VIII-II-1. Project Research

Project 6

PR6 Project Research on the Abnormal Aggregation of Proteins by UV and Gamma Ray-Irradiation 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 irradiation such as gamma-ray and UV and protein function. We also investigate the repair mechanism for the damaged protein by irradiation. This research program has started in 2008. In this year, the 7 research subjects were carried out. The allotted research subjects (ARS) are as follows;

ARS-1: Structural investigation of the 20S proteasome and its related particles (Y. Morimoto, K. Nishio and M. Sugiyama)

ARS-2: Study on structure of complex consisting of αA-Crystallin and βB_2-crystallin (M. Sugiyama, N. Fujii and N. Fujii)

ARS-3: 3-Dimensional structure observation of mammalian D-aspartyl endopeptidase by high-resolution atomic force microscopy (T. Kinouchi and N. Fujii)

ARS-4: Damage of biological molecules induced by ionizing radiation and biological defense mechanisms II (T. Saito and N. Fujii)

ARS-5: Establishment of analytical method for the recombinant Asp-isomerized Human α-A crystalline using reversed-phase HPLC (Y. Sadakane and N. Fujii)

ARS-6: Conformational change of tryptophanase on the basis of comparison with γ-tryptophanase (A. Shimada, N. Fujii and T. Saito)

ARS-7: Inversion and isomerization of an aspartyl residue affect the properties of peptides (N. Fujii and N. Fujii)

Main Results and Contents of This Project

ARS-1: Morimoto, *et al.* have investigated a structural studies for a supramolecular complex and its biological behavier by X-ray and neutronscattering methods. In the project, a proteasome ubiquitine system whichfunctions on a degradation of un-needed proteins is focused in the structural characteristics. A crystal structure of deubiquitinatingenzyme (UCH37) binding to a 20S proteasome has been determined at 2.2 Å resolution, and a joint complex of the 20S proteasome and the enzyme will give us information for a combination and binding mechanism of their association.

ARS-2: Sugiyama *et al*. investigated the coupling effect between αA-crystallin and βB2-crystallin. With the mixture solution both of them, the proteins sizes were observed with Small-Angle X-ray Scattering (SAXS). The SAXS profiles show that there is no coupling between normal αA-crystallin and βB2-crystallin.

ARS-3: Kinouchi and Fujii analyzed the conformation of mammalian D-aspartyl endopeptidase (DAEP) by high-resolution atomic force microscopy (AFM) whose advantage is its ability to study biological macromolecules in an ambient air environment. Our data indicated that DAEP forms a ring-like structure with a diameter of approximately 40 nm, and suggest that DAEP topologicallybelongs to the AAA+ protease family such as proteasome.

ARS-4: Saito *et al.* found that 8.5 x 10^{-5} M beta-carotene significantly suppressed the peroxidation of linolenic acid caused by gamma-irradiation, whereas 5 x 10^{-5} and 5 x 10^{-6} M beta-carotene significantly promoted the peroxidation of linolenic acid caused by gamma-irradiation. These results suggest that carotenoid pigments are involved in the defense mechanism against gamma-radiation-induced cellular damage.

ARS-5: Sadakane *et al*. developed the method for analyzing the structural alteration of Asp residues in α A-crystallin protein using RP-HPLC, which will be used for analyzing the effect of radiation on the structural alteration of Asp residue in the protein.

ARS-6: Shimada *et al.* studied on a secondary structural change of tryptophanase through CD spectrum on the basis of with conformational change between tryptophanase and γ-tryptophanase. The secondary stereostructural change of γ-tryptophanase was very large, but that of tryptophanase was almost negligible.

ARS-7: Fujii *et al*. synthesized peptides corresponding to 70-88 (KFVIFLDVKHFSPEDLTVK) of human αA-crystallin and its corresponding diastereoisomers in which Lα-Asp was replaced with Lβ-Asp, Dα-Asp, and Dβ-Asp residue at position 76 and compared their biochemical properties with that of normal peptide. The results clearly indicated that that a single substitution of an Asp isomer in a peptide induces a large change to the properties of the peptide.

This modification can be the result of the partial unfolding of protein leading to various age-related ocular diseases.

採択課題番号 21P6　放射線や紫外線照射によるタンパク質の異常凝集と　プロジェクト
その防御及び修復機構に関する研究

（京大原子炉）藤井紀子

PR6-1 Structural Investigation of the 20S Proteasome and Its Related Particles

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INTRODUCTION: Proteasomes (or multicatalytic protease complexes) are widely distributed in eukaryotes, ranging from human to yeasts. In higher eukaryotes, proteasomes are known to act as antigen-processing enzymes responsible for the generation of peptide ligands presented by major histocompatibility complex (MHC) class I molecules. Proteasomes have molecular masses of approximately 750 kDa and sedimentation coefficients of approximately 20S. They are barrel-like particles formed by the axial stacking of four rings made up of two outer α-rings and two inner β-rings, being associated in the order of αββα Furthermore, a 26S particle has two large terminal components at both ends of the 20S core particle. An ubiquitin-proteasome system regulates some small (~30KDa) enzymes which catalyze ubiqutin association or dissociation against the 20S proteasome core. How the mechanism of a formation about 20S, 26S and other enzymes are intrinsic and strict in the living cells. We have tried the determination of the structure of the enzyme (UCH37) and its formation into the 20S core or 26S whole particle.

EXPERIMENTS: The crystal structure of the deubiquitinating enzyme (UCH37) has been determined, and a conjugating site into the 20S core particle is examined. The UCH37 could be expressed as a GST fusion protein and purified using affinity chromatographic method. The purified protein was used for crystallization using a polyethylene glycol as a precipitant. The native dataset was harvested at BL44XU beam line, SPring-8.

RESULTS: A total of 237 amino acids out of 329 residues was solved at 2A resolution, the following figure 1, but the conjugating site was undetermined because of the C-terminal deletion.

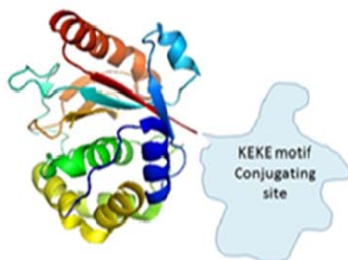

Fig.1.Crystal structure of the UCH37N-terminal domain.

It is very important that how the enzyme does to bind 20S core particle, and where and what happened on the 20S subunits (Fig.2). We hope to clarify a quaternary structure of the 20S subunits with the enzyme, and a small-angle neutron scattering technique could reveal that in a solution like *in vivo* state. Therefore we have planning to examine the quaternary structure and its formation of 20S proteasome subunits and related other protein molecules.

20S proteasome was prepared from a yeast tuned by genetic modification, then 8L cultivation gives us a final sample amount of 5mg. Sample preparation and various-sample condition (concentration, pH and so on) are carried out at the KUR D-shop Lab using a yeast expression system. The observed SANS data were corrected for background, cell, buffer scatterings and the transmissions.

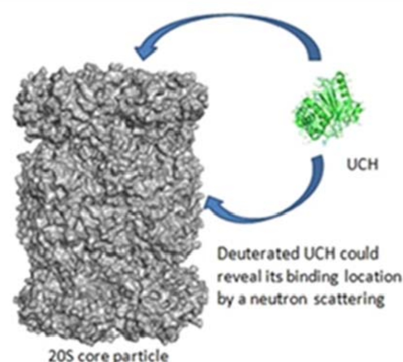

Fig.2. Whole Particle of the 20S proteasome and the UCH.

Typical small-angle X-ray scattering profiles (Fig.3) of the 20S proteasome and other related protein clearly show distinct differences at a low angle curvature.

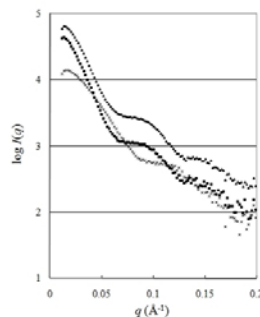

Fig.3. SAXS Scattering Curves of the 20S proteasome and activator particle.

Since the deuterium effect on the neutron scattering could amplify those characteristics according to an H/D exchange in a solution, particle components or binding proteins would be made them to admit location. Such a technique will be powerful to demonstrate a formation or combination of subunits in a protein complex research.

採択課題番号 21P6-1 巨大で複雑なタンパク質複合体の動態と作動機構 プロジェクト
（京大・原子炉）森本幸生、藤井紀子

PR6-2 Study on Structure of Complex Consisting of αA-Crystallin and βB_2-Crystallin

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INTRODUCTION: Eye lens intrinsically has two features, high refractive index and high transparency. In order to achieve these features, there are *high* concentrated proteins, α-, β-, γ-crystallin, in eye lens. However, the high concentration has possibility to make aggregation of the proteins. Therefore, α-crystallin with the largest molecular weight of ca 800 kDa has a chaperone activity to prevent from anomalous huge aggregation of the crystallins in the human eye lens, and therefore maintain the transparency.

External stresses make proteins denatured and the denatured protein aggregates up to huge size: in the case of crystallin, the aggregated crystallin finally causes *Cataract*, which could be considered as one of protein aggregated deceases. Therefore, it is very important to reveal a repairing mechanism of α-crystallin. It is supposed that α-crystallin makes a complex with a denatured protein, for example denatured β-crystallin, when the α-crystallin fix the denatured protein. In order to observe making a complex with α-crystallin and the denatured protein, we adopt a small-angle X-ray scattering (SAXS). As the first step, we observe the solution with normal α-crystallin and normal β-crystallin. Here, we report the result of this preliminary experiment

EXPERIMENTS: Human αA-crystallin and βB_2-crystallin expressed by *E.Coli* were used as samples, and both mixed solution was also prepared. The concentrations of samples were tuned to be 1.0 mg/ml and the solvent was 20 mM Tris/HCl (pH 7.8) + 150 mM NaCl.
The SAXS experiments were carried out at room temperature with a SAXS apparatus (SAXES) installed at BL10C of Photon Factory in Institute of Materials Structure Science (IMSS), High Energy Accelerator Research Organization (KEK), Tsukuba, Japan. An X-ray beam (1.488 Å in wavelength) was used as a light source of SAXES and the intensity distribution of the scattered X-ray was measured by a one-dimensional position sensitive proportional counter. The magnitude of the scattering vector ($q=(4\pi/\lambda)\sin(\theta/2)$, where λ is the wavelength and θ is the angle of scatter) ranged from 7.0×10^{-3} to 1.5×10^{-1} Å^{-1}. The observed X-ray intensity was corrected for the buffer scattering and absorption, and then normalized with respect to the thickness of the sample (1 mm) and irradiation beam intensity. Typical irradiation time for sample was 1800 sec.

RESULTS: Figure 1 shows Guinier plots of SAXS profiles of αA-, βB_2-crystallins and their mixed solution, respectively. Gyration radius was calculated with Guinier formula, in which gyration radius is expressed by the slope of the straight line. The gyration radii of αA- and βB_2-crystallins were found to be 53 Å and 25 Å, respectively. These values are reasonable for their molecular weight. The mixed sample has an intermediates gyration radius (40Å) and the SAXS profile is expressed with a linear combination of those of αA and βB_2-crystallins. It means that there is no interaction between normal αA-and βB_2-crystallins. In the next, we will examine if there is the interaction between normal αA-crystallin and denatured βB_2-crystallin.

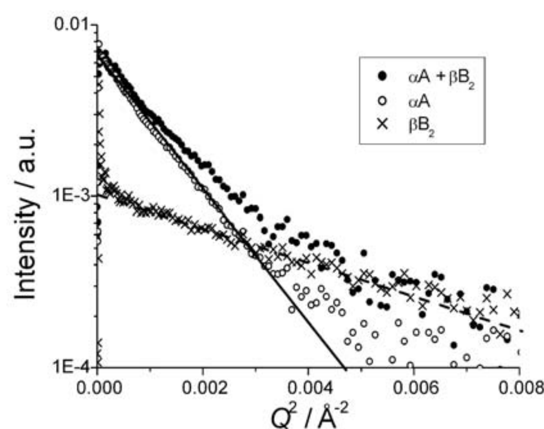

Fig. 1. Guinier plots of SAXS profiles of αA, βB_2-crystallins and the mixed solution. Open circles, crosses and closed circles exhibit αA, βB_2-crystallins and the mixed solution. In addition, straight lines show the result of least square fitting with Guinier formula.

採択課題番号 21P6-2　　タンパク質の凝集／会合過程の観測と動態解析　　　プロジェクト
（京大・原子炉）杉山正明、藤井紀子

PR6-3 3-Dimensional Structure Observation of Mammalian D-Aspartyl Endopeptidase by High-Resolution Atomic Force Microscopy

T. Kinouchi and N. Fujii

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INTRODUCTION

It is suggested that D-isomers of aspartic acid (D-Asp) containing protein are spontaneously generated by oxidative stress and would cause many aging-related protein-misfolding diseases such as Alzheimer's disease. We have identified a D-Asp-containing protein-specific protease, D-aspartyl endopeptidase (DAEP) from mammalian mitochondria, therefore DAEP would serve as a defensive system against the noxious D-Asp-containing protein. Previous study suggests that the conformation of DAEP forms a barrel-shaped oligomeric complex, however, it is still unclear how DAEP exerts its unique enzymatic function, because its higher order structure remains quite unsolved [1]. Therefore, in this study, we analyzed the structure by high-resolution atomic force microscopy (AFM), because high-resolution AFM is a powerful technique for naturally examining conformations and, stoichiometries of protein-protein complexes. We discuss here the topological analysis of DAEP by AFM.

EXPERIMENTS

Sample preparation: The purification of DAEP from mouse (DDY) liver was previously described [2]. The purified DAEP solution of 3.3 μg/ml protein in 50 μl of TEC (50 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.1% CHAPS) was deposited and incubated on freshly-cleaved mica for 2 min at room temperature, rinsed 5 times with 2 ml Milli-Q water and dried under nitrogen for 30 min.

Atomic force microscopy imaging: AFM scanning images were captured by a modified Nanoscope IIIa microscope (Veeco Instruments Inc., Santa Barbara, CA, USA) with D-NCH-10V etched silicon probes (Nanoworld AG, Neuchâtel, Switzerland) operating in tapping mode under ambient atmospheric pressure and temperature.

RESULTS & DISCUSSION

To gain a better understanding of the unique features of DAEP, we analyzed a higher order structure of DAEP by high-resolution AFM. As shown in Fig. 1, several particles (as indicated by arrows) with an average diameter of approximately 40 nm, were observed. Fig. 2 shows a higher magnified image of the particle whose shape is a ring-like structure consisting of at least 6-7 components. These data suggest that DAEP topologically belongs to the AAA+ protease family such as proteasome, Lon, and mitochondrial membrane-bound i-/m-AAA protease (a homologue of the bacterial FtsH protease). Insight from further structural analyses of DAEP may lead to a better understanding of its physiological function.

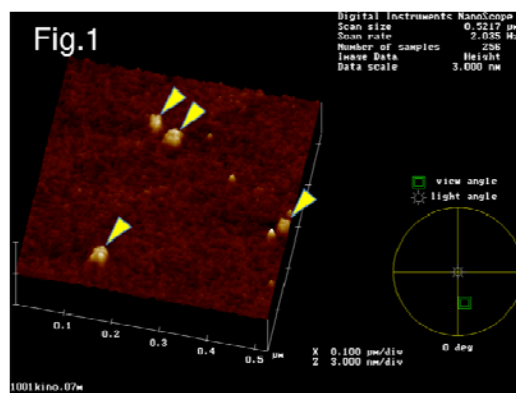

Fig. 1. 3-D image of DAEP particles analyzed by AFM.

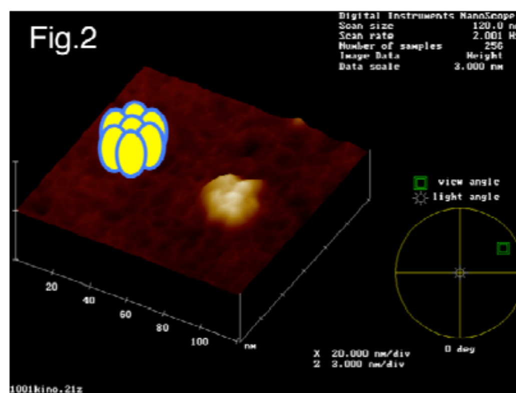

Fig. 2. 3-D close-up image of DAEP particle.

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[2] T. Kinouchi et al. *Biochem. Biophys. Res. Commun.*, **314** (2004) 730-736.

採択課題番号 21P6-3 D-アスパラギン酸含有蛋白質の蓄積モデルの構築とそれに特異的な分解酵素による品質管理機構の研究 プロジェクト（京大・原子炉）木野内忠稔、藤井紀子

PR6-4

Damage of Biological Molecules Induced by Ionizing Radiation and Biological Defense Mechanisms II

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IINTRODUCTION: Some bacteria show considerably high resistance to ionizing radiation [1]. A common feature of these bacteria is that these bacteria contain red carotenoid pigments [1, 2, 3]. Colorless mutants of these radioresistant bacteria are more sensitive than wild types to gamma-irradiation [1]. Therefore, these red carotenoid pigments are thought to contribute to defense mechanisms of these radioresistant bacteria against ionizing radiation. It is well known that biological effects induced by low LET ionizing radiation are caused mainly by radicals that is generated by radiolysis. Carotenoid pigments possess high radical scavenging activity. Carotenoid pigments are localized within the lipids of the cell surfaces in procaryote. From these things, we consider that red carotenoid pigments in radioresistant bacteria must defense the lipids of the cell surfaces of these bacteria against ionizing radiation.

When this biological defense mechanism of these radioresistant bacteria against ionizing radiation is considered, elucidating effects of carotenoid pigment in damage of biological molecules caused by ionizing radiation is important. We have reported that linolenic acid is peroxidized by gamma-irradiation in dose-dependent manner [4]. In this study, the effect of beta-carotene, which is the typical carotenoid pigment, in peroxidation of linolenic acid caused by gamma-irradiation was analyzed.

EXPERIMENTS: Sample Preparation: The linolenic acid was dissolved in benzene at a final concentration of 0.5 M and beta-carotene was added at final concentrations of 5 x 10^{-6} to 8.5 x 10^{-3} M. Gamma-Irradiation: The prepared solutions were irradiated with ^{60}Co gamma-rays at a dose of 30 kGy and a dose-rate of 30 kGy/h. Assay of Peroxidation of Linolenic Acid: The modified method of Buege and Aust was used [5]. TCA-TBA-BHT-HCl regent which contained 15% trichloroacetic acid, 0.38% thiobarbituric acid, 0.04% butylated hydroxytoluene, and 0.25 N hydrochloric acid was prepared. Gamma-irradiated sample was diluted 50 times with benzene. Three ml of diluted solution was evaporated in vacuo. The residue was dispersed in 9 ml of PBS(-) by sonicator. One ml of the dispersed solution was combined with 2.0 ml of TCA-TBA-BHT-HCl regent and mixed thoroughly. The mixed solution was heated for 15 min in a boiling water bath. The absorption at 532 nm of the reaction solution was measured. The amount of generated malondialdehyde (MDA) was calculated using the molar absorption coefficient (ε) of MDA, which is 1.56 x 10^{5} at 532 nm. In this study, the linolenic acid peroxidation was evaluated by the MDA amount.

RESULTS: In this experimental condition, 8.5 x 10^{-5} M beta-carotene significantly suppressed the peroxidation of linolenic acid caused by gamma-irradiation. On the other hand, 5 x 10^{-5} and 5 x 10^{-6} M beta-carotene significantly promoted the peroxidation of linolenic acid caused by gamma-irradiation. These results suggested that carotenoid pigments play a role in the protection against damage of lipid structure cased by gamma-irradiation in these radioresistant bacteria and concentrations of these carotenoid pigments in the cells are strictly regulated.

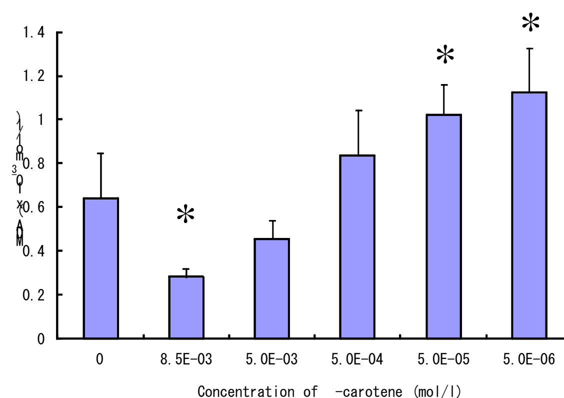

Fig. 1. The effect of beta-carotene on the amount of generated MDA by gamma-irradiation to linolenic acid at a dose of 30 kGy. Concentration of beta-carotene is shown on the horizontal axis, and the generated MDA amount is shown on the vertical axis. Each value is expressed as an average ± S.E. of three independent experiments. *: samples with a significant difference as compared with sample not added beta-carotene ($P < 0.05$).

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[1] T. Saito, Viva Origino, **30** (2007) 85-92.
[2] T. Saito *et al*, Arch. Microbiol., **162**, (1994) 414-421.
[3] T. Saito *et al*, Microbios, **95**, (1998) 79-90.
[4] T. Saito *et al*, KURRI Prog. Rep., (2008) 141.
[5] J. A. Buege and S. D. Aust, Meth. Enzymol., **52**, (1978) 302-310.

PR6-5 Establishment of Analytical Method for the Recombinant Asp-Isomerized Human α-A Crystallin Using Reversed-Phase HPLC

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School of Pharmaceutical Sciences, Kyushu Univ. Heal. Welf.
[1]*Research Reactor Institute, Kyoto University*

INTRODUCTION: α-Crystallin, which is the major protein of the mammalian lens and is necessary for the focusing of the light on the retina, is one of the well-known proteins in which Asp residues are altered to D-form. The structural alterations of L-aspartic acid (Asp) residues including isomerization and racemization occur spontaneously at a physiological temperature and pH, and resulted in generation of three isomerized Asp residues such as D-Asp, L-isoAsp, and D-isoAsp. The side chain hydroxyl linkage in Asp residue forms a succinimide intermediate by intramolecular rearrangement. The intermediate is quickly hydrolyzed, and a mixture of L-Asp and L-isoAsp is produced. The L-succinimide intermediate also undergoes reversible stereoinversion, which results in the generation of a D-succinimide intermediate. This intermediate is also quickly hydrolyzed, and a mixture of D-Asp and D-isoAsp is produced. The hydrolysis of the Asp residue in proteins or peptides, which also occurs spontaneously at physiological conditions, generates L-isoAsp, D-Asp or D-isoAsp residues through succinimide intermediates. Such modification of α-crystallin would affect the three-dimensional packing of the lens protein and may affect the transparency of the lens. Fujii *et al* (co-author of the article) have shown that a specific Asp residue located at 151 from N-terminus is highly changed to D-form in aged αA-crystallin protein, and more interestingly, D/L ratio of the Asp residues is more than 1.

Various treatments such as heating, UV irradiation and radiation have reported to affect the rate of structure alteration of Asp residue in the protein and peptide. To investigate and elucidate these phenomena, we have to establish the easy and reproducible analytical method for the structural alteration of Asp residue in the αA-crystallin protein, since the determination of the isomerization/stereoinversion of amino acid residue is time consuming and not reproducible. Traditionally, the determination performed by a combination of amino acid sequencing analysis, mass spectrometry, and enantiomeric analysis. In the enantiomeric analysis, the hydrolysis of proteins is required and resulted in the generation of additional D-enantiomers. We have established a simple method to quantify the structural alterations of Asp residues in proteins or peptides by reversed-phase (RP) HPLC with a standard octadecylsilane (ODS) column. In the present analytical method, the estimation of the four structurally altered Asp residues (L-Asp, D-Asp, L-isoAsp, and D-isoAsp) can be simultaneously estimated by carrying out a single HPLC analysis of quantification of each peptide constructing αA-crystallin protein.

RESULTS and DISCUSSION: Recombinant human αA-crystallin was expressed in *E. coli* and purified by subsequent column operations. The purified protein (2 mg/mL) was dissolved in 50 mM phosphate buffer (pH 7.4) and incubated at 37 °C for 140 days. The aged protein was digested by TPCK-treated trypsin, and the digestion product was separated by RP-HPLC with a linear gradient of 0 – 30 % acetonitrile containing 0.1 % TFA (Fig. 1A). The fractions that included the four types of T6 ([55]TVLDSGISEVR[65]) and T10 ([79]HFSPEDLTVK[88]) peptides were recovered (indicated by line), concentrated, and used as a sample for analyzing the isomerization of Asp residues. The samples were separated by RP-HPLC with acetonitrile-sodium phosphate solution as a mobile phase (Fig.1B). The standard peptides that contained L-Asp and L-isoAsp residues were analyzed under same condition as a standard (Fig. 1C). We developed a RP-HPLC technique that enabled the quantitative and sensitive analysis of the isomerized Asp residues in two digested peptides of αA-crystallin protein. The present method will be used for analyzing the effect of radiation on the structural alteration of Asp residue in the protein.

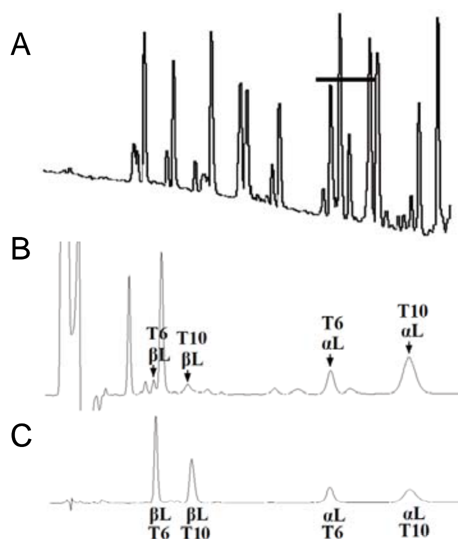

Fig.1. HPLC profiles of the peptide αA-crystallin protein.

採択課題番号 21P6-5 タンパク質中のアスパラギン酸残基付近の放射線による構造と機能変化の解析 プロジェクト
（九州保福大・薬）定金 豊 （京大・原子炉）藤井紀子

PR6-6 Conformational Change of Tryptophanase on the Basis of Comparison with γ-Tryptophanase

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[2]*Research Reactor Institute, Kyoto University*

INTRODUCTION: Enzyme stereospecificity on optical isomers is generally considered absolute, which is the basic principle for birth of life. Tryptophanase is also known as one of the enzymes with extremely rigid stereospecificity, cleaving L-tryptophan into indole, pyruvate and ammonium by catalyzing hydrolytic β-elimination, or synthesizing L-tryptophan from indole and L-serine by β-replacement. It completely has no activity on either D-tryptophan or D-serine in ordinary reaction condition. However, when tryptophanase is in ammonium phosphate solution, it becomes active toward D-tryptophan or D-serine — quite different from what is expected. However, it is reversibly reverted to the original enantioselectivity if ammonium phosphate is removed. The only salts inducing the activity are diammonium phosphate, triammonium phosphate and ammonium sulfate, although other salts don't have the activity. Why does such reversible interconversion of enzyme stereospecificity occurred? What enables it? Various questions will naturally spring from our results. The first thing that comes to our mind is that a main cause of such stereospecific change may be attributable to drastic stereostructual change. For example, Figure.1 imaginarily illustrates how drastically tryptophanase changes in the presence of diammoniumhydrogen phosphate. Here, let's say this is an original conformation of tryptophanase. Here we assume tryptophanase has grobule structure. Its tertiary structure receives progressively large changes through the effect of DAP. The conformational change is drastic very much, altering tryptophanase stereoselectivity to express the activity on D-amino acids.

Fig.1. An imaginary illustration of drastic conformational change of tryptophanase in ammonium phosphates solution.

Thus, the conformational change of tryptophanase is here measured on the basis of circular dichroism spectra to confirm whether such drastic change occurs.

EXPERIMENTS: Source intensity and absorption dose of cobalt 60 was 151 TBq and 11 kGy/h. Apotryptophanase purchased from Sigma Chem. Co. (St. Louis, USA) was prepared to a concentration of 200 μg/ml with 0.1 M potassium phosphate buffer solution of pH 8.3 including 1.1 mM pyridoxal 5′-phosphate. Tryptophanase was irradiated with dose of 0 – 11 kGy at room temperature. CD spectrum of γ-tryptophanase was measured in a 200 μℓ cell with 0.1 cm path length or a 3 mℓ call with 1 cm path length to analyze secondary structural change. Spectra were recorded at wavelengths from 200 to 400 nm at room temperature. Scans were repeated five times per a spectrum, averaged, and expressed as molar ellipticity in degrees cm^2 $dmol^{-1}$. The CD spectra were analyzed with a CDPro software package and calculated by subtracting a blank prepared identically but without Tryptophanase. They were compared with the CD spectra of Tryptophanase in three ammoniumphosphate solutions of 50 % saturation. Unless otherwise stated, reagents were obtained from Wako Pure Chem. Co. Ltd. (Osaka).

RESULTS: γ-tryptophanase is measured with CD spectrophotometer. γ-rays were irradiated with 1 kGy and 2 KGy. Secondary structure of tryptophanase largely changed between 200 and 250 nm, with tertiary structural changes from 250 to 300 nm. Drastic changes of secondary structure were seen in γ-tryptophanase despite low irradiation.

On the other hand, circular dichroism on tryptophanase in ammonium phosphate solution showed the small degree of conformational change. In these spectra, tryptophanase in diammoniumhydrogen phosphate was almost similar to one in phosphate buffer between 200 and 350 nm, indicating secondary and tertiary structure didn't change at all, or only allowed any slight alteration, though detectable change was observed in monoammonium dihydrogen phosphate because it denatured tryptophanase. Diammoniumhydrogen phosphate probably gave only minor reversible change to tryptophanase stereostructure, judging from spectral analytic results of circular dichroism spectra. It responsively conducted D-tryptophan degradation or L-tryptophan synthesis from D-serine. This result indicates enzyme stereospecificity is more flexible than we think. We could say the exclusive selection of L-amino acid may be underpinned by a delicate balance between subtle streic structural difference of enzyme.

採択課題番号 21P6-6　　酵素の立体選択性の変化に及ぼす放射線照射の影響　　　プロジェクト
（筑波大・生命環境科学研究科）島田秋彦　(京大・原子炉)藤井紀子，齋藤　毅

PR6-7 Inversion and Isomerization of an Aspartyl Residue Affect the Properties of Peptides

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IINTRODUCTION: Proteins have been considered to consist exclusively of L-amino acids in living tissues. However, our previous studies showed that two specific aspartyl (Asp) residues in αA- and αB-crystallins, respectively, from human eye lenses invert to the D-isomers to a high degree during aging. The reaction is also accompanied by isomerization into a form containing β-Asp (isoaspartate) residues. The appearance of D- and β-Asp in protein potentially induces large changes to the higher order structure of the protein as well as its function. However, it remains unclear whether the formation of the Asp isomer is the direct trigger of the change to the higher order structure and function. In this study, we synthesized peptides corresponding to 70-88 (KFVIFLDVKHFSPEDLTVK) of human αA-crystallin and its corresponding diastereoisomers in which Lα-Asp was replaced with Lβ-Asp, Dα-Asp, and Dβ-Asp residue at position 76 and compared their biochemical properties with that of normal peptide.

EXPERIMENTS: The four isomers of the peptide KFVIFLDVKHFSPEDLTVK, corresponding to the amino acid sequence 70-88 of human αA-crystallin, were synthesized with the Lα-, Dα-, Lβ- and Dβ-isomers of Asp-76 by Peptide 2.0 Inc. (USA). We performed the biochemical properties of these four isomers (αAP-Lα, αAP-Dα, αAP-Lβ, αAP-Dβ) using the HPLC analysis, MALDI-TOFMS analysis and circular dichroism (CD) analysis. The aggregation of insulin in the presence of these four isomers was monitored using the ThT fluorescence intensity at 415 nm.

RESULTS: As shown in Fig. 1, the abnormal isomers (αAP-Dα, αAP-Lβ, αAP-Dβ) were more hydrophilic than the normal peptide (αAP-Lα). CD spectrum showed that their β-sheet structures lost and changed to random structures. The normal peptide promoted the aggregation of insulin while the other three isomers suppressed the aggregation of insulin (Fig. 2).

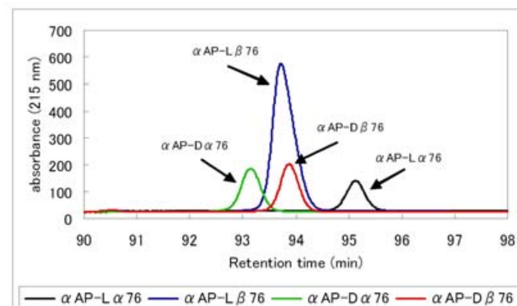

Fig. 1. Elution profile of the αA-crystallin 70-88 peptide isomers in which the normal Lα of Asp-76 was replaced with Dα- Lβ- and Dβ- isomers.

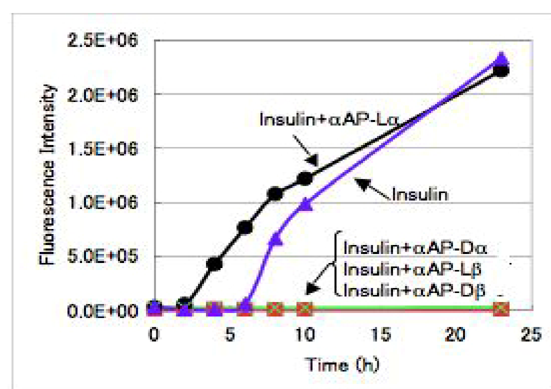

Fig. 2. ThT fluorescence assay for the effects of the peptide isomers (0.25 mg/mL) on the aggregation of insulin (2 mg/mL insulin).

CONCLUSION: Generally, one point mutation in a protein seldom occurs within living tissues of mammals, however, the isomerization of Asp residues in protein or peptide occurs spontaneously and easily through a succinimide intermediate under physiological conditions. This study clearly indicates that a single substitution of an Asp isomer in a peptide induces a large change to the properties of the peptide.

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