生体物質関連化学 ゲノム化学に基づくインテリジェント分子の創製

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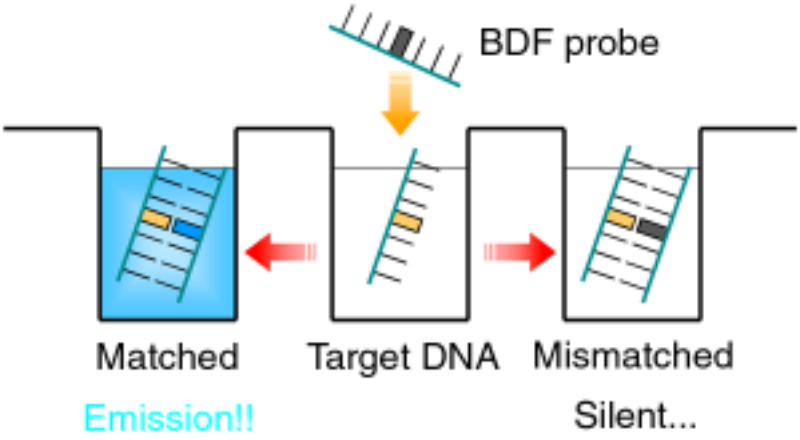
齋藤　烈（さいとういさお）
1941 年福島生まれ。1968 年京都大学大学院工学研究科博士課程修了。京都大学工学部助手、助教授を経て 1991 年京都大学工学部教授。専門　生物有機化学。この 10 年間はゲノム化学の研究が中心。1996 年－2001 年科学技術振興事業団 CREST 研究代表者、2002 年－SORST 研究代表者。日本化学会、光化学協会、近畿化学協会、日本光医学・光生物学会などの理事、評議員。IUPAC 有機化学部門 TITLAR MEMBER, アメリカ光生物学会 COUNCILOR。受賞、日本化学会進歩賞、光化学協会賞、日本化学会賞、米国光生物学協会功績賞、IUPAC Fellow, AAAS Fellow.

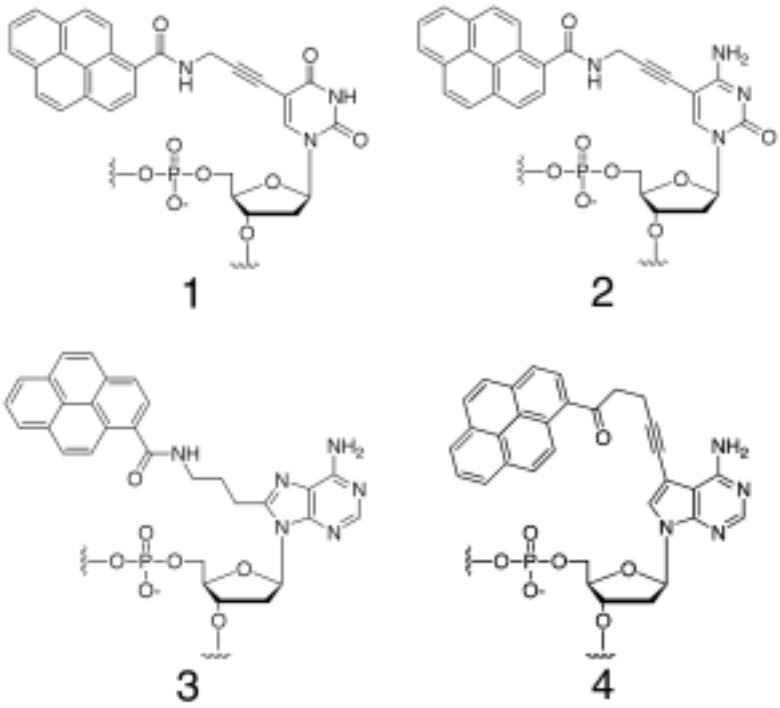
ゲノム化学（Chemical Genomics）とは、ゲノム、プロテオミクスなどバイオ全般に関して化学をベースとして行う研究を指し、ゲノム創薬、遺伝子診断、バイオチップ、バイオセンサー、バイオナノ材料などきわめて広い応用分野がある。将来の巨大なゲノム産業創出のためには、“物の作り出せる”ゲノム化学の研究がきわめて重要である。本 COE 拠点研究での目的は、最新の化学と分子生物学的手法を結集して、ゲノム科学やバイオナノテク産業に役立つインテリジェント分子を創製することにある。具体的には、１）DNA の１塩基多型（SNPs）を検出するための画期的手法の開発、２）DNA を経る電子移動のモニタリングと DNA ナノワイヤーの開発、３）電気的な DNA 変異検出法の開発である。

（１）相手塩基を見分けるインテリジェント蛍光性核酸塩基の設計と SNP チップの開発[1-5]

簡便かつハイスループットな SNPs（１塩基多型）検出法を開発することは、将来不可欠な遺伝子診断法を確立するためには不可欠であり、世界中で凌ぎを削っている分野である。我々は、従来とは全く異なる新しいコンセプトに基づいて、相補鎖上の相手塩基を見分け特定の相手塩基のときに選択的に蛍光を発する新しいインテリジェント核酸塩基 **BDF** 塩基（**B**ase-**D**iscriminating **F**luorescent Base）を４種類開発した。これらの塩基は、二本鎖 DNA の内部でそれぞれ特定の塩基と対合したときに選択的に強い蛍光を発光させる事ができた。これら BDF 塩基を DNA オリゴマーに導入した BDF プローブを用いる homogeneous SNP typing キットならびにこれらをチップにのせた DNA チップの開発研究を行っている。

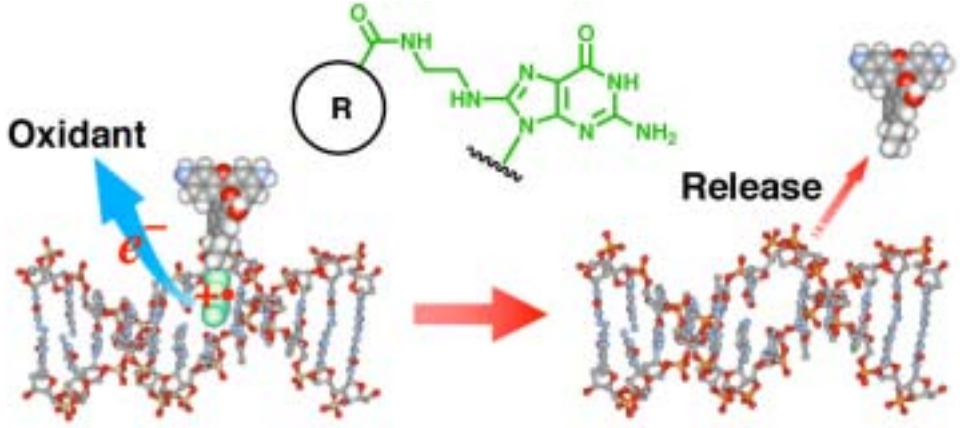
開発した BDF 塩基は、周りが AT 塩基対の時には、全ての場合に選択的に強い蛍光を発するが、BDF 塩基 **3** および **4** は GC 塩基対が隣接していると蛍光が弱くなるので、現在塩基配列に依存しない BDF 塩基を新たに開発中である。





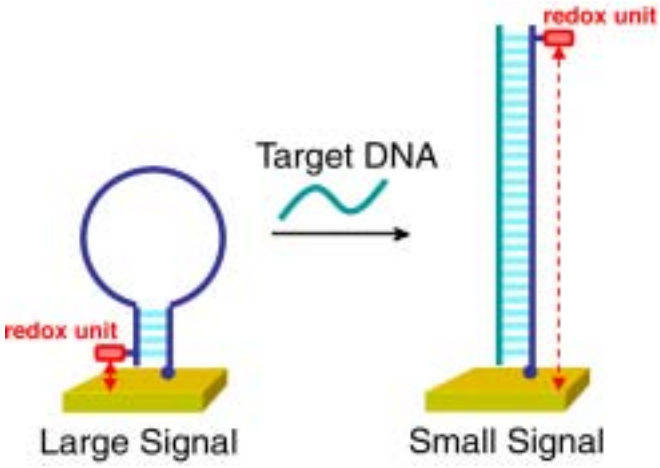
（２）一電子酸化によりレポータータグを放出するヌクレオシドの開発と応用[6]

ＤＮＡの酸化によってレポータータグを放出する核酸塩基を開発した。近年、迅速かつシンプルな方法で標的ＤＮＡから有用な情報を得る手法の開発が極めて重要になってきている。しかしながら、これまで酸化や光照射などをトリッガーとして、有用な機能をもつレポータータグ分子をＤＮＡから放出させられるようなＤＮＡプローブは、全く研究されていなかった。酸化や光照射のような外部からの刺激によって引き起こすことができる分子放出系の構築は、ドラッグデリバリーシステムへの応用のみならず、遺伝子解析への展開においても重要なツールになるもの考えられる。我々は、この新しいコンセプトに基づき新規核酸塩基 ^{eda}G を開発した。この塩基は、一電子酸化によって分解して効率的にレポータータグを放出する機能を持つ。オリゴヌクレオチドに導入された ^{eda}G は、様々な穏和な酸化剤（リボフラビンやイリジウムイオンなど）やＤＮＡ内ホール移動によって分解され、系を着色する色素など様々な分子を酸化剤の量に応じて放出する。したがって、この技術は、ドラッグリリースシステムとしてだけでなく、遺伝子操作における蛍光性タグの放出・検出のために有効である。



（３）ＤＮＡ変異の電気化学的検出

電気的なＤＮＡ検出法の開発は、多くの電気メーカーはじめベンチャーが争って行っている研究である。しかしながら、いずれもその基本となるコンセプトが不鮮明でこれまであまり成功しているとは言い難い。我々は、末端にレドックスユニット（アントラキノンやルミフラビンなど）を連結したヘアピン型のＤＮＡによって修飾した新しい電極チップを開発した。この電極では、微分パルスボルタンメトリーで電気的にＤＮＡを検出することができる。目的のＤＮＡが系中に存在していない状態では強い電流応答を示すが、目的のＤＮＡが系中に加えられると、二本鎖を形成し、電気信号は顕著に小さくなった。



Development of Intelligent Molecules Based on Chemical Genomics

Isao Saito

Born in 1941. He received his Ph.D. degree from Kyoto University. He was an assistant professor in Department of Synthetic Chemistry, Faculty of Engineering, Kyoto University (1968-1987), a postdoctoral fellow in USA and Canada (1972-1973), an associate professor in Department of Synthetic Chemistry, Faculty of Engineering, Kyoto University (1987-1991) and a professor in Department of Synthetic Chemistry, Faculty of Engineering, Kyoto University (1991-1993). Now he has been a professor in Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University (1993-). He was also Councilor of American Society for Photobiology (1993-1995), IUPAC Titlar Member (Organic Chemistry Division) (1994-1998), CREST Research Project Leader, Japan Science and Technology Corporation (JST) (1996-2001), IUPAC Fellow (International Advisor) (1999-), and SORST Research Project Leader, Japan Science and Technology Corporation (JST) (2002-). He got Naito Foundation Award, Japan Photochemistry Association Award, Asahi Glass Research Fund Award, the Japan Chemical Society Award, and the AAAS Fellow

(1) Base-discriminating Fluorescent Nucleobases and Their Application to Single Nucleotide Polymorphism Typing：We designed novel base-discriminating fluorescent (BDF) nucleobases, and applied to single nucleotide polymorphism (SNP) typing. We devised novel BDF nucleosides, ^{Py}**U** (**1**) and ^{Py}**C** (**2**), which contain a pyrenecarboxamide chromophore connected by a propargyl linker. The fluorescence spectrum of the duplex containing a ^{Py}**U**/A base pair showed a strong emission at 397 nm on 327 nm excitation. In contrast, the fluorescence of duplexes containing ^{Py}**U**/N base pairs (N = C, G, or T) was considerably weaker. The proposed structure of the duplex containing a matched ^{Py}**U**/A base pair suggests that the high polarity near the pyrenecarboxamide group is responsible for the strong A-selective fluorescence emission. Moreover, the fluorescence of the duplex containing a ^{Py}**U**/A base pair was not quenched by a flanking C/G base pair. The fluorescence properties are quite different from previous BDF nucleobases, where fluorescence is quenchable by flanking C/G base pairs. The duplex containing the C derivative, ^{Py}**C**, selectively emitted fluorescence when the base opposite ^{Py}**C** was G. The drastic change of fluorescence intensity by the nature of the complementary base is

extremely useful for SNP typing. ^{Py}**U**- and ^{Py}**C**– containing oligodeoxynucleotides acted as effective reporter probes for homogeneous SNP typing of DNA samples containing c-Ha-*ras* and BRCA2 SNP sites.

(2) A Novel Nucleobase that Releases Reporter Tags upon DNA Oxidation: DNA biosensors offer considerable promise for extracting information from target genes in a quick and simple manner. Various DNA probes that give signals in a sequence-specific fashion, as represented by molecular beacons, have been widely used. However, there are very few DNA probes that can release useful functional molecules. A molecular releasing system that is triggered by external stimulation such as oxidation or photoirradiation would be a useful tool for gene analysis. We developed a novel nucleosbase, ^{eda}G, that efficiently releases reporter tags upon one-electron oxidation. The ^{eda}G-selective degradation of ODNs was achieved by various mild oxidizing agents (riboflavin and iridium (IV) salt). This oxidant-dependent molecular releasing technique is useful not only for drug releasing systems but also for the release of fluorescent tag after gene analysis.

(3) Electrochemical DNA Detection Using Redox-active Hairpin DNA: DNA hybridization biosensors offer considerable promise for obtaining sequence information of genes in a fast and simple manner. Various DNA probes that give signals in a sequence-specific fashion, as represented by molecular beacons, have been widely used. Recently, the molecular beacon-like hairpin DNA probes immobilized on a gold surface has been examined for gene diagnosis. By the hybridization with target sequence, the structure of these probes changes from the hairpin structure to the double strand state. With this structural change, the distance between a gold surface and a reporter tag of the probe end, such as redox-active units and fluorophore, extends, and thus, the change of the response, such as decrease of current or increase in the fluorescence intensity, is generated. If a diversity of the reporter tag extends further, detection and distinction of various sequence will become easy using this technique. We devised new electrochemical DNA probes for DNA detection. We synthesized hairpin DNA probes labeled by flavin and anthraquinone. The flavin- and anthraquinone-labeled DNA probes were readily synthesized, and immobilized on gold electrodes. The current signal of DNA probe was sensitively changed by the hybridization with a target DNA. This electrochemical probe was useful for SNP typing.

Selected Publications

· Presentation & Lectures

1. I. Saito, “Design of Base-Discriminating Flurescent Nucleobase for SNP Typing”, 14 The International Congress on Photobiology (ICP 2004), 2004.6.10-6.15, Jeju, Korea (Invited)

· Articles

1. "Clear Distinction of Purine Bases on the Complementary Strand by a Fluorescence Change of a Novel Fluorescent Nucleoside", A. Okamoto, K. Tainaka, I. Saito, *J. Am. Chem. Soc*., **125**, 4972-4973 (2003)
2. "Design of Base-discriminating Fluorescent Nucleoside and Its Application to T/C SNP Typing", A. Okamoto, K. Tanaka, T. Fukuta, I. Saito, *J. Am. Chem. Soc.*, **125**, 9296-9297 (2003)
3. "Pyrene-labled Base-discriminating Fluorescent DNA Probes for Homogeneous SNP Typing", A. Okamoto, K. Kanatani, I. Saito, *J. Am. Chem. Soc.*, **126**, 4820-4827 (2004)
4. "Cytosine Detection by a Fluorescein-labeled probe Containing Base-discriminating Fluorescent Nucleobase", A. Okamoto, K. Tanaka, T. Fukuta, I. Saito, *ChemBioChem*, **5**, 958-963 (2004)
5. "Pyrene-labeled Oligodeoxynucleotide Probe for Detecting Base Insertion by Excimer Fluorescece Emission", A. Okamoto, T. Ichiba, I. Saito, *J. Am. Chem. Soc.*, **126**, 8364-8365 (2004)
6. "A Novel Nucleobase that Releases Reporter Tags upon DNA Oxidation", A. Okamoto, K. Tanaka, I. Saito, *J. Am. Chem. Soc.*, **126**, 416-417 (2004)

生体関連物質化学 金属置換基による４π電子環状反応の制御

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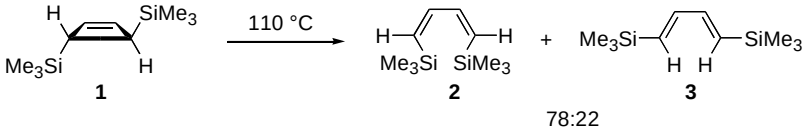
1956年生まれ。1979年東京大学理学部卒。1984同大大学院博士課程修了。理学博士（東京大学）。1984年東京大学理学部助手、1987年京都大学工学部助手、1993年同助教授を経て、2002年１月より同大学大学院工学研究科合成・生物化学専攻教授、現在に至る。1989年日本化学会進歩賞。

（１）*trans*-3,4-bis(trimethylsilyl)cyclobuteneの熱的開環反応

trans-3,4-bis(trimethylsilyl)cyclobutene **1** はかさ高い置換基のために inward 旋回における立体反発は増大するが、電子的効果による安定化がこれにうち勝つかどうか検討した。**1** を *n*-decane 中 110 °C で加熱したところ、熱的開環反応が同旋的に進行し、*Z,Z*-diene **2** と *E,E*-diene **3** の２種類の開環生成物を与えた。興味深いことに inward 旋回における非常に大きな立体障害にも関わらず *Z,Z*-diene が 78：22 の比で有利に生成した（式 1）。これは以前報告したモノシリル体よりも高い選択性である。



(1)

これについて理論計算を行った結果、inward旋回の遷移状態はoutward旋回よりも0.51 kcal/mol低く、実験結果との一致が見られた(Figure 1)。興味深いことにinward旋回の反応経路には*s*-cis配座がエネルギー最小値として存在しない。これはトリメチルシリル基の立体障害がいかに大きいかを示している。Figure 2にNatural Bond Orbital解析の結果を視覚化した遷移状態を示す。これによって二つのシリル基のσ^*軌道とHOMO電子との相互作用がinward旋回の遷移状態安定化に作用していることが示唆された。この二重の安定化効果が今回の異常な*Z,Z*-diene選択性を発現していると考えられる。本反応はケイ素の電子的な効果が立体化学の制御因子として働く典型的な例である。

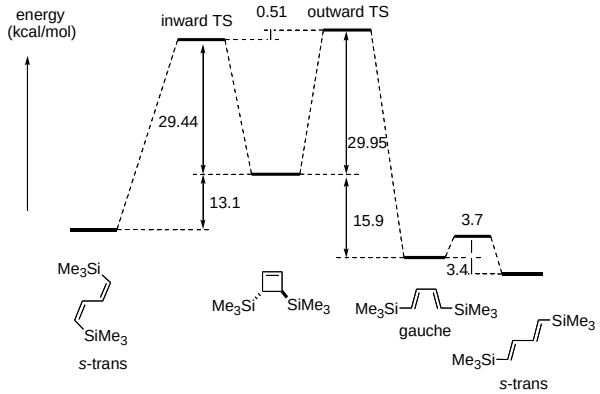


Fig. 1

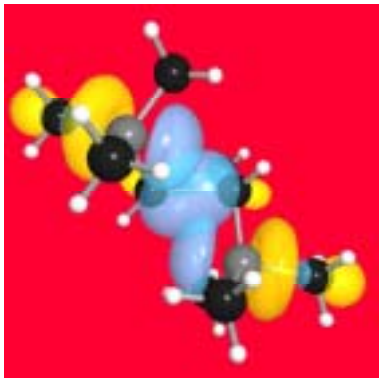
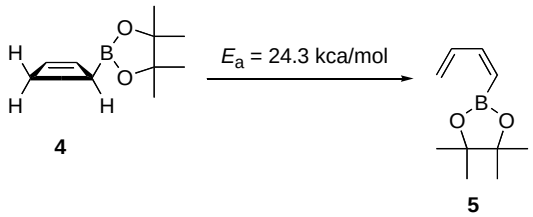


Fig. 2

（２）3-borylcyclobuteneの熱的開環反応

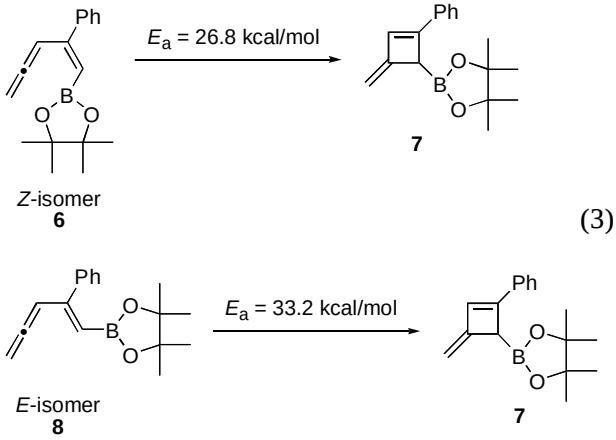
ホウ素はケイ素に比べて電気陰性である一方、エネルギー準位の低い空の p 軌道を有するため Lewis 酸として働く。今回、この空の p 軌道をシクロブテンの開環反応における HOMO 電子の受容軌道として着目し、3-borylcyclobutene **4** の熱的開環反応について検討を行った。**4** は toluene-d_8 中 90 ℃で開環し、驚くべきことにインワード旋回による Z 体のジエン **5** のみを与えた（式２）。シクロブテンの開環反応において、ホウ素の空の p 軌道が関与する協奏反応は珍しく、重要な知見である。



4 **5** (2)

（３）vinylalleneの熱的閉環反応におけるホウ素置換基の効果

1,2,4-pentatriene (vinylallene) と methylenecyclobutene は同等の熱力学的安定性を有しているため、熱反応においてはそれらの平衡混合物を与えることが知られている。上述の電子環状反応におけるホウ素の置換基効果の知見をもとに、5-boryl-1,2,4-triene の *Z* 体 **6** および *E* 体 **8** の熱的閉環反応の検討を行った。*Z* 体を *m*-xylene 中、140 °C で加熱したところ、3 時間で反応は完結し対応する閉環化合物 **7** を与えた。さらに速度論的研究により、*Z* 体の活性化エネルギーは 26.8 kcal/mol であることが明らかになった（式３）。*E* 体の閉環反応も同条件で完結し、**7** を与えた。しかしその反応速度は非常に遅く、*E* 体の活性化エネルギーは 33.2 kcal/mol で、*Z* 体に比べて 6.4 kcal/mol も大きいことがわかった。この結果もまたホウ素置換基の p 軌道の電子受容効果によって解釈できる。



(3)

4π Electrocyclic Reactions Dictated by Metal Substituents

Masahiro Murakami

Born in 1956. He received his Ph.D. from The University of Tokyo under the direction of Professor T. Mukaiyama in 1987. He held the position of Assistant Professor at the University of Tokyo (1984-1987) and Kyoto University (1987-1993). From 1991 to 1992, he worked as a postdoctoral fellow with Professor A. Eschenmoser at ETH Zürich, Switzerland. He was promoted to Associate Professor in 1993 and full Professor in 2002 at Kyoto University. He received The Chemical Society of Japan Award for Young Chemists in 1989.

(1) Thermal Ring-Opening Reaction of trans-3,4-Bis(trimethylsilyl)cyclobutene: *Trans*-3,4-bis (trimethylsilyl) cyclobutene (**1**), synthesized in four steps from cyclobutene-3,4-dione, was heated at 110 ° C. It underwent ring-opening in a conrotatory fashion to afford a mixture of*Z,Z*-1,3-diene **2** and

the corresponding *E*,*E* isomer **3** (eq 1). Remarkably, the *Z*,*Z* isomer **2** predominated over **3** by a ratio of 78:22. The two trimethylsilyl substituents preferred inward rotation despite the significantly greater steric constraints on this reaction pathway. We attribute this counterintuitive rotational behavior to the electron-accepting nature of the antibonding orbitals on the silicon atoms. The calculated inward transition state is shown Fig. 1, together with the natural bond orbital (NBO) overlap image for the two antibonds on the silicon atoms, and the breaking σ bond, i.e., the HOMO of the opening cyclobutene skeleton (Fig. 2). This figure clearly shows the overlap of the antibonding orbitals with the HOMO. Electronic stabilization arising from the dual delocalization of the HOMO electron density into the two antibonding orbitals overcomes the steric congestion at the inward transition state, leading to the predominance of **2**.

(2) Contrasteric Stereochemical Dictation of the Cyclobutene Ring-Opening Reaction by a Vacant Boron p Orbital: Whereas boron is even less electronegative than silicon, it has a vacant p orbital which is energetically low-lying. It can accept electron density from another molecule (Lewis acid– base complexation), or the inside of the molecule (delocalization or conjugation). We expected more dramatic effects for a boron substituent than a silyl substituent. 3-Borylcyclobutene **4** was synthesized in nine steps starting from dimethylphenylvinylsilane. The cyclobutene **4** was heated in toluene-d_8. The ring-opening reaction proceeded even at 90 ° C and led to completion in 15 h. (*Z*)-1-Borylbuta-1,3-diene **5** was exclusively formed by inward rotation (eq 2). Thus, the effects of a boryl substituent predicted by Rondan and Houk were experimentally confirmed. Electronic participation of the vacant p orbital of the boryl substituent explains these results well.

(3) Effects of Boryl Substituents on Thermal Ring-Closing Reaction of Vinylallenes: Both thermodynamic and kinetic properties of the electrocyclic ring-opening and -closing reactions between cyclobutene derivatives and conjugated dienes change according to the structural variation with additional unsaturation. 1,2,4-Pentatriene (vinylallene) and methylenecyclobutene possess comparable thermodynamic stabilities such that thermal treatment produces an equilibrium mixture of them. *Z*- and *E*-isomers of 5-boryl-1,2,4-triene were synthesized and their reactivities were compared. Heating the *Z*-isomer in xylene at 140 ° C affected the electrocyclic ring-closing reaction in 3 h, providing the ring-closed product in 96% yield (eq 3). The kinetic studies showed that the reaction was first order with an activation energy of 26.8 kcal/mol. The *E*-isomer also underwent ring-closure leading to **7** unidirectionally. However, the reaction was much slower than that of **6**. An activation energy was 33.2 kcal/mol. It is of note that the activation energy for **8** is 6.4 kcal/mol greater than that of the *Z*-isomer **6**. Thus, a large difference between the activation energies for the *Z*- and *E*-stereoisomers was observed. These results can be understood by assuming electronic participation of the vacant p orbital of the boryl substituent.

Selected Publications

Presentation & Lectures

"The Stereochemistry of Electrocyclic Reactions Dominated by Hyperconjugatio Rather Than Sterics" 38th ESF/Euchem Conference on Stereochemistry, April 30, 2003, Bürgenstock Resort, Switzerland

"Hyperconjugation Rather Than Sterics Dominates the Ring-Opening Stereochemistry of Cyclobutene" CMDS Symposium 2003, October 10, 2003, KAIST (Korea Advanced Institute of Science and Technology), Korea

"Torque Control by Metal-Orbital Interactions" The Second Kansai-CMDS Meeting on OMCOS, 2004, September 26, 2004, Haevichi Resort, Korea

Articles

1. "Aminoboranes as "Compatible" Iminium Ion Generators in Aminative C– C Bond Formations", M. Suginome, L. Uehlin, M. Murakami, *J. Am. Chem. Soc.,* **126,** 13196 (2004)
2. "Synthesis and Thermal Ring-Opening of trans-3,4-Disilylcyclobutene", M. Murakami and M. Hasegawa, *Angew. Chem. Int. Ed.,* **43**, 4874 (2004)
3. "Dramatic Effects of Boryl Substituents on Thermal Ring-Closing Reaction of Vinylallenes", M. Murakami, S. Ashida, and T. Matsuda, *J. Am. Chem. Soc.,* 126, 10838 (2004)
4. "Eight-Membered Ring Formation via Olefin Insertion into a Carbon– Carbon Single Bond", T. Matsuda, A. Fujimoto, M. Ishibashi, and M. Murakami, *Chem. Lett.,* **33**, 876 (2004)
5. "Stereoselective Synthesis of (Z)-1-Silyl-2-stannylethene by Palladium-Catalyzed Silastannation of Ethyne and Its Synthetic Transformations, M. Murakami, T. Matsuda, K. Itami, S. Ashida, and M. Terayama, *Synthesis*, 1522, (2004)
6. "Synthesis of Tertiary Propargylamines by Sequential Reactions of in Situ Generated Thioiminium Salts with Organolithium and -magnesium Reagents", T. Murai, Y. Mutoh, Y. Ohta, and M. Murakami, *J. Am. Chem. Soc.,* **126**, 5968 (2004)
7. "Rhodium-Catalyzed Addition/Ring-Opening Reaction of Arylboronic Acids with Cyclobutanones", T. Matsuda, M. Makino, and M. Murakami, *Org. Lett.,* **6**, 1257 (2004)
8. "A New Look at Boron Enolate Chemistry: Aminative C– C Bond Formation Using Diaminoboron Enolate with Aldehyde", M. Suginome, L. Uehlin, A. Yamamoto, and M. Murakami, *Org. Lett.,* **6**, 1167 (2004)
9. "Theoretical and Experimental Studies on the Thermal Ring-Opening Reaction of Cyclobutene Having a Stannyl Substituent at the 3-Position", M. Murakami, M. Hasegawa, and H. Igawa, *J. Org. Chem.,* **69**, 587 (2004)
10. "Enantioface-Selective Palladium-Catalyzed Silaboration of Allenes via Double Asymmetric Induction", M. Suginome, T. Ohmura, Y. Miyake, S. Mitani, Y. Ito, and M. Murakami, *J. Am. Chem. Soc.,* **125**, 11174 (2003)
11. "Palladium- and Nickel-Catalyzed Intramolecular Cyanoboration of Alkynes", M. Suginome, A. Yamamoto, and M. Murakami, *J. Am. Chem. Soc.,* **125**, 6358 (2003)
12. "Ruthenium-Mediated Regio- and Stereoselective Alkenylation of Pyridine", M. Murakami and S. Hori, *J. Am. Chem. Soc.,* **125**, 4720 (**2003**)
13. "New Catalyzed Three-Component Cycloadditions for the Synthesis of Eight-Membered Carbocycles", M. Murakami, *Angew. Chem., Int. Ed.,* **42**, 718 (2003)

Book

"Cycloadditions of Allenes", M. Murakami and T. Matsuda, In *Modern Allene Chemistry*; Klause, N., Hashmi, A. S. K., Eds.; Wiley-VCH: Weinheim, 2004; Chapter 12

生体関連物質化学 ヘム蛋白質の分子工学

工学研究科　分子工学専攻　森島　　績・石森浩一郎

森島　績（もりしま　いさお）
1940 年名古屋市生。1963 年京都大学工学部燃料化学科卒業。1965 年同大学院燃料化学専攻修士課程修了。同年同博士課程中退。工学博士（京都大学）。1965 年京都大学工学部石油化学化助手、1971 年同助教授を経て 1989 年同大学院工学研究科分子工学専攻教授、2004 年定年退官。現在京都大学名誉教授。この間、1975 年から 1 年間米国 NIH 客員研究員、1995 年から 1 年間分子科学研究所客員教授併任。アメリカ生化学・分子生物学会名誉会員。一貫して金属酵素蛋白質の構造と機能ならびに分子設計に関する分子生物化学、生物物理化学、ならびに蛋白質分子工学的研究に従事している。平成 12 年度から 15 年度まで文部科学省科学研究費補助金特別推進研究「構造および機能単位としてのモジュールを組み合わせた新規蛋白質の分子設計と創製」を遂行した。1996 年より日本学術振興会審査会専門委員、学術審議会専門委員などを歴任。

石森　浩一郎（いしもり　こういちろう）
1961 年京都市生。1984 年京都大学工学部石油化学科卒業。1989 年同大学院分子工学専攻博士課程修了。工学博士（京都大学）。1989 年同大学院工学研究科分子工学専攻助手、1995 年同助教授、2004 年より分子科学研究所客員助教授併任、現在に至る。この間、1987 年から 2 年間日本学術振興会特別研究員、1993 年から 1 年間文部省在外研究員として米国ウィスコンシン大学マジソン校客員研究員。金属蛋白質の構造と機能を物理化学的、分子生物化学的手法により分子レベルで解明し、その応用として人工的な蛋白質の設計を試みている。

生体内で種々の重要な働きを果たしている金属酵素蛋白質を中心に、分子の特異的な性質と機能の発現には蛋白質構造や活性中心の微細分子構造のみならず電子の挙動が重要であるとの観点に立ち、分子のミクロ構造、電子構造や分子的、電子的諸過程の実験的解明、これらを制御する静的及び動的構造因子の探索を通じ、新しい特性を示す新規機能性蛋白質の分子設計を行ってきた。具体的な研究対象として、（１）蛋白質における分子、電子レベルでの構造機能相関の解明、（２）新規金属蛋白質の制御メカニズムの解明などを取り上げ、種々の物理化学的、生化学的、分子生物学的手法を縦横に駆使して研究を推し進めている。以下では、これらの研究の成果を紹介する。

（１）蛋白質における分子、電子レベルでの構造機能相関の解明

シトクロム P450 はヘムを活性中心とする酸素添加酵素で、種々の基質に対して温和な条件下、位置特異的に酸素原子を挿入することができる。このような酸素添加反応には 1 分子の分子状酸素に対し 2 等量の電子が必要で、その電子はそれぞれの P450 に特異的な電子伝達蛋白質によって供給される。水溶性の P450 である P450cam は基質として D-camphor を結合し、その 5-exo-位を水酸化するが、この際の電子の供給は鉄硫黄蛋白質であるプチダレドキシン（Pd）によって行われる。P450cam による水酸化反応では、Pd よりも還元力の強い還元剤を用いても反応が進行しないことから、Pd は単に電子を供給するだけではなく、Pd は P450cam に結合することで何らかの構造変化を誘起する「エフェクター」の役割を果たしていると想定されている。ヘム近傍の Leu358（Fig. 1）を置換した変異 P450cam を用いてこの「エフェクター」の役割を検討したところ、

Fig. 1. Heme Environmental Structure of P450cam

Pd 結合によって酸素添加反応を促進するような構造変化がヘム近傍に誘起されることを示すことができた。

（２）新規金属蛋白質の制御メカニズムの解明

生体内で酸素運搬機能を果たしているヘモグロビンの蛋白質部分であるグロビンは、網状赤血球中で合成されるが、その生成量は利用できうるヘムの量によって制限されている。すなわち、利用できうるヘムの量が少ないとグロビンの合成は阻害されるが、そのヘム濃度を感知するのが eIF2α キナーゼ（HRI）であると考えられている。また、この HRI は生体内のシグナル伝達分子である NO によっても制御されており、NO による細胞分裂停止などとの関連が注目されている。しかし、HRI の立体構造は知られておらず、ヘムがどのように結合するのかさえも十分検討されていない。我々はこの HRI について、その N 末端ドメインを単離することにより、ヘムの結合様式を明らかにすることを試みた。紫外可視吸収、ＣＤ、共鳴ラマン、ＥＰＲスペクトルなどの種々の分光法を駆使することにより、ヘムへの配位子を His78 と His123 と同定し、NO は His123 と置換して６配位型で配位することを示すことができた（Fig.2）。一方、HRI と同様に NO によってその機能が制御されるグアニル酸シクラーゼでは、NO 結合によって蛋白質由来の軸配位子との結合が解裂した５配位型が生成することから、これらの両蛋白質では NO 結合による機能の活性化機構に大きな違いがあることが示唆された。

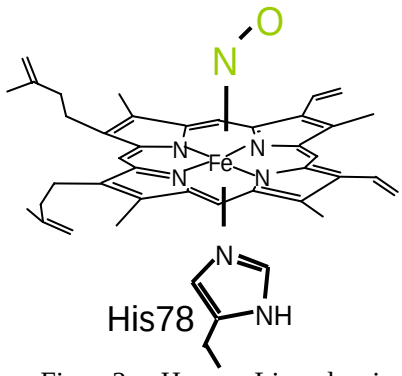


Fig. 2. Heme Ligands in NO-bound N-terminal Domain

Molecular Engineering of hemoproteins

Isao Morishima

Born in 1940. He received his Ph.D. degree from Kyoto University. He was a research associate (1965-1971), an associate professor in the department of Hydrocarbon Chemistry, Faculty of Engineering, (1971-1983), an associate professor in the division of Molecular Engineering, Graduate School of Engineering, Kyoto University(1983-1989) and a professor in the department of Molecular Engineering, Graduate School of Engineering, Kyoto University (1989-2004). Now he is an emeritus professor of Kyoto University. He was also a visiting professor in NIH (1975-1976). He has been interested in the molecular mechanism in hemoproteins and their functional regulations.

Koichiro Ishimori

Born in 1961. He received his Ph.D. degree from Kyoto University. He was a research associate in the division of Molecular Engineering, Graduate School of Engineering, Kyoto University (1989-1995) and has been an associate professor in the department of Molecular Engineering, Graduate School of Engineering, Kyoto University (1995-). He was also a visiting researcher in University of Wisconsin-Madison (1993-1994) and a visiting associate professor in the Institute of Molecular Science (2004-). His research interests are the molecular mechanisms in metalloproteins and their functional designs.

(1) L358P Mutation on Cytochrome P450cam Simulates Structural Changes upon Putidaredoxin Binding：To investigate the functional and structural characterization of a crucial cytochrome P450cam (P450cam)-putidaredoxin (Pdx) complex, we utilized a mutant whose spectroscopic property corresponds to the properties of the wild type P450cam in the presence of Pdx. The 1H NMR spectrum of the carbonmonoxy adduct of the mutant, the Leu-358 → Pro mutant (L358P), in the absence of Pdx showed that the ring current-shifted signals arising from D-camphor were upfield-shifted and observed as resolved signals, which are typical for the wild type enzyme in the presence of Pdx. Signals from the β-proton of the axial cysteine and the γ-methyl group of Thr-252 were also shifted upfield and

downfield, respectively, in the L358P mutant as observed for Pdx-bound wild type P450cam. The close similarity in the NMR spectra suggests that the heme environment of the L358P mutant mimics that of the Pdx-bound enzyme. The functional analysis of the L358P mutant has revealed that the oxygen adduct of the L358P mutant can promote the oxygenation reaction for D-camphor with nonphysiological electron donors such as dithionite and ascorbic acid, showing that oxygenated L358P is "activated" to receive electron from the donor. Based on the structural and functional characterization of the L358P mutant, we conclude that the Pdx-induced structural changes in P450cam would facilitate the electron transfer from the electron donor, and the Pdx binding to P450cam would be a trigger for the electron transfer to oxygenated P450cam.

(2) Identification of Crucial Histidines for Heme Binding in the N-terminal Domain of the Heme-regulated eIF2α Kinase : The heme-regulated eukaryotic initiation factor-2α (eIF2α) kinase (HRI) regulates the initiation of protein synthesis in reticulocytes. The binding of NO to the Nterminal heme-binding domain (NTD) of HRI positively modulates its kinase activity. By utilizing UV-visible absorption, resonance Raman, EPR and CD spectroscopies, two histidine residues have been identified that are crucial for the binding of heme to the NTD. The UV-visible absorption and resonance Raman spectra of all the histidine to alanine mutants constructed were similar to those of the unmutated NTD. However, the change in the CD spectra of the NTD construct containing mutation of His78 to Ala (H78A) indicated loss of the specific binding of heme. The EPR spectrum for the ferric H78A mutant was also substantially perturbed. Thus, His78 is one of the axial ligands for the NTD of HRI. Significant changes in the EPR spectrum of the H123A mutant were also observed, and heme readily dissociated from both the H123A and the H78A NTD mutants, suggesting that His123 was also an axial heme ligand. However, the CD spectrum for the Soret region of the H123A mutant indicated that this mutant still bound heme specifically. Thus, while both His78 and His123 are crucial for stable heme binding, the effects of their mutations on the structure of the NTD differed. His78 appears to play the primary role in the specific binding of heme to the NTD, acting analogously to the "proximal histidine" ligand of globins, while His123 appears to act as the "distal" heme ligand.

Selected Publications

· Presentation & Lectures

1. I. Morishima, "Some New Aspects of Molecular Engineering of Heme Proteins to Manipulate Their Functions", *11th International Conference of Bioinorganic Chemistry*, July 18-23, 2003, Carins, Australia (Invited)
2. I. Morishima, "Molecular Mechanism of P450cam Catalyzed Oxygenation Reaction Regulated by the Association with Putidaredoxin", *Gordon Research Conference, Metal in Biology,* January 18-23, 2004, Ventura, USA (Invited)
3. I. Morishima, "Heme as a Regulatory Molecule in Biological Systems: Structural Characterization of Heme Binding in Heme-Regulated Proteins", *3rd International Conference on Porphyrin and Phthalocyanines*, July 11-16, 2004, New Orleans, USA (Invited)
4. K. Ishimori, "A New Family of Hemoproteins: Heme-regulated Sensor Proteins", *The First Asian Meeting of Bioinorganic Chemistry*, March 7-10, 2003, Okazaki, Japan (Invited)
5. K. Ishimori, "A New Family of Hemoproteins: Heme-regulated Sensor Proteins", *Symposium on Biological Inorganic and Related Chemistry*, August 13, 2003, Kyoto, Japan (Invited)
6. K. Ishimori, "Spectroscopic and Crystallographic Characterization of Redox Partner Induced Structural Changes in P450cam: Molecular Mechanism for Structural Changes Required for Electron Transfer and Oxygen Activation", *The 9th Southeast Asian-Western Pacific Regional Meeting of Pharmacologists*, August 19-23, 2003, Busan, Korea (Invited)
7. K. Ishimori, "Heme-regulated Sensor Proteins: Novel Functions of Heme", *Chemical Biology of Metal Sensors with Switching Function. The 3rd Symposium"*, October 10-11, 2003, Kyoto, Japan (Invited)

· Articles

1. Activation of Hydrogen Peroxide in Horseradish Peroxidase Occurs within ~ 200 μs Observed by a New Freeze-Quench Device, Tanaka, M., Matsuura, K., Hori, H., Takahashi, S., Ishimori, K., Morishima, I. *Biophys. J, 81*, 1998-2004 (2003).

2. Isolation of an Ubiquitin-Protein Ligase that Recognizes Oxidized IRP2, Yamanaka, K., Ishikawa, H., Megumi, Y., Tokunaga, F., Kanie, M., Rouault, T., Morishima, I., Minato, N., Ishimori, K., Iwai, K. *Nature Cell Biol. 5*, 336-340 (2003).

3. NMR Study on the Structural Changes of Cytochrome P450cam upon the Complex Formation with Putidaredoxin: Functional Significance of the Putidaredoxin-induced Structural Changes, Tosha, T., Yoshioka, S., Takahashi, S., Ishimori, K., Shimada, H., Morishima, I. *J. Biol. Chem.*, *278*, 39809-39821 (2003).

4. Kinetic and Spectroscopic Characterization of a Hydroperoxy Compound in the Reaction of Native Myoglobin with Hydrogen Peroxide, Egawa, T., Yoshioka, S., Takahashi, S., Hori, H., Nagano, S., Shimada, H., Ishimori**,** K., Morishima, I., Suematsu, M., Ishimura, Y. *J. Biol. Chem.*, *278*, 41597-41606 (2003).

5. Collapse and Search Dynamics of Apomyoglobin Folding by Submillisecond-resolved CD and SAXS Observations, Uzawa, T., Kimura, T., Takahashi, S., Ishimori, K., Morishima, M., Akiyama, S., Fujisawa, T. *Proc. Natl. Acad. Sci. USA*, 101, 1171-1176 (2004).

6. Identification of Crucial Histidines for Heme Binding in the N-terminal Domain of the Heme-regulated eIF2α Kinase, Inuzuka, T., Yun, B. G., Ishikawa, H., Takahashi, S., Hori, H., Matts, R. L., Ishimori, K. Morishima, I. *J. Biol. Chem.*, *279*, 6778-6782 (2004).

7. Dioxygen Reduction by *bo*-Type Quinol Oxidase from *Escherichia coli* Studied by Submillisecond-Resolved Freeze-Quench EPR Spectroscopy, Matsuura, K., Yoshioka, S., Takahashi, S., Ishimori, K., Mogi, T., Hori, H., Morishima, I. *Biochemistry, 43*, 2288-2296 (2004).

8. Small-angle X-ray Scattering Reveals a Bent-conformation of the Transcriptional Activator CooA in Solution Conditions: New Insights into Activation Mechanisms of CooA, Akiyama, A., Fujisawa, T., Ishimori, K., Morishima, I., Aono, S. *J. Mol. Biol.*, *341*, 651-668 (2004).

9. L358P Mutation on P450cam Simulates Structural Changes upon Putidaredoxin Binding. The Structural Changes Trigger Electron Transfer to Oxy-P450cam from Electron Donors, Tosha, T., Yoshioka, S., Ishimori, K., Morishima, I. *J. Biol. Chem. 279*, 42836-42843 (2004).

10. Crystal Structure of the Cytochrome P450cam Mutant That Exhibits the Same Spectral Perturbations Caused by Putidaredoxin Binding, Nagano, S., Tosha, T., Ishimori, K., Morishima, I., Poulos,.T. L. *J. Biol. Chem. 279*, 42844-42849 (2004).

11. Electron Transfer Reaction in Single Protein Molecule Observed by Total Internal Reflection Fluorescence Microscopy, Furukawa, Y., Ban, T., Hamada, D., Ishimori, K., Goto, Y., Morishima, I. *J. Am. Chem. Soc*. in press (2004).

12. Structural and Functional Characterization of “Laboratory Evolved” Cytochrome P450cam Mutants Showing Enhanced Naphthalene Oxygenation Activity”, Matsuura, K., Tosha, T., Yoshioka, S., Takahashi, S., Ishimori, K., Morishima, I. *Biochem. Biophys. Res. Commun.*, *323*, 1209-1215 (2004).

13. Steric Effects of Isoleucine 107 on Heme Reorientation Reaction in Human Myoglobin: Evidence for Heme Rotation in “Protein Cage”, Ishikawa, H., Takahashi, S., Ishimori, K., Morishima, I. *Biochem. Biophys. Res. Commun.*, *324*, 1095-1100 (2004).

14. Association of human neuroglobin with cystatin C, a cysteine proteinase inhibitor, Wakasugi, K., Nakano, T., Morishima, I. *Biochemistry 43*, 5119-5125 (2004).

15. Human neuroglobin interacts with flotillin-1, a lipid raft microdomain-associated protein. *Biochem Biophys Res Commun*. Wakasugi, K., Nakano, T., Kitatsuji, C., Morishima, I. *318*, 453-460 (2004).

生体関連物質化学　生体機能物質の創製と機能

化学研究所　杉浦　幸雄

杉浦　幸雄（すぎうら ゆきお）
昭和 17 年生まれ。昭和 39 年京都大学薬学部薬学科卒業。昭和 40 年京都大学薬学部助手、昭和 57 年同助教授を経て、昭和 63 年京都大学化学研究所教授、現在に至る。この間、平成 10 年～12 年に京都大学化学研究所長、平成 12 年～14 年京都大学付属図書館宇治分館長を務める。主として遺伝子の発現に深くかかわっている亜鉛フィンガータンパク質の構造と機能を明らかにするとともに、新しい人工亜鉛フィンガータンパク質を設計・創製し、遺伝子発現の制御を展開する。昭和 59 年日本薬学会奨励賞、平成 4 年アップジョン科学研究賞、平成 12 年日本薬学会賞を受賞。

触媒機能を有する亜鉛フィンガー創製

亜鉛フィンガーは、真核細胞によく見いだされる DNA 結合モチーフであり、その代表格である C_2H_2 型亜鉛フィンガーは約 30 アミノ酸残基から成るペプチドの繰り返し構造から構成されている。亜鉛フィンガーは、亜鉛の配位によりコンパクトなββα構造が誘起され、高い DNA 結合能を獲得している。亜鉛フィンガーは亜鉛結合能と DNA 結合能の二つの精密な分子認識能を併せもち、機能性金属ペプチドの基本骨格として優れていると考えられる。本亜鉛フィンガーモチーフを基にして、新規な機能を有する人工亜鉛フィンガーペプチドの設計を行い、新たに触媒機能を有する亜鉛フィンガーペプチドの創製に成功した。タンパク質内に見いだされる金属イオンは、タンパク質構造の安定化に寄与する構造金属イオンと酵素機能発現などにかかわる機能金属イオンの二つに大別される。亜鉛フィンガー内の金属イオンは DNA との結合には直接的に関与しておらず、フォールディング構造の安定化のみに関与する構造金属イオンの代表的なものである。金属タンパク質における金属イオンの配位構造と機能の違いに着目し、亜鉛フィンガーの構造金属イオンの機能金属イオンへの変換を試みた。すなわち、亜鉛フィンガー型転写因子 Sp1 の四つの配位アミノ酸残基のうち、一つを配位不可能なグリシンやアラニンに置換した 3 配位型亜鉛フィンガーペプチドを作製した。各変異体における亜鉛イオンは分子設計通り配位空座を有し、その配位構造が配位アミノ酸残基の種類に大きく依存していた。配位空座を有する亜鉛フィンガー変異体の機能発現の一つとして酢酸 4-ニトロフェニルエステル加水分解能を検討した結果、野生型ペプチドには触媒作用が全く認められなかったが、配位空座を有する変異型ペプチドはいずれも加水分解反応を促進した。その反応速度は、配位アミノ酸残基の電子供与性の増大に伴い減少した。また、亜鉛フィンガーペプチドの立体構造も反応性に鋭敏に反映し、高度にフォールディングした立体構造が重要と考えられた。これらの知見をもとに、すべての配位アミノ酸残基をヒスチジンに置換した亜鉛フィンガーペプチドを新たに設計した結果、反応性が大きく向上し、酢酸エステルのみならずリン酸エステルや DNA の加水分解も引き起こすことが発見された。

Search for Biorelated Material Chemistry Area
Investigation of Creation and Function of Biofunctional Materials

Yukio Sugiura

Born in 1942. He received his Ph. D. degree from Kyoto University. He was a Postdoctoral fellow in Wayne State University and University of North Corolina (USA), a research associate (1965) and an associate professor (1982) in the Faculty of pharmaceutical Sciences, Kyoto University, and a professor (1988) in the Institute for chemical Research, Kyoto University. He was also a visiting professor (1998~2004) in the University of Manchester, UK. He got the Upjohn Scientific Research Award (1992) and the Award of Pharmaceutical Society of Japan (2000). He has been active in the fields of bioinorganic chemistry and biofunctional chemistry.

Creation of Catalytic Zinc Finger Mutants

This project investigates the creation and function of new zinc finger proteins. To redesign a metal site originally required for the stabilization of a folded protein structure into a functional metal site, we constructed a series of zinc finger mutant peptides such as zf(CCHG) and zf(GCHH), in which one zinccoordinating residue is substituted into a noncoordinating one. The mutant peptides having water bound to the zinc ion catalyzed the hydrolysis of 4-nitrophenyl acetate as well as the enantioselective hydrolysis of amino acid esters. All the zinc complexes of the mutant peptides showed hydrolytic activity, depending on their peptide sequences. In contrast, the zinc complex of the wild-type, zf(CCHH), and zinc ion alone exhibited no hydrolytic ability. These results clearly indicate that the catalytic abilities are predominantly attributed to the zinc center in the zinc complexes of the mutant peptides. Kinetic studies of the mutant peptides demonstrated that the catalytic hydrolysis is affected by the electron-donating ability of the protein ligands and the coordination environment. In addition, the pH dependence of the hydrolysis strongly suggests that the zinc-coordinated hydroxide ion participates the catalytic reaction. This report is the first successful study of catalytically active zinc finger peptides. In an effort to design an artificial nuclease for DNA hydrolysis, the zinc finger mutant peptide was designed, in which one zinc-coordinating residue is substituted into a noncoordinating one. The zinc finger contributes DNA-binding affinity, and the zinc ion containing the vacant site(s) provides reactivity. The zinc finger mutant peptides demonstrated to convert supercoild plasmid DNA to nicked circular form. Zinc-dependent cleavage confirms that the zinc ion coordinating the zinc finger is essential for the reaction. Ionic strength dependent and no site-specific hydrolysis were found; therefore, the zinc finger mutant peptides associate with DNA through electrostatic interaction. This report illustrates a novel approach to creating an artificial nuclease using metallopeptides.

Selected Publications

・ Reports in Academic Meeting

1. Yukio Sugiura, “Design and Function of Zinc Finger Proteins”, 29th Biomoleculer Science Meeting, 2002. 7. 11-12, Okazaki (Special Lecture)
2. Wataru Nomura, Yukio Sugiura, “Nine-Zinc Finger Proteins”, 11th International Conference on Biological Inorganic Chemistry, 2003. 7. 19-23, Cairns, Australia (Invited Lecture)
3. Shiroh Futaki, Miki Niwa, Naoya Wakako, Ikuhiko Nakase, Yasuhisa Shiraishi, Yukio Sugiura, “Intercellurar Delivery of Histidine-Tagged Protein”, Fifth AFMC International Medicinal Chemistry Symposium, 2003. 10. 14-17, Kyoto, Japan
4. Yukio Sugiura, “Artificial Zinc Finger Proteins: Designs and Functions”, Special Seminar of Resbon University, 2004. 3. 15, Risbon, Portogal (Special Lecture)

5. Yukio Sugiura, “Structure and Function of Zinc Finger Protein”, 124th Annual Meeting of Pharmaceutical Sciences of Japan, 2004. 3. 29-31, Osaka (Invited Lecture)
6. Yukio Sugiura, “Creation and Function of Zinc Finger Proteins”, 31th Organic Reaction Meeting, 2004. 7. 31, Kyoto (Invited Lecture)

・ Articles

1. M. Imanishi, Y. Sugiura, Artificial DNA-Bending Six-Zinc Finger Peptides with Different Charged Linkers: Distinct Kinetic Properties of DNA Bindings, *Biochemistry,* **41(4)**, 1328-1334 (2002)
2. S. Futaki, I. Nakase, T. Suzuki, Y. Zhang, Y. Sugiura, Translocation of Branched-Chain Arginine Peptides through Cell Membranes: Flexibility in the Spatial Disposition of Positive Charges in Membrane-Permeable Peptides, *Biochemistry,* **41(25)**, 7925-7930 (2002)
3. M. Nagaoka, Y. Shiraishi, Y. Uno, W. Nomura, Y. Sugiura, Interconversion between Serine and Aspartic Acid in the α Helix of the N-Terminal Zinc Finger of Sp1: Implication for General Recognition Code and for Design of Novel Zinc Finger Peptide Recognizing Complementary Strand, *Biochemistry,* **41(28)**, 8819-8825 (2002)
4. A. Nomura, Y. Sugiura, Contribution of Individual Zinc Ligands to Metal Binding and Peptide Folding of Zinc Finger Peptides, *Inorg. Chem.,* **41(14)**, 3693-3698 (2002)
5. M. Nagaoka, Y. Doi, J. Kuwahara, Y. Sugiura, Novel Strategy for the Design of a New Zinc Finger: Creation of a Zinc Finger for the AT-Rich Sequence by α-Helix Substitution, *J. Am. Chem. Soc.*, **124(23)**, 6526-6527 (2002)
6. Y. Hori, Y. Sugiura, Conversion of Antennapedia Homeodomain to Zinc Finger-Like Domain: Zn(II)-Induced Change in Protein Conformation and DNA Binding, *J. Am. Chem. Soc.*, **124(32)**, 9362-9363 (2002)
7. W. Nomura, M. Nagaoka, Y. Shiraishi, Y. Sugiura, Influence of TFIIIA-Type Linker at the N- or C-Terminal of Nine-Zinc Finger Protein on DNA-Binding Site, *Biochem. Biophys. Res. Commun.*, **300(1)**, 87-92 (2003)
8. W. Nomura, Y. Sugiura, Effects of Length and Position of an Extended Linker on Sequence-Selective DNA Recognition of Zinc Finger Peptides, *Biochemistry,* **42(50)**, 14805-14813 (2003)
9. K. Matsushita, Y. Sugiura, GFP-Linked Zinc Finger Protein Sp1: Fluorescence Study and Implication for N-Terminal Zinc Finger 1 as Hinge Finger, *Bioorg. Med. Chem.*, **11(1)**, 53-58 (2003)
10. Y. Hori, Y. Sugiura, Effects of Zn(II) Binding and Apoprotein Structural Stability on the Conformation Change of Designed Antennafinger Proteins, *Biochemistry,* **43(11)**, 3068-3074 (2004)
11. Y. Shiraishi, M. Imanishi, Y. Sugiura, Exchange of Histidine Spacing between Sp1 and GLI Zinc Fingers: Distinct Effect of Histidine Spacing-Linker Region on DNA Binding, *Biochemistry,* **43(20)**, 6352-6359 (2004)
12. S. Futaki, M. Niwa, I. Nakase, A. Tadokoro, Y. Zhang, M. Nagaoka, N. Wakako, Y. Sugiura, Arginine Carrier Peptide Bearing Ni(II) Chelator to Promote Cellular Uptake of Histidine-Tagged Proteins, *Bioconjugate Chemistry,* **15(3)**, 475-481 (2004)
13. A. Nomura, Y. Sugiura, Hydrolytic Reaction by Zinc Finger Mutant Peptides: Successful Redesign of Structural Zinc Sites into Catalytic Zinc Sites, *Inorg. Chem.,* **43(5)**, 1708-1713 (2004)
14. A. Nomura, Y. Sugiura, Sequence Selective and Hydrobytic Cleavage of DNA by Zinc Finger Mutants, *J. Am. Chem. Soc.,* **126,** 15374-15375(2004)
15. S. Oka, Y. Shiraishi, T. Yoshida, T. Okubo, Y. Sugiura, Y. Kobayashi, NMR Structure of Transcription Factor Sp1 DNA Binding Domain, *Biochemistry,* **43,** (51), 16027-16035(2004)

生体関連物質化学 超好熱始原菌のゲノム情報を用いた未知遺伝子の機能解析

工学研究科　合成生物化学専攻　今中　忠行

今中　忠行（いまなかただゆき）
昭和 20 年生。学歴：昭和 42 年 3 月大阪大学工学部発酵工学科卒業、昭和 44 年 3 月大阪大学大学院工学研究科修士課程修了、昭和 44 年 12 月大阪大学大学院工学研究科博士課程中退。学位：工学博士（昭和 48 年 2 月大阪大学）職歴：昭和 45 年 1 月大阪大学工学部助手、昭和 48 年 2 月 MIT 博士研究員（出張）～49 年 10 月。昭和 56 年 11 月大阪大学工学部助教授。平成元年 3 月大阪大学工学部教授。平成 7 年 4 月大阪大学大学院工学研究科応用生物工学専攻教授（組織替え）。平成 8 年 7 月京都大学大学院工学研究科合成・生物化学専攻教授。専門：生物工学（遺伝子工学、タンパク質工学、応用微生物学、極限環境微生物学、環境バイオテクノロジー）。

（1）新型 Fructose 1,6-bisphosphatase (FBPase) の同定：

我々は超好熱始原菌 *Thermococcus kodakaraensis* を対象として、解糖や糖新生に関与する酵素のうち、遺伝子が同定されていないものを中心に研究を進めている。その結果、新型の FBPase を同定した。FBPase はあらゆる生物の糖新生系の鍵酵素でありながら、超好熱菌ゲノム上には従来の FBPase と相同性を示す遺伝子は存在しなかった。メタン生成超好熱始原菌 *Methanococcus* では異なる遺伝子の産物である inositol monophosphatase（IMPase）が *in vitro* で高い FBPase 活性を示したことから超好熱菌においては IMPase が FBPase の役割を果たしていることが提唱されていた。我々は *T. kodakaraensis* から酵素活性を指標として FBPase を精製し、遺伝子を同定することにより超好熱菌固有の新型 FBPase を発見した(Class V)。また本遺伝子（TK2164）および IMPase に対応する遺伝子(TK0878)それぞれの破壊株の形質を評価することにより、*in vivo* で FBPase の機能を果たしているのは TK2164 であることを遺伝学的に証明した(Fig. 1)。TK2164 の ortholog はほとんどの超好熱菌ゲノムに存在することから本遺伝子産物が超好熱菌の真の FBPase であることが予想された。

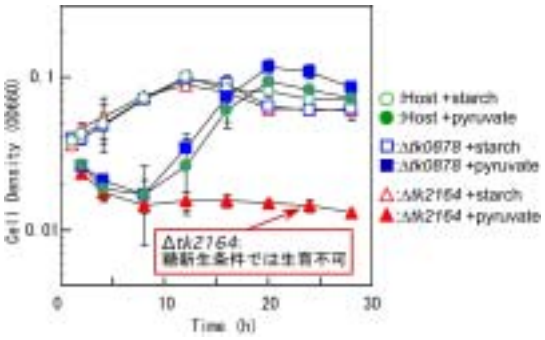


Fig. 1 Growth curve (open symbols: glycolytic growth, closed symbols: gluconeogenetic growth)

（2）Phosphopentomutase (PPM)および Phosphoglucomutase (PGM)の同定：

多くの細菌や哺乳細胞では、ヌクレオシド合成・資化代謝系酵素である PPM と deoxyribose phosphate aldolase (DERA)をもつ。*T. kodakaraensis* 細胞内では、DERA および PPM 活性が共に検出されているものの、遺伝子としては DERA 遺伝子のみしか見つかっていない。我々は PPM 活性を指標に、当該酵素の精製を行った結果、一次構造としては phosphomannomutase (PMM, COG1109 family)に類似した新規の PPM を発見した(TK1777)。一方、ゲノム解析結果より、本菌には COG1109 に属する遺伝子がさらに３つ存在していた（TK1108, TK1404 および TK2185）(Fig. 2)。これらの遺伝子産物を大腸菌で発現し、特性解析を行った結果、TK1108 がコードするタンパクが非常に高い PGM および PMM 活性を有していた。一

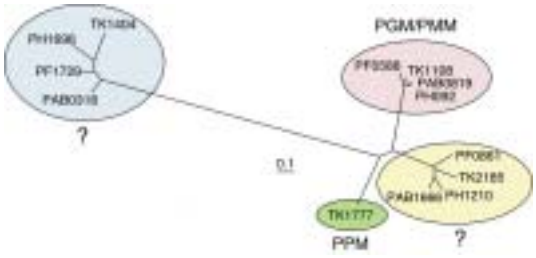


Fig. 2 Phylogenetic analysis of COG1109 family

方、TK1404 および TK2185 の両遺伝子産物は PGM/PMM 活性を共に有していなかった。以上の結果より、本菌において PPM および PGM/PMM を担うタンパクの同定に成功した。

Functional analysis of unanotatable genes from hyperthermophilic archaea

Tadayuki Imanaka

Born in 1945. He received his Ph.D. degree from Osaka University. He was an assistant professor (1970-1981), an associate professor (1981-1989) and a professor (1989-1996) in the Department of Fermentation Technology, Faculty of Engineering, Osaka University. He was also a postdoctoral fellow at MIT, USA (1973-1974). Now he has been a professor in the Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University (1996-).

(1) Fructose 1,6-bisphosphatase (FBPase)： FBPase is one of the key enzymes in gluconeogenesis. Although FBPase activity has been detected in several hyperthermophiles, no orthologs corresponding to the classical FBPases have been identified in their genomes. An inositol monophosphatase (IMPase) from *Methanococcus jannaschii* which displayed both FBPase and IMPase activities and a structurally novel FBPase (*Tk*-Fbp) from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 have been proposed as the “missing” FBPase. The IMPase/FBPase ortholog in *T. kodakaraensis*, *Tk*-Imp, was confirmed to possess high FBPase activity along with IMPase activity. We therefore constructed Δ*fbp* and Δ*imp* strains and investigated their phenotypes. The Δ*fbp* strain could not grow under gluconeogenic conditions while glycolytic growth was unimpaired (Fig. 1), and the disruption resulted in the complete abolishment of intracellular FBPase activity. Evidently, *Tk-fbp* is an indispensable gene for gluconeogenesis and is responsible for almost all intracellular FBPase activity. In contrast, the endogenous *Tk-imp* could not complement the defect of the *fbp* deletion, and its disruption did not lead to any detectable phenotypic changes under the conditions examined. These facts indicated that *Tk-imp* is irrelevant to gluconeogenesis, despite the high FBPase activity of its protein product. Our results provide strong evidence that the true FBPase for gluconeogenesis in *T. kodakaraensis* is the *Tk*-Fbp ortholog, not the IMPase/FBPase ortholog.

(2) Phosphopentomutase (PPM) and Phosphoglucomutase (PGM): Numerous bacteria and mammalian cells harbor two enzymes, PPM and 2-deoxyribose 5-phosphate aldolase (DERA), involved in the interconversion between nucleosides and central carbon metabolism. A search of the *T. kodakaraensis* genome sequence revealed the presence of a closely related ortholog of bacterial DERA genes while no ortholog of previously characterized PPM genes could be detected. As PPM activity was detected in *T. kodakaraensis* cells, partial purification of PPM was performed. A new class of PPM gene (TK1777) was identified, similar to phosphomannomutase (PMM) within COG1109 but not COG1015, which includes all previously identified PPMs. Our results clearly indicate the presence of a metabolic link between pentoses and central carbon metabolism in *T. kodakaraensis*. As for COG1109 family, four orthologous genes (TK1108, TK1404, TK1777, and TK2185) have been identified in the genome of *T. kodakaraensis* KOD1 (Fig. 2). In order to determine which of the remaining three orthologues encodes a PGM, we examined the PGM activity in *T. kodakaraensis* cells and identified the gene responsible for this activity. Characterization of the recombinant protein indicated that TK1108 encoded a protein with high levels of PGM activity (690 U mg/1), along with high levels of PMM activity (401 U mg/1). Similar analyses of the remaining two orthologs revealed that their protein products exhibited neither PGM nor PMM activity. Our results clearly indicate that, among the four PMM gene orthologues in *T. kodakaraensis*, only one gene, TK1108, actually encodes a protein with PGM and PMM activities.

Selected Publications

・ Presentation & Lectures

1. T. Imanaka, “Complete genome analysis and application of the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1”, 5th. International Conference on Extremophiles (Extremophiles 2004), 2004.9.19-23, Cambridge, Maryland, USA (Invited)

· Articles

1. "Among multiple phosphomannomutase orthologues, only one gene encodes a protein with phosphoglucomutase and phosphomannomutase activities in *Thermococcus kodakaraensis*", N. Rashid, T. Kanai, H. Atomi, & T. Imanaka, *J. Bacteriol.*, **186,** 6070-6076 (2004)
2. "Genetic evidence identifying the true gluconeogenic fructose-1,6-bisphosphatase in *Thermococcus kodakaraensis* and other hyperthermophiles", T. Sato, H. Imanaka, N. Rashid, T. Fukui, H. Atomi, & T. Imanaka, *J. Bacteriol.*, **186,** 5799-5807 (2004)
3. "Concerted action of diacetylchitobiose deacetylase and exo-beta-D-glucosaminidase in a novel chitinolytic pathway in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1", T. Tanaka, T. Fukui, S. Fujiwara, H. Atomi, & T. Imanaka, *J. Biol. Chem.*, **279,** 30021-30027 (2004)
4. "Reverse gyrase is not a prerequisite for hyperthermophilic life", H. Atomi, R. Matsumi, & T. Imanaka, *J. Bacteriol.*, **186,** 4829-4833 (2004)
5. "Presence of a novel phosphopentomutase and a 2-deoxyribose 5-phosphate aldolase reveals a metabolic link between pentoses and central carbon metabolism in the hyperthermophilic archaeon *Thermococcus kodakaraensis"*, N. Rashid, H. Imanaka, T. Fukui, H. Atomi, & T. Imanaka, *J. Bacteriol.*, **186,** 4185-4191 (2004)
6. "First characterization of an archaeal GTP-dependent phosphoenolpyruvate carboxykinase from the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1", W. Fukuda, T. Fukui, H. Atomi, & T. Imanaka, *J. Bacteriol.*, **186,** 4620-4627 (2004)
7. "Description of *Thermococcus kodakaraensis* sp. nov., a well studied hyperthermophilic archaeon previously reported as *Pyrococcus* sp. KOD1", H. Atomi, T. Fukui, T. Kanai, M. Morikawa, & T. Imanaka, *Archaea*, **1,** 263-267 (2004)
8. “Surface histidine residue of archaeal histone affects DNA compaction and thermostability.”, H. Higashibata, M. A. Siddiqui, M. Takagi, T. Imanaka, & S. Fujiwara, *FEMS Microbiol. Lett,*. **224,** 17-22 (2003).
9. “Gene cloning and function analysis of replication factor C from *Thermococcus kodakaraensis* KOD1.”, M. Kitabayashi, Y. Nishiya, M. Esaka, M. Itakura, & T. Imanaka, *Biosci. Biotechnol. Biochem,*. **67,** 2373-2380 (2003).
10. “Prevention of thermal inactivation and aggregation of lysozyme by polyamines.”, M. Kudou, K. Shiraki, S. Fujiwara, T. Imanaka, & M. Takagi., *Eur. J. Biochem.*, **270,** 4547-4554 (2003).
11. “Production and characterization of biosurfactants from *Bacillus licheniformis* F2.2.” Thaniyavarn, J., N. Roongsawang, T. Kameyama, M. Haruki, T. Imanaka, M. Morikawa, and S. Kanaya., *Biosci. Biotechnol. Biochem.*, **67,** 1239-1244 (2003).
12. “Cloning and characterization of the gene cluster encoding arthrofactin synthetase from *Pseudomonas* sp. MIS38.” N. Roongsawang, K. Hase, M. Haruki, T. Imanaka, M. Morikawa, & S. Kanaya., *Chem. Biol.*, **10,** 869-880 (2003).
13. “Genetic, enzymatic, and structural analyses of phenylalanyl-tRNA synthetase from *Thermococcus kodakaraensis* KOD1”. K. Shiraki, M. Tsuji, Y. Hashimoto, K. Fujimoto, S. Fujiwara, M. Takagi, & T. Imanaka., *J. Biochem. (Tokyo)*, **134,** 567-574 (2003).
14. "Temperature dependent modulation of farnesyl diphosphate/geranylgeranyl diphosphate synthase from hyperthermophilic archaea", S. Fujiwara, A. Yamanaka, K. Hirooka, A. Kobayashi, T. Imanaka, & E. Fukusaki, *Biochem. Biophys. Res. Commun.*, **325,** 1066-1074 (2004)
15. "Arginine ethylester prevents thermal inactivation and aggregation of lysozyme", K. Shiraki, M. Kudou, S. Nishikori, H. Kitagawa, T. Imanaka, & M. Takagi, *Eur. J. Biochem.*, **271,** 3242-3247 (2004)

生体関連物質化学 遺伝子の検出・運搬・発現システムの制御

工学研究科　合成・生物化学専攻　青山　安宏

青山　安宏（あおやまやすひろ）
昭和 20 年生。昭和 49 年京都大学工学研究科博士課程終了。同年九州大学助手（工学部）、昭和 56 年長岡技術科学大学助教授（工学部）、昭和 63 年同教授、平成 7 年九州大学教授（有機化学基礎研究センター）、平成 13 年より現職。日本化学会学術賞、フルカ賞、新潟日報学術賞を受賞。有機化合物の構造と物性に関する第２回ゴードンコンファレンス（平成１１年）、超分子化学に関する第１１回国際シンポジウム議長（平成１２年）。ラジカル反応機構、生物有機・無機化学、分子認識・構造有機化学、結晶工学・固体触媒・糖鎖工学などを経て現在の専門分野は生体認識化学・化学生物学。

（１）遺伝子をいかに細胞内に導入するか（サイズの制御）：

約 50 nm サイズの"人工グリコウイルス"は遺伝子の効率的な運搬体となるが、会合により運搬活性は急激に低下する。遺伝子複合体を取り込む細胞の飲作用（エンドサイトーシス）が 100 nm 以上の粒子に対しては有効でないことを示している。では、50 nm より小さな粒子はどうなのだろう。この点を明らかにするために CdSe 量子ドットを糖クラスターで覆った 15 nm の量子ドット糖コンジュゲート（glycoQD）を作成した。5 nm の糖被覆ミセル様ナノ粒子（GNP）、50 nm のグリコウイルスを用いた競争取り込みの実験（図１）から 50 nm の"ウイルスサイズ"がエンドサイトーシスに最適であることが明らかになった。この結果は、単に遺伝子のみならず、小分子、特に自発的には細胞膜を透過できない親水性物質を効率よく細胞内に導入する新手法になるものと期待される。

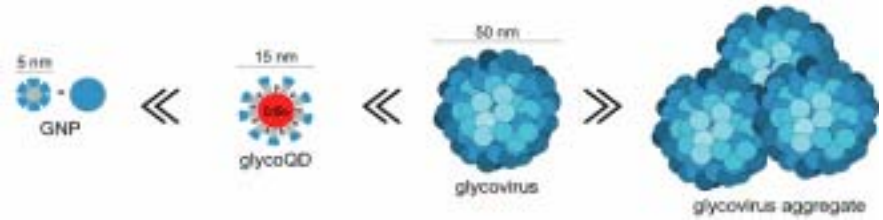


Fig. 1 Relative endocytosis susceptibility of GNP (5 nm), glycoQD (15 nm), glycovirus (50 nm), and glycovirus aggregate.

（２）細胞内の遺伝子をいかに診断するか（遺伝子の増幅センシング）：

遺伝子診断が細胞そのものに適用できれば、細胞の SNP 診断、生きたバクテリアのタイピング、薬物の細胞内スクリーニングなど、その意義は大きい。しかしながら大きな問題がある。通常の遺伝子診断では微量のサンプル（ターゲット）を PCR で増幅・単離できるが、PCR 法はいうまでもなく細胞内で用いることはできない。そこで、何らかの方法で遺伝子の増幅（触媒的）診断法の確立が必要になる。我々は、TASC (Taeget-Assisted Self-Cleavage) 法によりこれを可能にした。原理（図２）はプローブがターゲットとハイブリダイズした時にのみ自己切断に適した DNAzyme 構造をとるようにしむけた

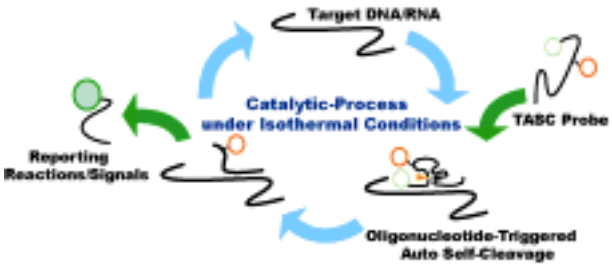


Fig. 2 Signal amplification scheme for a FRET-TASC probe with its target.

ものである。

(3) タンパクへの非天然アミノ酸など非天然基質の導入：

リボソーム系を用いた非天然基質のタンパク質への導入法の一つに non-sense suppression 法がある。これは、人工基質を用いて化学的に合成したアシル化 tRNA を用いて終始コドンをその基質にあてはめるものである。この場合、終始コドンへの人工基質の導入（read-through）は終結因子（release factor; RF）との競争であり、RF を除去した系では read-through 効率は向上するが、これは細胞内では適用できない。人工基質に対するアシル化 tRNA 合成酵素を進化法に基づいて作成することが検討されているが、我々は新しい視点からこの問題にアプローチし、一般のタンパクや配列制御オリゴマーに適用できる方法を開発した。

Manipulation of gene detection, delivery, and expression

Yasuhiro Aoyama

Born in 1945. He received his Ph.D degree from Kyoto University. He was a research associate in Kyushu University (1974-1981), an associate professor in Nagaoka University of Technology (1981-1988), and professor there (1988-1995). He then moved to Kyushu University as professor in the Institute for Fundamental Research of Organic Chemistry (1995-2001) and moved again to Kyoto University, where he is now professor (2001-) in the Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering. He received the Divisional Award of the Chemical Society of Japan, the Fluka Prize, and the Daily Niigata Cultural Award. He served as Chairman in the 2nd Gordon Research Conference on the Structure and Property of Organic Compounds as well as in the 11th International Synposium on Supramolecular Chemistry. He used to work on reaction mechanisms, bioorganic/bioinorganic chemistry, molecular recognition and structural organic chemistry, crystal engineering, solid catalysis, and glycoengineering and his current research activity is directed to biorecognics and chemical biology.

(1) Cellular uptake of genes (size regulation): Monomeric glycovirus of a size of ~50 nm serve as effective gene carriers but their gene-delivery activities are dramatically lowered upon their aggregation. This clearly indicates that endocytosis (cellular drinking), by which gene-conjugates are taken in the cells, is not effective for big (≥100 nm) particles. What, then, about small particles of a size of ≤50 nm? We prepared a glycoconjugate of CdSe quantum dot (QD) of a size of 15 nm and carried out a set of comtetitive cellular uptake experiments using the glycol-QD together with micellar glycocluster nanoparticle (5 nm) and glycovirus (50 nm) (Fig. 1). The size-controlled endocytosis is expected to provide a new strategy for delivering small polar molecules which, when alone, are not hydrophobic enough to get through the cell membrane.

(2) Intracellular detection of gene (amplified gene sensing): Intracellular sensing of genes potentially leads to direct SNP discrimination of living cells, detection and typing of living bacteria and viruses, and in vivo screening of drugs. A serious problem is that the PCR technique by which a tiny amount of target DNA/RNA can be amplified and isolated is by no means applicable to intracellular events. We need a new method of amplified or catalytic sensing of DNA/RNA. We devised the TASC (target-Assisted Self-Cleavage) strategy, which is based on signal amplification upon catalytic self-cleavage of the probe when hybridized with the target (Fig. 2).

(3) Incorporation of unnatural substrates into proteins: Unnatural substrates can be introduced into proteins by using the nonsense suppression method, which utilizes chemically acylated tRNA adapted to the stop codon. Nevertheless, the suppression or read-through of the stop codon is in competition with termination as effected by the release factor (RF). RF-free or – diminished systems promote read-through efficiency but this is not directly applicable in the cells, while evolution of tRNA synthetases working on artificial substrates is under active investigation. We devised a new strategy

of suppression working on normal (unmodified) mRNA target to give point-mutated proteins as well as highly sequence-regulated oligomers.

Selected Publications

· Presentation & Lectures

1. Y. Aoyama, "Calix[resorcarene-Based Glycocluster. From Amphiphile through nanoparticle to Glycovirus", 7th International Conference on Calixarenes (ICC 2003), 2003.8.12-16, Vancouver, Canada (Invited)
2. Y. Aoyama, "Number- and Size-Controlled Macromolecular Oligomerization. Hierarchical Growth of Glycocluster Amphiphile through Micellar Nanoparticle to Transfectious Glycovirus and Its Saccharide-Dependent Aggregates", 4th International Forum on Functional Organic Compounds (IFOC 4), 2003.11.15-17, Tokyo, Japan (Invited)
3. Y. Aoyama, "Endocytosis as a Macrobiomolecular Host-Guest Complexation: the Size Complementarity Effect", 13th International Symposium on Supramolecular Chemistry (ISSC 13), 2004.7.25-30, Notre Dame, Indiana, USA (Invited)
4. Y. Aoyama, "Cellular Uptake of Artificial Viruses and Related Nanoparticles", 12th International Symposium on Advanced Materials (ISAM 12), 2004.12.7-10, Tsukuba, Japan (Invited)

· Articles

1. "Heat Shock Protein-Like Activity of Artificial Molecular Chaperone: Thermo-Responsive Controlled Association of Protein with a Dynamic Nanogel of Hydrophobized Polysaccharide and Cyclodextrin", Y. Nomura, Y. Sasaki, M. Takagi, T. Narita, Y. Aoyama, K. Akiyoshi, *Biomacromolecules*, in press (2004)
2. "A Qunatum Dot Conjugated Sugar Ball and Its Cellular Uptake. On the Size Effects of Endocytosis in the Subviral Region"F. Osaki, T. Kanamori, S. Sando, T. Sera, Y. Aoyama, *J. Am. Chem. Soc*., **126,** 6520-6521 (2004)
3. "Macrocyclic Proteoglycan Mimics. Potent Inhibition of Cell Adhesion by a Bundle of Chondroitin Sulfate Chains Assembled on the Calix[4] resorcarene Platform", N. Tomita, S. Sando, T. Sera, Y. Aoyama, *Bioorg. Med. Chem. Lett.*, **14,** 2087-2090 (2004)
4. "Photo-responsive Nanogels Formed by the Self-Assembly of Spiropyrane -Bearing Pullulane That Act as Artificial Molecular Chaperones", *Biomacromolecules,* **5,** 1804-1809 (2004)
5. "Aptamer Selection for the Inhibition of Cell Adhesion with Fibronectin as Target", A. Ogawa, N. Tomita, N. Kikuchi, S. Sando, Y. Aoyama, *Bioorg. Med. Chem. Lett.*, **14,** 4001-4004 (2004)
6. "A Facile Route to Dynamic Glycopeptide Libraries Based on Disulfide-linked Sugar-Peptide Coupling", S. Sando, A. Narita, Y. Aoyama, *Bioorg. Med. Chem. Lett.,* **14,** 2835-2838 (2004)
7. "Protein Refolding Assisted by Self-Assembled Nanogels as Novel Molecular Chaperones", Y. Nomura, M. Ikeda, N. Yamaguchi, Y. Aoyama, K. Akiyoshi, *FEBS Lett.,* **553,** 271-276 (2003)
8. "Macrocyclic Glycoclusters: From Amphiphile through Nanoparticles to Glycoviruses", Y. Aoyama, *Chem. Eur. J.* **10,** 588-593 (2004)
9. "Amplified Nucleic Acid Sensing using Programmed Self-Cleaving DNAzyme", S. Sando, T. Sasaki, K. Kanatani, Y. Aoyama, *J. Am. Chem. Soc.*, **125,** 15720-15721 (2003)

· Patents

1. ”細胞内遺伝子診断手法とそのプロー”、青山安宏、山東信介、佐々木要徳、特願 20 03-329619。
2. ”アプタマーのスクリーニング方法”、青山安宏、山東信介、小川敦司、特願 2004-0 57956。
3. ”核酸プローブ、核酸チップ、標的核酸検出方法、薬剤スクリーニング方法、標的核酸検出装置及び遺伝子診断方法”、青山安宏、山東信介、佐々木要徳、成田敦、国際出願 PCT/JP2004/013740。

生体関連物質化学 カルコゲン機能素子の物性と構築

化学研究所　江崎　信芳

江崎　信芳（えさき　のぶよし）
1949 年大阪市生まれ。 1973 年京都大学農学部農芸化学科卒業、 1979 年同大学院農学研究科博士課程修了。京都大学農学博士。同年京都大学化学研究所助手、 1989 年同助教授を経て、1996 年同教授､現在に至る。1998～2000 年および 2002～2004 年、日本生化学会理事。 1999～2003 年、日本農芸化学会理事。 1999～2003 年、日本生物工学会理事。 2000～2003 年、日本学術会議第 18 期生物工学研究連絡委員。 2001～2004、科学研究費特定領域研究を推進。

鉄硫黄タンパク質は、鉄と無機硫黄原子から成る鉄硫黄クラスター（図 1）を持つタンパク質の総称であり、細菌から高等植物までほとんど全ての生物に存在する。鉄硫黄クラスターは、生体内で電子伝達、代謝物変換、遺伝子の発現制御など多様で必須な役割を担っている。鉄硫黄クラスターの生合成には、*isc* (iron-sulfur cluster) オペロンにコードされるタンパク質が重要な働きをすることがわかっている。*isc* オペロンにコードされるタンパク質の一つである IscS は、システインデスルフラーゼ活性を有しておりL-システインから硫黄とアラニンを生成する。この反応で生じた硫黄が鉄硫黄クラスターに組み込まれる。*iscS* を欠失させた大腸菌変異株は、鉄硫黄クラスター形成に支障をきたし、生育速度が減少する。そこで、鉄硫黄クラスター形成能を欠く大腸菌 *iscS* 欠損株と、野生株から得られるタンパク質を比較することにより実際に *iscS* 欠損が影響を与えるタンパク質を同定、解析することで得られた成果を紹介する。

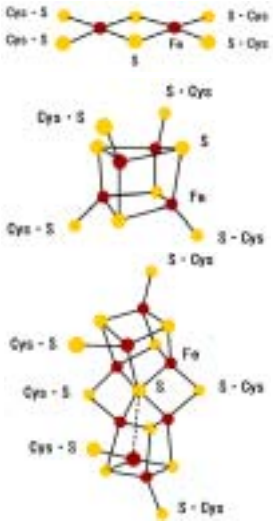


Fig. 1. [2Fe-2S] , [4Fe-4S] , [8Fe-7S] type Iron-Sulfur clusters

Escherichia coli MG1655 野生株および *iscS* 欠損株の細胞抽出液を Native-PAGE に供し、鉄染色を行うことにより、*iscS* 遺伝子に依存して鉄硫黄クラスターが形成されると考えられるタンパク質のバンドが複数認められた (図 2)。特に違いの顕著なバンド（図 2 において矢印で示したバンド）についてプロテインシーケンサーにより N 末端アミノ酸配列を決定した。得られたタンパク質の N 末端アミノ酸配列より、2 つのタンパク質 b2146 と YeiA が含まれることが判明した。*b2146* と *yeiA* は大腸菌ゲノム DNA 上でオペロン様構造を形成する。また、アミノ酸配列の解析より b2146 と YeiA はともに鉄硫黄クラスターを形成し得る配列モチーフを有していた。相同性検索の結果、b2146 と YeiA は、哺乳類のピリミジン代謝に関わる鉄硫黄タンパク質である Dihydropyrimidine dehydrogenase (NADPH)（DPD, EC 1.3.1.2）（図 4）の N 末端側と C 末端側の配列にそれぞれ相同性を示した。

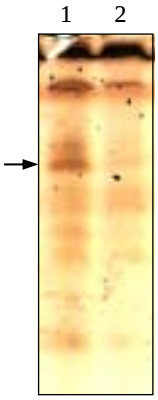


Fig. 2. Screening of Iron-Sulfur proteins by Fe-staining.
1. *E. coli* MG1655 (wild type)
2. *E. coli* MG1655 (*iscS*⁻)

b2146 と YeiA それぞれの発現系を構築し、精製した b2146 と YeiA を混合しても DPD 活性は確認できなかった。しかし、b2146 と YeiA を混合し、嫌気かつ還元的条件下で鉄硫黄クラ

スターを再構成させたところ、NADH 依存性の DPD 活性を検出することができた。また再構成後の b2146、YeiA は 330 nm, 410 nm 付近に鉄硫黄タンパク質特有の吸収の肩を示した（図 3）。

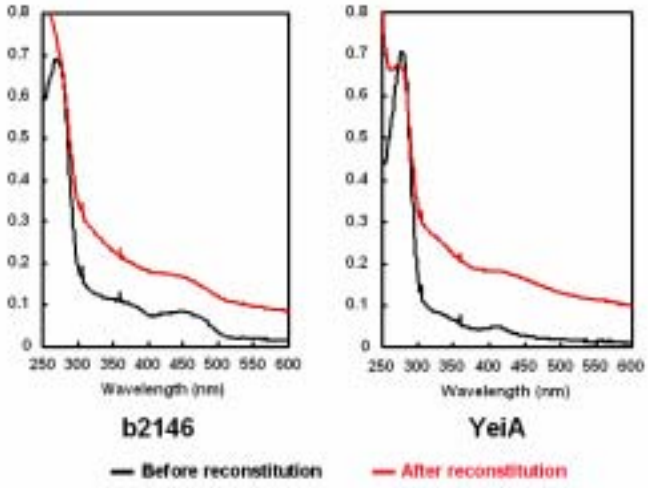


Fig. 3. Reconstitution of Iron-Sulfur clusters on b2146 and YeiA

さらに、*E. coli* MG1655 野生株の細胞抽出液と鉄硫黄クラスター形成能を欠く *iscS* 欠損株の細胞抽出液を 2 次元電気泳動により分離・分析した結果、*iscS* 欠損株において発現量が著しく低下している 2 つのタンパク質を見出した。これらのタンパク質を同定すると、DPD と同様にピリミジン代謝に関連する酵素である Cytidine deaminase と Uridine phosphorylase であった (図 4)。これは *iscS* 遺伝子の欠損により DPD の鉄硫黄クラスターが欠損し、それが DPD の不活性化を招くに伴い菌体内のウラシル濃度が上昇する。そのことがピリミジン代謝関連酵素である Cytidine deaminase と Uridine phosphorylase の発現量に影響を及ぼしたと考えられる。

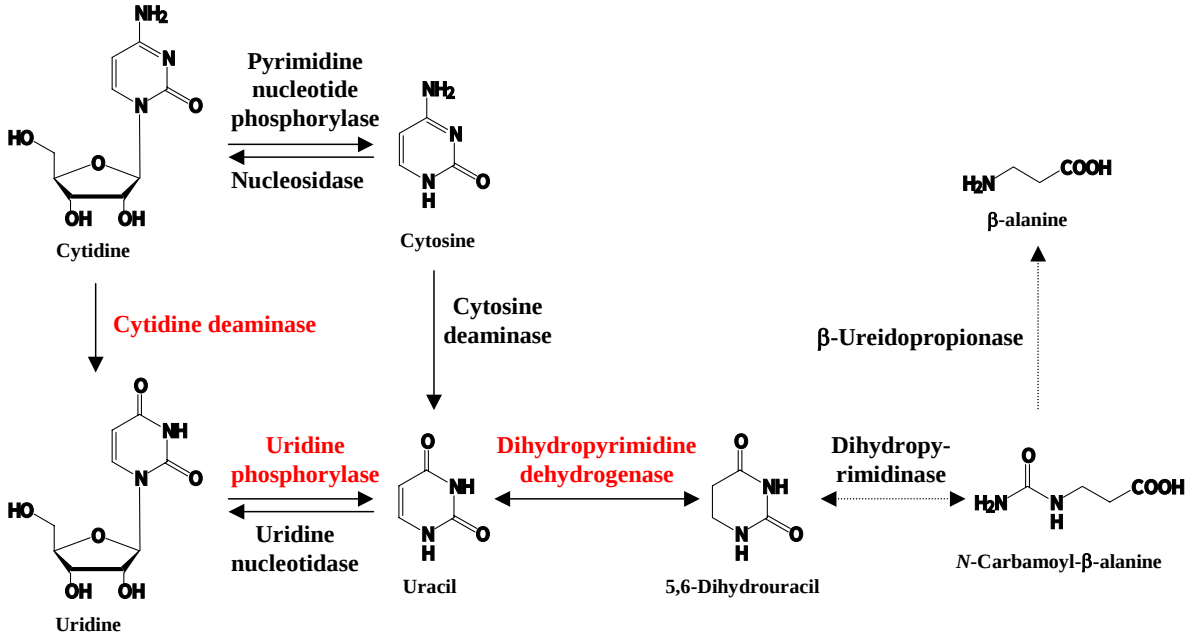


Fig. 4. Pyrimidine metabolic pathway

Red character shows the enzyme whose expression was affected by the deletion of *iscS* gene.

以上のように、本プロジェクトにおいて、*iscS* 遺伝子の欠損が実際に生体内において鉄硫黄クラスター構築能の欠損を導くことを明らかにした。さらに、鉄硫黄クラスターの欠損による鉄硫黄タンパク質の不活性化が、それが関与する代謝系全体に大きく影響を及ぼすことを明らかにした。本プロジェクトにおいて、共に研究に携わった学生諸君の研究能力も向上し、教育的効果も大きかったと評価できる。

Property and construction of chalcogenic functional materials

Nobuyoshi Esaki

Born in 1949. He received his Ph.D. degree from Kyoto University. He was a research associate (1979-1989), an associate professor (1989-1996) and a professor in the Institute of Chemical Research of Kyoto University (1996-). He was a director of The Japanese Chemical Society (1998-2000, 2002-2004), a director of Japan Society for Bioscience, Biotechnology, and Agrochemistry (1999-2003) and a director of The Society for Biotechnology, Japan (1999-2003). He promoted researches supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Scientific Research on Priority Area (2001-2004).

Escherichia coli IscS catalyzes the conversion of L-cysteine to L-alanine and sulfur. IscS provides the inorganic sulfur required for the formation of Fe/S clusters in Fe/S proteins. We analyzed extracts of *E. coli* MG1655 and an *iscS*-null strain by native polyacrylamide gel electrophoresis and iron-staining technique (Fig. 2). A protein band that gave the most significant difference in the band intensity between the two strains was subjected to N-terminal sequence analysis. We found that the stained band contained two polypeptides encoded by *b2146* and *yeiA*. Amino acid sequences of b2146 and YeiA, respectively, showed similarities (22% identity) to N- and C-terminal halves of mammalian dihydropyrimidine dehydrogenase (DPD). Each protein was expressed in *E. coli* as a histidine-tagged protein and purified. The absorption spectra of b2146 and YeiA exhibited characteristic maxima at 370 nm and 448 nm and 320 nm and 455 nm, respectively, suggesting the presence of Fe/S clusters (Fig. 3). By 2-DE analysis two different proteins were detected as the proteins affected by *iscS* deficiency and subsequently identified by protein sequencing. Expression levels of cytidine deaminase (CDD) and uridine phosphorylase (UDP) decreased owing to the *iscS* deficiency. CDD and UDP are also involved in pyrimidine salvage pathways (Fig. 4). IscS deficiency results in the deficiency of the iron-sulfur cluster of DPD, which causes the inactivation of DPD. Therefore, the accumulation of excess uracil might repress *cdd* and *udp* expression. Taken together, these results suggest that the IscS deficiency affects the pyrimidine metabolism, implying that the prymidine reductive degradation pathway involving DPD is operating in *E. coli* K12 cells, as well as mammals.

Selected Publications

· Presentation & Lectures

1. Analysis of expression level of selenocystein lyase in mouse, Kurokawa S, Mihara H, Kurihara T, Esaki N, 2004 Annual Meeting, Jpn. Soc. Biomed. Res. Trace Elem., 1 July
2. Functional analysis of MnmA, a tRNA-modifying enzyme, Kwak M-S, Mihara H, Kurihara T, Yoshimura T, Esaki N, 2004 Annual Meeting, Jpn. Soc. Biosci. Biotech. and Agrochem., 30 March
3. Functional analysis of Suf proteins, Ano K, Kazuoka T, Mihara H, Kurihara T, Esaki N, 2004 Annual Meeting, Jpn. Soc. Biosci. Biotech. and Agrochem., 30 March
4. Characteristics of Dihydropyrimidine Dehydrogenase from *E. coli*, Yamane M, Mihara H, Kurihara T, Esaki N, 2004 Annual Meeting, Jpn. Soc. Biosci. Biotech. and Agrochem., 30 March
5. Identification of Iron-Sulfur Proteins by Differential Screening Using an IscS-Deficient Strain, Yamane M, Mihara H, Kurihara T, Takahashi Y, Yoshimura T, Esaki N, 2003 Annual Meeting, Jpn. Biochem. Soc., 16 October.
6. Characterization of chloroplast-type cysteine desulfurase from *Synechocystis* sp. PCC6803, Maruoka N, Igarashi M, Kato S, Kurihara T, Mihara H, Esaki N, 2003 Annual Meeting, Jpn. Soc. Biosci. Biotech. and Agrochem., 2 April.

· Articles

1. “Enhanced Selenium Tolerance and Accumulation in Transgenic Arabidopsis Expressing a Mouse Selenocysteine Lyase”, Pilon M, Owen JD, Garifullina GF, Kurihara T, Mihara H, Esaki N, Pilon-Smits EAH, *Plant Physiol,* 2003, **131,** 1250-1257.
2. “A novel regulatory function of selenocysteine lyase, a unique catalyst to modulate major urinary protein”, Kwak MS, Mihara H, Esaki N, *J Mol Catal B: Enzymatic*, 2003, 23367-23372.
3. “Effects of Selenium Deficiency on the Expression of Selenoprotein mRNAs in Mouse Brain”, Kuwana E, Kurokawa S, Mihara H, Kurihara T, Yoshimura T, Esaki N *Biomed Res Trace Elem*, 2003, 14293-14296.
4. “Assembly of Iron-sulfur clusters mediated by cysteine desulfurases, IscS, CsdB and CSD, from *Escherichia coil*”, Kurihara T, Mihara H, Kato S, Yoshimura T, Esaki N *Biochim Biophys Acta*, 2003, **1647,** 303-309.
5. “D-amino acid aminotransferase: Fragmentation at a flexible loop is an efficient metond to generate mutant enzymes with new substrate specificities and elevated activities”, Fuchikami Y, Yoshimura T, Esaki N, *J Mol Catal B: Enzymatic*, 2003, 23321-328.
6. “Network of Protein-Protein Interactions among Iron-Sulfur Cluster Assembly Proteins in *Eschrichia coli*”, Tokumoto U, Nomura S, Minami Y, Mihara H, Kato S, Kurihara T, Esaki N, Kanazawa H, Matsubara H, Takahashi Y, *J Biochem*, 2002, **131,** 713-719.
7. “Characterization of a NifS-Like Chloroplast Protein from Arabidopsis. Implications for Its Role in Sulfur and Selenium Metabolism”, Pilon-Smits E, Garifullina G, Abdel-Ghany S, Kato S, Mihara H, Hale K, Burkhead J, Esaki N, Kurihara T, Pilon M, *Plant Physiol*, 2002, **130,** 1309-1318.
8. “Bacterial cysteine desulfurases: their function and mechanisms”, Mihara H, Esaki N, *Appl Microbio. Biotechnol*, 2002, **60,** 12-23.
9. “Hsc62, Hsc56, and GrpE, the third Hsp70 chaperone system of *Escherichia coli*”, Yoshimune K, Yoshimura T, Nakayama T, Nishino T, Esaki N, *Biochem Biophys Res Commun*, 2002, **293,** 1389-1395.
10. “Crystallization and preliminary X-ray analysis of substrate complexes of leucine dehydrogenase from *Thermoactinomyces intermedius*”, Muranova TA, Ruzheinikov SN, Sedelnikova SE, Baker PJ, Pasquo A, Galkin A, Esaki N, Ohshima T, Soda K, Rice DW, *Acta Crystallogr D Biol Crystallogr*, 2002, **58,** 1059-1062.
11. “The *iscS* gene is essential for the biosynthesis of 2-selenouridine in tRNA and the selenocysteine-containing formate dehydrogenase H”, Mihara H, Kato S, Lacourciere GM, Stadtman TC, Kennedy RA, Kurihara T, Tokumoto U, Takahashi Y, Esaki N, *Proc Natl Acad Sci U S A*, 2002, **99,** 6679-6683.
12. “Structure of External Aldimine of *Escherichia coli* CsdB, an IscS/NifS Homolog: Implications for Its Specificity toward Selenocysteine”, Mihara H, Fujii T, Kato S, Kurihara T, Hata Y, Esaki N, *J Biochem (Tokyo)* 2002, **131,** 679-685.
13. “Network of Protein-Protein Interactions among Iron-Sulfur Cluster Assembly Proteins in *Escherichia coli*”, Tokumoto U, Nomura S, Minami Y, Mihara H, Kato S, Kurihara T, Esaki N, Kanazawa H, Matsubara H, Takahashi Y, *J Biochem (Tokyo)*, 2002, **131,** 713-719.
14. “Cys-328 of IscS and Cys-63 of IscU are the sites of disulfide bridge formation in a covalently bound IscS/IscU complex: Implications for the mechanism of iron-sulfur cluster assembly”, Kato S, Mihara H, Kurihara T, Takahashi Y, Tokumoto U, Yoshimura T, Esaki N, *Proc Natl Acad Sci U S A*, 2002, **99,** 5948-5952.
15. “Selenocysteine lyase from mouse liver”, Mihara H, Esaki N, *Methods Enzymol*, 2002, **347,** 198-203.

生体関連物質化学 生体分子間相互作用の構造生物学

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1952 年生. 1978 年同大学院工学研究科博士後期課程中退, 1981 年工学博士(大阪大学), 1978 年大阪大学工学部助手(1982～1983 年マックスプランク生化学研究所博士研究員), 1991 年東京工業大学資源化学研究所助教授, 1994 年京都大学理学部教授, 1995 年より現職. 1999 年より理化学研究所播磨研究所主任研究員（兼務）. 日本化学会進歩賞, 日本結晶学会学術賞. Zeitschrift fuer Kristallographie 誌, Editor, PROTEINS: Structure, Function and Bioinformatics 誌, Editorial Board. 現在, 文部科学省タンパク 3000 プロジェクト・個別的解析プロジェクト中核拠点代表者. タンパク質結晶学の手法に基づいた構造生物学の研究を行っている.

生体分子間相互作用の構造生物学的研究として，以下のようなタンパク質の立体構造を，シンクロトロン放射光を用いたＸ線結晶解析によって決定し，その詳細な三次元構造を得るとともに，その構造に基づいてそれぞれの機能の解明を目指した.

(1) フコース特異的認識レクチン

ヒイロチャワンタケレクチン（AAL）は，六炭糖の一つであるフコースを高い特異性で認識する．AAL のフコース特異的認識機構を解明するため, その結晶構造を決定した．AAL の立体構造は，アミノ酸配列の６回繰り返し構造を反映してβシート６枚が疑似６回対称に配置した構造であった. この構造はβプロペラ構造と呼ばれており，AAL は６枚のβプロペラ構造を持つ初めてのレクチンである. 解析した結晶構造では，レクチン単量体あたり３分子のフコースがプロペラの間の窪みに結合していた. βプロペラ構造の６箇所の窪みのうち５箇所がフコース結合部位であると考えられ, そのうちの３箇所にフコースが結合していた.

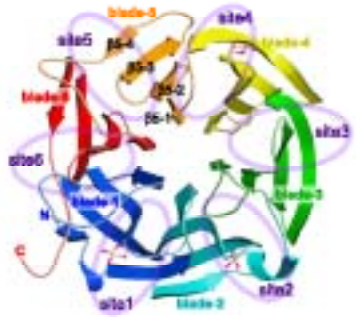

Fig.1. Crystal structure of AAL (lectin from *Aleuria aurantia*)

(2) グループ II シャペロニン

シャペロニンは生体内の多くのタンパク質の成熟や修復に関与するきわめて重要なタンパク質である．その立体構造上の特徴としては，相手のタンパク質を取り込むための内部の大きな空洞と，その中への出入りを制御する蓋の存在があげられる．グループⅡに属している古細菌由来のシャペロニンは，大腸菌由来の GroEL に代表されるグループⅠシャペロニンと比較して，その構造と機能には不明な点が多かった．２種類のサブユニット（αおよびβ）で構成される古細菌由来のシャペロニン Thermosome のαサブユニットホモオリゴマー（16 量体）の結晶構造を決定し，いくつかの変異体が構造に及ぼす影響や，“open form”と“close form”の構造変化の分子機構について議論した.

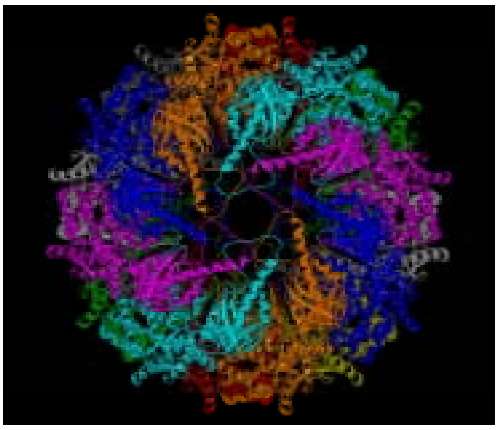

Fig.2. Crystal Structures of the *Thermococcus* Group II Chaperonin

（３）KP43 アルカリセリンプロテアーゼ

KP-43 は, 酸化剤耐性のサチライシン型のアルカリセリンプロテアーゼである．この KP-43 プロテアーゼの結晶構造を，native 型と酸化型で決定し，他のサチライシン様酵素と同様, 活性セリンに隣接するメチオニン残基が酸化されることを明らかにした．この KP-43 の構造は，タンパク質前駆体プロセッシングプロテアーゼである kexin や furin とその構築が類似していること，aqualysin I の予測二次構造との比較から，KP-43 の C-末端側延長配列部分のβ-バレルドメインがフォールディングに関わっている可能性があることを考察した.

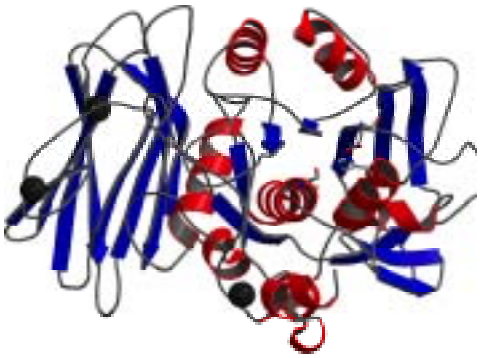

Fig.3. Crystal structure of an oxidatively stable subtilisin-like alkaline serine protease, KP-43

Structural biology of biological intermolecular interactions

Kunio Miki

Born in 1952. He received his Ph.D. degree from Osaka University. He was a research associate in the Faculty of Engineering, Osaka University (1978-1990), an associate professor in the Research Laboratory of Resources Utilization, Tokyo Institute of Technology (1991-1994), and a professor in the Department of Chemistry, Faculty of Science, Kyoto University (1994-1995). Now he has been a professor in the Graduate School of Science, Kyoto University (1995-) and also a chief scientist (a director of laboratory) in the RIKEN Harima Institute at SPring-8 (1999-). He was awarded the Progress Prize of the Chemical Society of Japan and the Academic Prize of the Crystallographic Society of Japan. He has been devoted in the studies of structural biology by means of X-ray crystallography.

(1) Crystal structure of a fucose specific lectin from *Aleuria aurantia* in complex with three fucose molecules: *Aleuria Aurantia* has a fucose-specific lectin (AAL) that is widely used as a specific probe for fucose. Since the fucosylated sugars often play pivotal roles in many cellular processes, we have determined the crystal structure of AAL with fucose molecules in order to understand the specific recognition mechanism. The three-dimensional structure of AAL is a six β-blade structure, reflecting the six internal homologous regions in the sequences. These blades form six clefts and are related by pseudo six-fold symmetry. Based on the similarities of both sequence and three-dimensional structure, five of the six clefts are thought to be fucose-binding sites. It was found that three of the five sites were bound with fucose molecules. These findings suggest that AAL has five fucose-binding sites per a subunit and ten sites in a physiological dimer. The multiplicity is higher than that of any known lectins and may contribute to the high affinity to fucosylated residues.

(2) Crystal Structures of the *Thermococcus* Group II Chaperonin: The crystal structures of the group II chaperonin consisting of α subunit with amino acid substitutions of G65C and/or I125T from the hyperthermophilic archaeum, *Thermococcus* strain KS-1, were determined. These mutants have been shown to be active in ATP hydrolyzing but inactive in protein folding. The structures were shown to be double-ring hexadecamers in an extremely closed form, which was consistent with the crystal structure of native $\alpha_8\beta_8$-chaperonin from *Thermoplasma acidophilum*. Comparisons of the present structures with the atomic structures of the $GroEL_{14}$-$GroES_7$-$(ADP)_7$ complex revealed that the deficiency in protein-folding activity of G65C amino acid substitutants is caused by the steric hindrance of the local conformational change in an equatorial domain. We concluded that this mutant chaperonin with G65C substitution is deprived of the smooth conformational change in the refolding-reaction cycle. We also obtained a new form of crystal with the distinct space group at the

lower sulfate ion concentration in the presence of nucleotide. The crystal structure obtained at the lower sulfate ion concentration tilts outward, and has much looser inter-subunit contacts compared with those in the presence of a higher concentration of sulfate ion. Such subunit rotation that has never been characterized in group II chaperonins. The crystal structure obtained at the lower sulfate ion concentration tilts outward, and has much looser inter-subunit contacts compared with those in the presence of a higher concentration of sulfate ion.

(3) Crystal structure of an oxidatively-stable subtilisin-like alkaline serine protease, KP-43: The crystal structure of an oxidatively stable subtilisin-like alkaline serine protease, KP-43 from *Bacillus* sp. KSM-KP43, with a C-terminal extension domain, was determined by the multiple isomorphous replacements method with anomalous scattering. The structure of the native form showed that KP-43 consists of two domains, a subtilisin-like α/β domain and a C-terminal jelly roll β-barrel domain. The topological architecture of the molecule is similar to that of kexin and furin, which belong to the subtilisin-like proprotein convertases (SPCs), whereas the amino acid sequence and the binding orientation of the C-terminal β-barrel domain both differ in each case. Since the C-terminal domains of SPCs are essential for folding themselves, the domain of KP-43 is also thought to play such a role. KP-43 is known to be an oxidation-resistant protease among the general subtilisin-like proteases. The structure of the oxidized form revealed that Met-256, adjacent to catalytic Ser-255, was oxidized similarly to an equivalent residue in subtilisin BPN'. Although KP-43, as well as proteinase K and subtilisin Carlsberg, lose their hydrolyzing activity against synthetic peptides after oxidation treatment, all of them retain 70-80% activity against proteinaceous substrates. These results, as well as the β-casein digestion pattern analysis, have indicated that the oxidation of the methionine adjacent to the catalytic serine is not a dominant modification, but might alter the substrate specificities.

Selected Publications

Presentation & Lectures

1. K. Miki, "Structural Basis of Bacterial Lipoprotein Localization System", 2003 POSTECH Symposium of Structure and Folding in Protein Science, 2003.10.2, Pohang, Korea (Invited)
2. K. Miki, "Structural Genomics in Japanese University Community", 3rd Tsinghua International Conference of Protein Sciences, TICPS 3, 2003.10.21-23, Tsinghua University, Beijing, China (Invited)
3. K. Miki, "Structural Genomics in the Field of Formation of Protein Higher-order Structures and Expression of Protein Functions", Workshop France-Japan on Structural Genomics, 2003.11.10-12, University of Tokyo, Tokyo, Japan (Invited)
4. K. Miki, "Structural Genomics and Proteomics in Japanese University Community", The 1st Pasific-Rim International Conference on Protein Science and the 4th Annual Meeting of the Protein Science Society of Japan, 2004. 4.14-18, Yokohama, Japan (Invited)
5. K. Miki, "Structural Genomics and Proteomics in Japanese University Community", The 9th Symposium on Recent Advances in Biophysics, 2004. 5.26-28, Academia Sinica, Taipei, Taiwan (Plenary lecture)
6. K. Takeda, H. Miyatake, N. Yokota, S. Matsuyama, H. Tokuda, and K. Miki, "Structural Basis of Bacterial Lipoprotein Transport", The Sixth Conference of the Asian Crystallographic Association, AsCA04, 2004.6.27-30, Hong Kong, China (Invited)
7. K. Miki, "Structural Basis of Bacterial Lipoprotein Localization", The Seventh R.O.C and Japan Joint Seminar on Crystallography, 2004.11.8-9, Tokyo, Japan (Invited)

Articles

1. "Crystal Structure of the (*R*)-Specific Enoyl-CoA Hydratase from *Aeromonas caviae* Involved in Polyhydroxyalkanoate Synthesis", T. Hisano, T. Tsuge, T. Fukui, T. Iwata, K. Miki, and Y. Doi, *J. Biol. Chem.*, 2003, **278**, 617-624.
2. "X-ray Crystallographic Characterization and Phasing of a Fucose Specific Lectin from *Aleuria Aurantia*", M. Fujihashi, D. H. Peapus, E. Nakajima, T. Yamada, J. Saito, A. Kita, Y. Higuchi, Y. Sugawara, A. Ando, N. Kamiya, Y. Nagata, and K. Miki, *Acta Crystallogr.*, 2003, **D59**, 378-380.

3. “Mutational Analysis of Allylic Substrate Binding Site of *Micrococcus luteus* B-P 26 Undecaprenyl Diphosphate Synthase”, K. Fujikura, Y.-W. Zhang, M. Fujihashi, K. Miki, and T. Koyama, *Biochemistry*, 2003, **42**, 4035-4041
4. “Crystallization and Preliminary Crystallographic Study of the Outer-Membrane Lipoprotein Receptor LolB, a Member of the Lipoprotein Localization Factors”, K. Takeda, H. Miyatake, N. Yokota, S. Matsuyama, H. Tokuda, and K. Miki, *Acta Crystallogr.*, 2003, **D59**, 1224-1226.
5. “Crystal Structures of Bacterial Lipoprotein Localization Factors, LolA and LolB”, K. Takeda, H. Miyatake, N. Yokota, S. Matsuyama, H. Tokuda, and K. Miki, *EMBO J.*, 2003, **22**, 3199-3209
6. “Crystal Structure of Calcium-free -Amylase from *Bacillus* sp. Strain KSM-K38 (AmyK38) and Its Sodium Ion Binding Sites”, T. Nonaka, M. Fujihashi, A. Kita, H. Hagihara, K. Ozaki, S. Ito, and K. Miki, *J. Biol. Chem.*, 2003, **278**, 24818-24824.
7. “A Practical Phasing Procedure Using the MAD Method without the Aid of XAFS Measurements: Successful Solution in the Structure Determination of the Outer-Membrane Lipoprotein Carrier LolA”, K. Takeda, H. Miyatake, N. Yokota, S. Matsuyama, H. Tokuda, and K. Miki, *Acta Crystallog*r., 2003, **D59**, 1440-1446.
8. “Crystal Structure of Dissimilatory Sulfite Reductase D (DsrD) Protein --- Possible Interaction with B- and Z-DNA by Its Winged-Helix Motif”, N. Mizuno, G. Voordouw, K. Miki, A. Sarai, and Y. Higuchi, *Structure*, 2003, **11**, 1133-1140.
9. “Crystal Structure of Fucose Specific Lectin from *Aleuria aurantia* Binding Ligands at Three of Its Five Sugar Recognition Sites”, M. Fujihashi, D. H. Peapus, N. Kamiya, Y. Nagata, and K. Miki, *Biochemistry*, 2003, **42**, 11093-11099.
10. “ATP Binding Is Critical for the Conformational Change from an Open to Closed State in Archaeal Group II Chaperonin”, R. Iizuka, T. Yoshida, Y. Shomura, K. Miki, T. Maruyama, M. Odaka, and M. Yohda, J. Biol. Chem., 2003, **278**, 44959-44965.
11. “Crystal Structure of Human Pirin: An Iron-Bonding Nuclear Protein and Transcription Cofactors”, H. Pang, M. Bartlam, Q. Zeng, H. Miyatake, T. Hisano, K. Miki, L.-L. Wong, G. F. Gao, and Z. Rao, J. Biol. Chem., 2004, **279**, 1491-1498.
12. “Crystal Structures of the Group II Chaperonin from *Thermococcus* strain KS-1: Steric Hindrance by the Substituted Amino Acid, and Inter-subunit Rearrangement between Two Crystal Forms”, Y. Shomura, T. Yoshida, R. Iizuka, T. Maruyama, M. Yohda, and K. Miki, 2004, J. Mol. Biol., **335**, 1265-1278.
13. “The N-terminal Domain of the Replication Initiator Protein RepE Is a Dimerization Domain Forming a Stable Dimer”, A. Nakamura, H. Komori, G. Kobayashi, A. Kita, C. Wada, and K. Miki, Biochem. Biophys. Res. Commun., 2004, **315**, 10-15.
14. “Crystallization and Preliminary X-ray Crystallographic Studies on the Small Form of Glucose-inhibited Division Protein A from *Thermus thermophilus* HB8”, W. Iwasaki, H. Miyatake, A. Ebihara, and K. Miki, Acta Crystallogr., 2004, **D60**, 515-517.
15. “Structure of the C subunit of V-type ATPase from *Thermus thermophilus* at 1.85 Å resolution”, N. Numoto, A. Kita, and K. Miki, 2004, Acta Crystallogr., **D60**, 810-815.
16. “DNA apophotolyase from *Anacystis nidulans*: 1.8 Å structure, 8-HDF reconstitution and X-ray induced FAD-reduction”, R. Kort H. Komori, S. Adachi, K. Miki, and A. P. M. Eker, 2004, Acta Crystallogr., **D60**, 1205-1213.
17. “The Crystal Structure of an Oxidatively-stable Subtilisin-like Alkaline Serine Protease, KP-43, with a C-terminal β-barrel Domain”, T. Nonaka, M. Fujihashi, A. Kita, K. Saeki, S. Ito, K. Horikoshi, and K. Miki, J. Biol. Chem., 2004, **279**, 47344-47351.
18. Multiwavelength Anomalous Diffraction Method Using Ultra-high Energy X-rays: Test Studies of I-MAD and X-MAD Experiments at BL41XU of SPring-8, K. Takeda, H. Miyatake, S.-Y. Park, M. Kawamoto, N. Kamiya, and K. Miki, J. Appl. Crystallogr., 2004, **37**, 925-933.