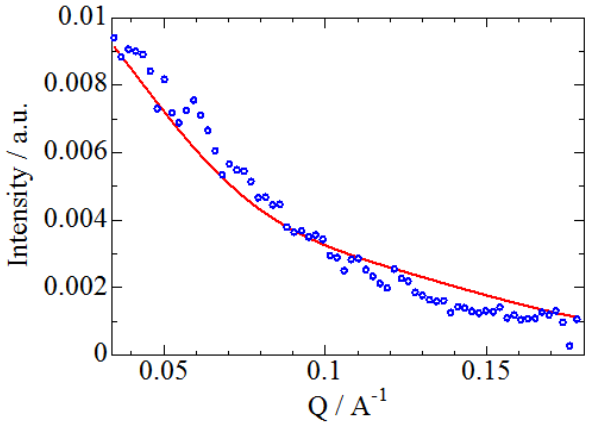

Fig. 2. SANS profile of βB_2-crystallins (open circles) and simulated one with PDB data (line).

PR11-2 Evolutional Consideration of D-Aspartyl Endopeptidase

T. Kinouchi and N. Fujii

Research Reactor Institute, Kyoto University

INTRODUCTION: We have been characterizing mammalian D-aspartyl endopeptidase (DAEP) [1-3]. Significant features of DAEP are as follows: DAEP forms a large ring-like complex structure, such as the AAA^+ protease [2], and stereoselectively recognizes and degrades its substrate at the internal D-α-aspartate (Asp) residue in an ATP-independent manner [3]. As can be seen from many reports, D-Asp is detected in abnormally aggregated proteins causing age-related diseases (i.e., cataract, prion disease and Alzheimer's disease), and it is strongly suggested that formation of D-isomer of Asp residue in human proteins is potentially noxious for the normal protein turnover in our cell. Therefore DAEP seems to physiologically serve as a kind of quality-control system against the misfolded D-Asp containg-protein and to consequently maintain the protein homeostasis in high body-temperature and long life span animals such as mammals. On the other hand, a distribution of DAEP in living things has not been examined, and the results would be highly informative for the further functional characterization and the discovery of a primitive homolog of DAEP. We therefore started examining the distribution of DAEP in various animals and microorganisms.

EXPERIENTS: Materials>Archaea: *Thermoplasma acidophilum*, *Archaeoglobus fulgidus*, *Pyrococcus horikoshii*, and *Thermococcus celericrescens* were purchased from NBRC (NITE Biological Resource Center). Prokaryotes: *E.coli, Thermus thermophiles* (was kindly gifted by Dr. Kunio Miki, Kyoto University), and *Deinococcus radiodurans* (was kindly gifted by Dr. Takeshi Saito, KURRI). Eukaryotes: yeast (*Saccharomyces cerevisiae*), nematoda (*Caenorhabditis elegans*), fishes: medaka (*Oryzias latipes,* was purchased from a local petshop), amphibians: African clawed frog (*Xenopus laevis,* was kindly gifted by Dr. Yuichi Tsuchiya, Toho University), birds: chickens (*Gallus gallus domesticus,* purchased from a local slaughterhouse).
Screening of DAEP activity>We developed an assay system for DAEP activity using the synthetic D-Asp containing substrate, Succinyl-D-Aspartic acid α-(4-methyl-coumaryl-7-amide) (Suc-D-Asp-MCA) [1]. Homogenate of the above biological materials 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 $MnCl_2$) at optimum temperature for each. The fluorescence of aminomethylcoumarin liberated from Suc-D-Asp-MCA by DAEP is measured at λex = 380 nm and λem = 460 nm.

RESULTS & DISCUSSION: DAEP activity was detected in fishes, amphibians and birds, but was not detectable in archaea, prokaryotes, yeast and nematode, which were examined in this study. As we reported, mammalian liver was shown to have the highest DAEP activity among tissues (fig. 1) [1]. Even in chickens, DAEP activity was distributed in their livers. On the other hand, the tissue distribution of DAEP activity in African clawed frog, *Xenopus laevis* was quite different from that in mammals and birds. As shown in fig. 1, high DAEP activity in *Xenopus laevis*, was remarkable in testis and unfertilized eggs, but was imperceptible in liver. Since *Xenopus laevis* is an animal ranking below mammals and birds in the viewpoint of evolutionary biology, a primitive physiological function of DAEP is considered to be associated with fertilization and/or oocyte maturation. Further analyses of *Xenopus* DAEP might elucidate its essential function.

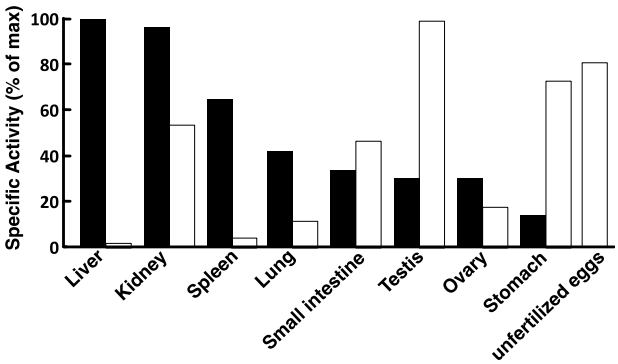

Fig. 1. DAEP activity in various tissues of rabbit and *Xenopus laevis*.
Y-axis indicates specific activity of DAEP (% of maximum). Closed and open bars represent rabbit and *Xenopus laevis*, respectively.

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採択課題番号 23P11-2　D-アスパラギン酸含有蛋白質に特異的な分解酵素の研究　プロジェクト（京大・原子炉）木野内忠稔、藤井紀子

PR11-3 Damage to Biological Molecules Induced by Ionizing Radiation and Biological Defense Mechanisms Provided by Radical Scavengers

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INTRODUCTION: Some bacteria exhibit very high resistance to ionizing radiation [1]. A common feature of these bacteria is that they contain red carotenoid pigments [1, 2, 3]. Because colorless mutants of these radioresistant bacteria are more sensitive to gamma irradiation than wild types, the red carotenoid pigments are thought to contribute to the bacteria's defense mechanisms against ionizing radiation [1]. It is well known that biological effects induced by low-LET ionizing radiation are caused mainly by radicals generated by radiolysis. Carotenoid pigments exhibit high radical scavenging activity and are localized within the lipids of the cell surfaces in prokaryotes. These facts indicate that red carotenoid pigments in radioresistant bacteria must defend the lipids of the cell surfaces of these 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 carotenoid pigment as regards damage to biological molecules. We have reported that linolenic acid is oxidatively degraded and peroxidized by gamma irradiation in a dose-dependent manner [4]. In this study, we analyzed the effect of beta-carotene, which is a typical carotenoid pigment, on the peroxidation of linolenic acid by gamma irradiation.

EXPERIMENTS: Sample Preparation: Linolenic acid was dissolved in benzene at a final concentration of 5.0 x 10^{-1} M and beta-carotene was added at final concentrations of 5.0 x 10^{-8} to 5.0 x 10^{-4} M. Gamma Irradiation: The prepared solutions were irradiated with ^{60}Co gamma rays at a dose of 30 kGy and a dose rate of 25 kGy/h. Linolenic Acid Peroxidation Assay: A modified version of the method described by Pryor and Castle was used [5]. The gamma-irradiated sample was diluted 600 times with benzene, and then 5 ml of the diluted solution was evaporated in vacuo. The residue was dissolved in 5 ml of *n*-hexane, and the average absorbance from 230-236 nm of this solution, which was derived from the generated conjugated diene, was measured. In this study, the level of linolenic acid peroxidation was evaluated by the amount of generated conjugated diene.

RESULTS: Under this experimental condition, beta-carotene had no significant effect on the peroxidation reaction of linolenic acid induced by gamma irradiation. We have previously reported that beta-carotene affects the oxidative degradation reaction of linolenic acid induced by gamma irradiation using an analysis in which the level of the oxidative degradation of linolenic acid was evaluated by the amount of generated malondialdehyde [6]. The lipid damage induced by radicals begins with a hydrogen abstraction reaction followed by a peroxidation reaction. Then there is a degradation reaction that includes a radical reaction. The above reveals that beta-carotene does not affect the linolenic acid peroxidation reaction during the initial radical reaction, which has a high reaction rate, but affects the linolenic acid degradation reaction that proceeds after the peroxidation reaction in the linolenic acid damage process induced by gamma irradiation.

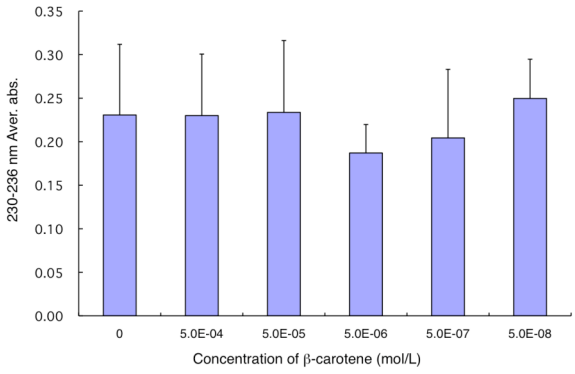

Fig. 1. The effect of beta-carotene on the amount of generated conjugated diene induced by the gamma irradiation of linolenic acid at a dose of 30 kGy. The horizontal axis shows the beta-carotene concentration, and the vertical axis shows the 230-236 nm average absorbance, which reveals the relative amount of generated conjugated diene. Each value is expressed as an average ± S.D. of three separate experiments.

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採択課題番号 23P11-3　放射線照射による生体分子の損傷とラジカルスカベンジャー　プロジェクトによる生体防御機構

（京大・原子炉）齊藤　毅、藤井　紀子

PR11-4 **Effect of Structual Alteration of Asparagine Residue in the Prion Peptide Fragment (106-126) on the Amyloid Fiber Formation**

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INTRODUCTION: The native L–asparagine (Asn) residue in the peptide and protein was converted to the L–succinimide intermediate, and it is quickly hydrolyzed and produced the mixture containing L–Asp, L–isoAsp, D-Asp and D-isoAsp residues (Fig. 1). The alternation of aspartic acid (Asp) or Asn residue also has been found in the key proteins related to neuronal disease such as prion protein (PrP). The deamidation of Asn residue in $PrP^{106\text{-}126}$ has been reported to affect its channel activity in planar lipid bilayer. In this study, we investigated the effect of such structural alteration of the PrP peptide on the amyloid fiber formation by the thioflavin fluorescent measurement and the solution viscosity observation.

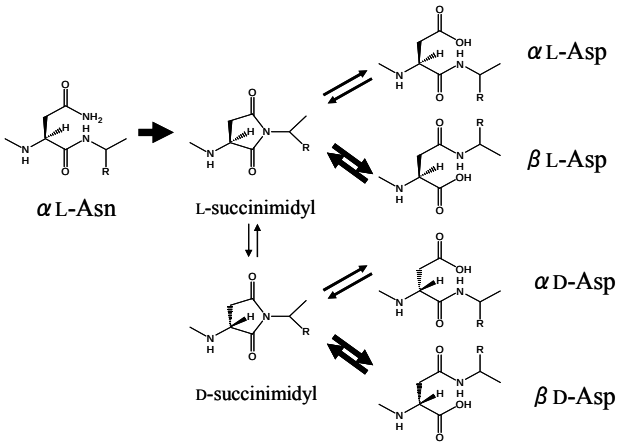

Fig. 1 The asparagine residue generates four aspartic acids which bear the beta–linkage bond and stereo-inverted D-form.

EXPERIMENTS: The original and four peptide isomers, (L-Asn^{108}, L -Asp^{108}, L-$isoAsp^{108}$, D-Asp^{108}, D-$isoAsp^{108}$, which were represented by N, αL, βL, αD, and βD in this study) corresponding to the $PrP^{106\text{-}126}$ sequence (KT^{108}NMKH MAGAAAAGAVVGGLG) were prepared by Fmoc chemistry [1]. The thioflavin fluorescent intensity was measured in the peptide solution which had been incubated at 37 °C for 5 days. The viscosity of peptide solution was observed by using microtubes (0.2 mL vol.). The peptide solution was incubated at 37 °C for a week in the tubes, then the tubes were turned upside down, and the solution state was observed.

RESULTS: The relative fluorescent intensity of thioflavin added in the peptide solution after incubating at 37 °C for 5 days were measured by fluorescent micro-plate reader (Fig. 2). The intensities of five peptide solution were shown no difference each other. The photograph of upside-down tubes containing peptide solution that had been incubated at 37 °C for a week was shown (Fig. 3). The peptide solutions bearing D-form Asp were leaved in the bottom of tubes. This result shows that the viscosity of D-form Asp-containing peptide solution increase in comparison with that of the others.

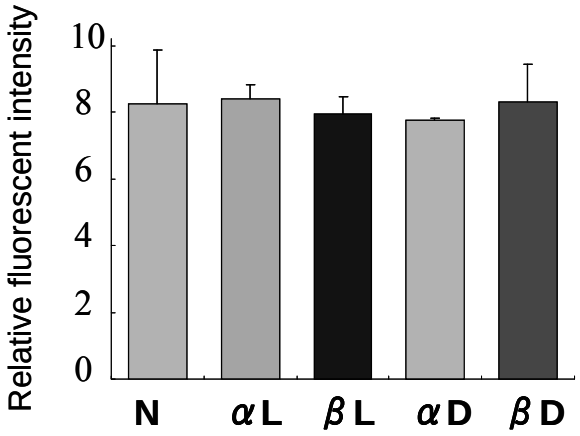

Fig. 2 Effect of structural alteration of asparagine residue on the thioflavin fluorescent intensity after 37 °C incubation for 5days. N, asparagine; αL, βL, αD, and βD, aspartic acid residues.

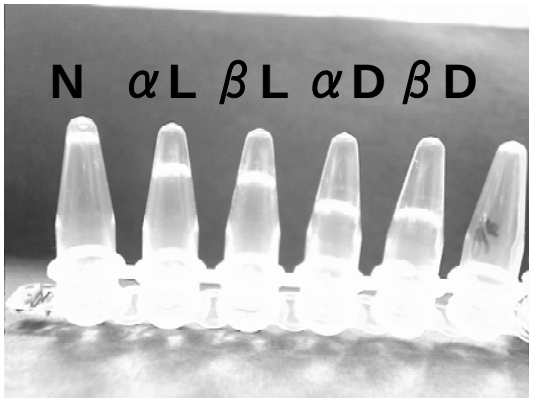

Fig. 3 Effect of structural alteration of asparazine residue on the viscosity of peptide solution after 37 °C incubation for 7 days. N, asparagine; αL, βL, αD, and βD, aspartic acid residues.

REFERENCE:

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採択課題番号 23P11−4　　タンパク質中のアスパラギン酸残基の異性化と機能変化に関する研究　　プロジェクト

（鈴鹿医療科学大学・薬学部）定金 豊、（京大・原子炉）藤井 紀子

PR11-5 Analysis of Environmental Stress-related Hearing Loss in Mice

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INTRODUCTION: Hearing level is affected by genetic, aging and environmental factors [1,2]. About 120 million people worldwide suffer from congenital (early-onset) hearing loss. Thirty percent of them have syndromic hearing loss and the remaining seventy percent have non-syndromic hearing loss. In addition, a large number of aged people worldwide suffer from age-related (late-onset) hearing loss [1,2]. These hearing losses have been generally categorized as distinct diseases due to different pathogeneses.

c-Ret encodes a receptor-tyrosine kinase. Glial cell line-derived neurotrophic factor (GDNF) is one of the ligands for c-Ret [3]. GDNF exerts its effect on target cells by binding to a glycosyl phosphatidylinositol (GPI)-anchored cell surface protein (GFRα1), which then allows the receptor tyrosine kinase c-Ret to form a multi-subunit signaling complex. Formation of this complex results in c-Ret autophosphorylation and a cascade of intracellular signaling to control cell survival [3]. Tyrosine 1062 (Y1062) in c-Ret is one of the most crucial autophosphorylation sites for its kinase activation and is a multi-docking site for several signaling molecules including Shc (Src Homology 2 domain-Containing transforming protein 1), a transmitter for c-Ret signaling [3].

We have recently shown that complete impairment of Y1062-phoshorylation in c-Ret causes congenital deafness with HSCR in homozygous *c-Ret* Y1062F knock-in mice (*c-Ret*-KI$^{Y1062F/Y1062F}$-mice) [4]. On the other hand, heterozygous *c-Ret* Y1062F knock-in mice (*c-Ret*-KI$^{Y1062F/+}$-mice) have been shown to have no HSCR-linked phenotype [3] except for hearing levels. In this study, we used *c-Ret*-KI$^{Y1062F/+}$-mice in order to analyze whether partial impairment of Y1062-phoshorylation in c-Ret causes age-related hearing loss.

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EXPERIMENTS: *c-Ret*-KI$^{Y1062F/+}$-mice used in this study were previously reported [3]. All experiments were authorized by the Institutional Animal Care and Use Committee in Chubu University (approval number: 2210038) and the Institutional Recombinant DNA Experiment Committee in Chubu University (approval number: 06-01) and followed the Japanese Government Regulations for Animal Experiments. Hearing levels in mice were measured by auditory brainstem responses (ABR) [4]. Inner ears were analyzed by light microscopy and scanning- and transmission electron microscopy [4]. Preparation of tissues for transmission electron microscopy basically followed the previous method [4].

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RESULTS: Partial impairment of Y1062-phosphorylation in c-Ret showed acceleration of age-related hearing loss in heterozygous *c-Ret* Y1062F knock-in mice (*c-Ret*-KI$^{Y1062F/+}$-mice) [5]. The hearing loss involved late-onset neurodegeneration of spiral ganglion neurons (SGNs) in *c-Ret*-KI$^{Y1062F/+}$-mice [5]. Morphological abnormalities in inner- and outer-hair cells and the stria vascularis in *c-Ret*-KI$^{Y1062F/+}$-mice were hardly detected under scanning electron microscopy and light microscopy [5]. We further performed detailed morphological analyses of SGNs from *c-Ret*-KI$^{Y1062F/+}$-mice by transmission electron microscopy [4]. Aged *c-Ret*-KI$^{Y1062F/+}$-mice showed gaps between SGNs and Schwann cells compared to those in littermate WT mice [5]. Nuclei of SGNs from aged *c-Ret*-KI$^{Y1062F/+}$-mice were obviously shrunken compared with those from littermate WT mice [5]. The nuclei of SGNs from aged *c-Ret*-KI$^{Y1062F/+}$-mice exhibited discontinuous nuclear membranes, while those from littermate WT mice had intact bilayer membranes of nuclei [5]. Degeneration at the auditory nerve fiber was observed in aged *c-Ret*-KI$^{Y1062F/+}$-mice [5]. Thus, our results suggest that partial impairment of *c-Ret* causes age-related hearing loss involving neurodegeneration of SGNs in mice [4]. Neurodegenerative disorders usually involve impairments of protein including aggregation. Further study is needed to investigate whether hearing loss involves protein aggregation.

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採択課題番号 23P11-5 騒音ストレスによる内耳タンパク質中のアスパラギン酸残基の異性化の解析 プロジェクト（中部大学）大神信孝（京大・原子炉）藤井紀子

PR11-6 Effect of γ-Ray Irradiaiton on the Activity of Tyrptophanase and Tryptophan Syntase

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INTRODUCTION: γ-ray-irradiated enzyme reduces its activity because γ-ray irradiation against the enzyme causes drastic tertiary conformational change with irreversible denaturation. When the enzyme is administrated at lower dose, the activity should make minute change responsive to small conformational change. Currently, we have just few data on the activity in low dose. We have presented many researches about interesting tryptophanase's activity on D-tryptophan in the past. So we are quite familiar with tryptophanase. Whereas tryptophan synthase is structurally different from tryptophanase, both enzymes have similar activity. Thereby it is of great interest to compare tryptophanase's activity with tryptophan synthase's one. The present study aims to investigate how tryptophanase (TPase) and tryptophan synthase (TSase) change in their activities when they receive a low radiation dose. In this study, we first investigate about tryptophanase's activity. Tryptophanase, an enzyme with extreme absolute stereospecificity for optically active stereoisomers, catalyzes the degradation of L-tryptophan into indole, pyruvate and ammonia through α,β-elimination mechanism, having no activity on D-tryptophan (D-Trp). Although tryptophanase is widely known as one of enzymes with strict stereospecificity, it becomes active for D-Trp in a highly concentrated diammoniumhydrogen phosphate solution. This reaction has never been reported before. This reaction activity, however, is very low. Activity on D-Trp has only 0.7 % of one of L-Trp at 37 ℃. We investigate here whether it is possible to elevate the activity by use of a low dose of γ-rays.

EXPERIMENTAL: Apotryptophanase was purchased from Sigma Chem. Co., prepared to a concentration of 200 μg/mℓ in 100 mM potassium phosphate buffer solution with a 20 % saturation concentration of diammoniumhydrogen phosphate (DAP) and 380 mM pyridoxal 5′-phosphate. The enzyme was exposed to γ-rays, for which a cobalt-60 source was used with a dose rate of 3 Gy/sec. Tryptophanase was irradiated at doses of 30 – 5400 Gy. Here we wrote about γ-ray-irradiated tryptophanase as γ-TPase. The activity of TPase and γ-TPase was assayed against D-Trp. Reaction mixture was composed of 20 % saturation DAP, 380 mM pyridoxal 5'-phosphate, 245 μM D-tryptophan and 200 μg/mℓ TPase or γ-TPase (pH 7.8). Reaction was performed at 37 °C for 4 h. The reaction was stopped by adding n-butanol, vigorously mixed, and then immediately centrifuged at approximately 1,000 g for 10 min. After the centrifugation, the supernatant including indole released from tryptohan degradation was extracted, colored with Ehrlich's reagent for 30 min at 60 °C, and then its amount was spectroscopically detected at 570 nm to be converted into concentration from calibration curve. The activity on γ-TPase was compared with that of TPase to analyze how a low irradiation of γ-ray influenced on tryptophanase's activity. Unless otherwise stated, reagents were obtained from Wako Pure Chem. Co. Ltd. All chemicals were reagent grade. All glasswares were washed by soaking more than 3 days in a special detergent, CLEAN 99CL (Clean Chemical Co. Ltd.), thoroughly rinsed in deionized and distilled water, and then dried in an oven.

RESULTS AND DISCUSSION: As generally-accepted notion, tryptophanase's activity decreased in response to increasing γ-ray dose from 600 to some 2000 Gy. Its reduction rate was slowed down over 2000 Gy, almost stopping between 4000 – 5400 Gy. Tryptophanase was susceptible to γ-ray in the same manner as other enzymes and protein. However, we observed tryptophanase's reaction was different from this result between 0 and 600 Gy. Prior to experiment, we forecasted the activity of γ-TPase was far below that of TPase there. However, there was either slightly below or little difference from TPase's activity between them against our expectation. In some cases, we could observe that γ-TPase's activity reached several percent above TPase's one. We interpret γ-TPase's activity in γ-ray irradiation between 0 and 600 Gy as follows. Protein, along with enzyme, is fragile to radioactive ray. Enzyme is especially sensitive to it because its active site needs very precise tertiary structure. When the enzyme is exposed to massive radiation exceeding some limit, the enzyme's stereostructure is broken to be inactivated or denatured. However, when radiation dose within the limit is administered to the enzyme, it has a small effect on the enzyme due to its radioresistance. It goes without saying that γ-ray almost always has wrong effect on the enzyme. However, there may be the remotest chance that it gives positive effect on the enzyme activity. It will thus become important to analyze the activity in detail when the enzyme is γ-irradiated at low dose of ~ 100 grays. We predict that tertiary structural change is probably responsible for this effect, and also we are now searching the sophisticated ways to detect it. We will make it clear in the future study.

択課題番号 23P11-6　　ガンマー線を照射したトリプトファンシンターゼの立体選択性の変化　　プロジェクト

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PR11-7 Simultaneous Stereoinversion and Isomerization at the Asp-4 Residue in βB2-Crystallin from the Aged Human Eye Lenses

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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 α-, β-, and γ-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. 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. Previous studies concerning the posttranslational modification of β-crystallin have been limited to analyzing the deamidation of asparagine (Asn) or glutamine (Gln) residues, and oxidation of methionine and tryptophan residues [1]. Here, we demonstrate for the first time stereoinversion and isomerization of the Asp residues at the N- terminal regions in βB2-crystallin isolated from elderly individuals [2].

EXPERIMENTS: Lens samples from elderly individuals were homogenized and the sample was subjected to size exclusion chromatography to obtain the β-crystallin. Subsequently, β-crystallin was further fractionated into βB2-crystallin and other β-crystallins by reverse-phase high-performance liquid chromatography (RP-HPLC) using a C4 column. The βB2-crystallin was digested with trypsin and the resulting peptides were separated by RP-HPLC using a C18 column. The tryptic peptides of βB2-crystallin were identified using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOFMS) (AXIMA-TOF2; Shimadzu, Kyoto, Japan). The peptides were hydrolyzed and to form diastereoisomers using *o*-phthalaldehyde (OPA) and n-*tert*-butyloxycarbonyl-L-cysteine (Boc-L-cys). The D/L ratio of the amino acids was determined using RP-HPLC.

RESULTS: We found that the aspartyl (Asp) residue at position 4 of βB2-crystallin in the lenses of elderly human individuals undergoes a significant degree of inversion and isomerization to the biologically uncommon residue D-β-Asp. Surprisingly, the D/L ratio of β-Asp at position 4 in βB2-crystallin from elderly donors (67-77 year-old) was 0.88-3.21. A D/L ratio of amino acids greater than 1.0 is defined as an inversion of configuration from the L- to the D-form, rather than a racemization.

DISCUSSION: Usually, the inversion proceeds with difficulty under physiological conditions, however, D-Asp formation occurs via a succinimide when the neighboring amino acid of the Asp residue has a small side chain, such as glycine, alanine or serine. The residue neighboring Asp-4 of βB2-crystallin is histidine, which has a bulky side chain, suggesting the unusual D/L ratio of Asp-4 is not influenced by the adjacent residue. Rather, inversion of Asp-4 in βB2-crystallin may proceed because of the chiral environment around this residue that promotes inversion of the L- to the D-form. Carver et al. clearly showed that the N- and C-terminal extensions of bovine βB2-crystallin are largely unstructured. These extensions were shown to be accessible to solvent and possess significantly greater flexibility compared with the domain core of the protein [3]. Thus, the structural data supports our hypothesis that the highly flexible region around Asp-4 could promote formation of the succinimide followed by inversion and isomerization.

Inversion of these Asp residues directly affects the higher order structure of the protein. Hence, this modification may change crystallin-crystallin interactions and disrupt the function of crystallins in the lens.

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採択課題番号 23P11-7　　タンパク質中のアスパラギン酸残基の異性化と異常凝集　　プロジェクト
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