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JB Reflections and Perspectives

Professor Tatsuo Miyazawa: from molecular structure to biological function

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The late Prof. Tatsuo Miyazawa was an outstanding physical chemist, who established a number of spectroscopic methods to analyse the structures of proteins, peptides and nucleotides, and used them to understand molecular functions. He developed an infrared spectroscopic method to quantitatively analyse the secondary structures, α-helices and β-strands, of proteins. He successfully utilized nuclear magnetic resonance (NMR) methods to determine the conformations of peptides and proteins, particularly with respect to the interactions with their target molecules, which served as a solid basis for the wide range of applications of NMR spectroscopy to life science research. For example, he found that physiologically active peptides are randomly flexible in solution, but assume a particular effective conformation upon binding to their functional environments, such as membranes. He also used NMR spectroscopy to quantitatively analyse the conformer equilibrium of nucleotides, and related the dynamic properties of the modified nucleosides naturally-occurring in transfer ribonucleic acids (tRNAs) to their roles in correct codon recognition in protein synthesis. Furthermore, he studied the mechanisms of protein biosynthesis systems, including tRNA and aminoacyl-tRNA synthetases. Inspired by the structural mechanism of amino acid recognition by aminoacyl-tRNA synthetases, as revealed by NMR spectroscopy, he initiated a new research area in which non-natural amino acids are site-specifically incorporated into proteins to achieve novel protein functions (alloprotein technology).

Keywords: alloprotein/macromolecular conformation/NMR spectroscopy/protein biosynthesis/vibrational spectroscopy.

Abbreviations: Ahx, L-2-aminohexanoic acid; CW, continuous wave; EF-Tu, elongation factor Tu; FT, Fourier transform; GluRS, glutamyl-tRNA synthetase; hEGF, human epidermal growth factor; IleRS, isoleucyl-tRNA synthetase; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PTC, phenylthiocarbamoyl; RNase, ribonuclease; tRNA, transfer ribonucleic acid; TRNOE, transferred nuclear Overhauser effect.

Infrared spectroscopy of polypeptides and proteins: the amide bands

Professor Tatsuo Miyazawa (Fig. 1) graduated from the University of Tokyo in 1950. He had already joined the laboratory of Prof. San-ichiro Mizushima, and studied peptide conformation by infrared spectroscopy when he was a graduate student. After he obtained his PhD in 1956, he received a Fulbright Grant to join the laboratory of Prof. Kenneth S. Pitzer, at the Department of Chemistry, University of California, Berkeley. In 1958, he joined the laboratory of Prof. Elkan R. Blout at Children's Cancer Research Foundation, Boston, and analysed the amide bands in the infrared spectra of polypeptides. He continued vibrational spectroscopic studies at the Institute of Protein Research, Osaka University, as an associate professor (1959–64) and a professor (1964–74). He published more than 100 papers during these periods, and was recognized as an outstanding physical chemist.

Infrared spectroscopy is a useful method for studying the chain conformations of polypeptides and proteins (*1*, *2*). Polypeptides exhibit characteristic infrared absorption bands: amides A, B and amides I–VII (*1*). It was previously shown that amide A (3,300 cm^{-1}), amide B (3,100 cm^{-1}), amide I (1,650 cm^{-1}) and amide II (1,550 cm^{-1}) are characteristic of the CONH group (*3*, *4*). Professor Miyazawa elucidated the nature of these bands in detail by analysing monosubstituted amides, including *N*-methylacetamide (CH_3–CONH–CH_3), and performing theoretical calculations of the vibrations. Professor Miyazawa showed quantitatively that the amide A and B bands are due to the Fermi resonance between the fundamental N–H stretching vibration and the first overtone of the amide II vibration (*5*). Professor Miyazawa also quantitatively described the nature of the amide I and II bands by a normal coordinate analysis (*6*), as well as by infrared and Raman studies of a series of monosubstituted amides (*7*). Figure 2 shows the calculated normal modes of the amide I–IV vibrations.

The frequencies of the amide I and II bands reportedly differ between the α-helical conformation and the β-conformations (*8*). Professor Miyazawa theoretically analysed the vibrational interactions among peptide groups, and established an elegant relationship between the frequencies and the chain conformations (*9*, *10*), as summarized in Table I. This method to analyse the amide I and II bands is practically useful for vibrational spectroscopic analyses of protein main-chain conformations. Therefore, these papers by Prof. Miyazawa have been extensively cited, and are cited even today, as the primary theoretical basis.

Featured Article

Fig. 1 Professor Miyazawa (1927–93).

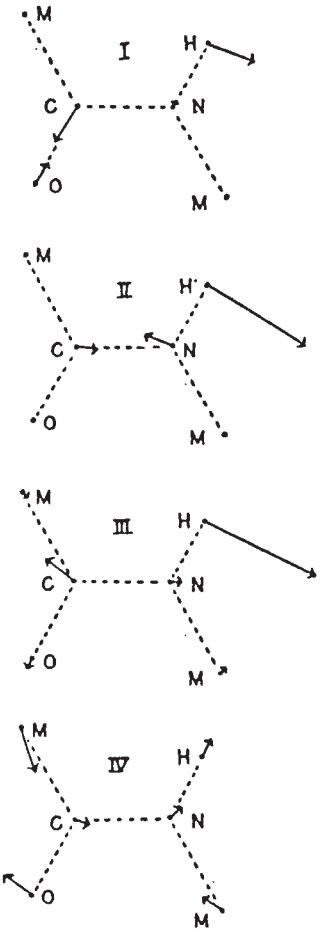

Fig. 2 Calculated normal modes of the amide I–IV vibrations of *N*-methylacetamide (*1*). Stahmann, Mark A. Polyamino Acids, Polypeptides, And Proteins. © 1962 by the Board of Regent of the University of Wisconsin System. Reprinted courtesy of The University of Wisconsin Press.

For proteins, various chain conformations may co-exist, and the amide bands IV–VI observed in the 800–500 cm^{-1} region can be used to estimate the fractions of various conformations (*2*). As for the side chain conformations, Prof. Miyazawa established the correlations of the cystine disulfide-bond conformations to the S–S and C–S stretching frequencies (*11*).

Since Prof. Miyazawa's achievements, vibrational spectroscopic methods have been greatly advanced by laser Raman and Fourier transform infrared spectroscopy, etc., and are now used to analyse the conformational properties of proteins, including protein unfolding and amyloids, for which other methods, such as X-ray crystallography, are not easily applied (*12*). The idea that the conformations of biomolecules, such as proteins, can be analysed by spectroscopic approaches has led to the current stage of NMR structural biology.

Table I. The frequencies (cm^{-1}) and relative intensities of the amide I and II bands of polypeptides in various conformations [based on ref. (*10*)].

Conformation	Amide I	Amide II
Random coil	1,655 (strong)	1,535 (strong)
α-Helix	1,650 (strong)	1,516 (weak)
	1,652 (medium)	1,546 (strong)
Parallel-chain β	1,645 (weak)	1,530 (strong)
	1,630 (strong)	1,550 (medium)
Anti-parallel-chain β	1,685 (weak)	1,530 (strong)
	1,632 (strong)	–
	–	1,550 (weak)

NMR analyses of nucleotides, peptides and proteins

In 1971, Prof. Miyazawa moved to the Department of Biophysics and Biochemistry, at the University of Tokyo. He performed NMR spectroscopy of biological molecules with Prof. Mitsuo Tasumi, an associate professor. In 1972, a 90-MHz NMR spectrometer (Hitachi R22) was installed, and was operated in a continuous wave (CW) mode and later in a Fourier transform (FT) mode. Then, in 1976, the first FT-NMR spectrometer with a superconducting magnet in Japan (Bruker WH-270) was installed in Miyazawa's laboratory. With these NMR spectrometers, new projects on nucleotides, peptides and small proteins were initiated.

Nucleotides and peptides in solution are flexible with respect to the conformations around single bonds, and thus multiple conformers may exist in equilibrium. By applying NMR spectroscopy, Prof. Miyazawa succeeded in the quantitative analysis of conformer

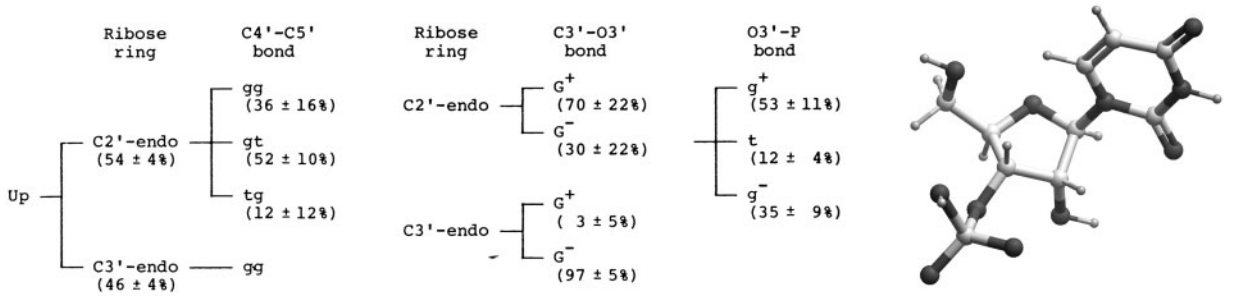

Fig. 3 Fractional populations of local conformations of Up (*16*). Reprinted in part with permission from *Biochemistry* **20**, 2981–2988. Copyright 1981 American Chemical Society. A boll-and-stick model of the most preferable conformation, C3′-*endo*-*gg*-G^{-}-g^{+}, of Up is also shown.

equilibria of molecules with plural flexible single bonds, for the first time. For this purpose, he chose the lanthanide-ion probe method, which utilizes the paramagnetic effects of lanthanide ions, bound at the nucleotide phosphate group, on the chemical shifts and the relaxation rates (*13*, *14*). The advantage of this method is that it can yield information about the distances and orientations of the observed nuclei relative to the lanthanide ion, spanning relatively long distances (~10 Å), or molecules as large as the nucleotide unit. By employing the lanthanide probe analyses in combination with shorter-distance analyses of vicinal spin-coupling constants and nuclear Overhauser effects (NOEs), Prof. Miyazawa successfully determined not only the structure but also the population of each conformer in a multi-conformational equilibrium (*15*, *16*). Figure 3 shows the fractional populations of the local conformations of uridine 3′-monophosphate (Up), a building block of ribonucleic acid (RNA). For example, the conformation around the C3′–O3′ bond of Up exists in an equilibrium between the G^{+} (major) and G^{-} (minor) forms when the ribose moiety assumes the C2′-*endo* form, but the C3′–O3′ conformation is exclusively in the G^{-} form when the ribose moiety assumes the C3′-*endo* form. This concept of 'conformational interrelations controlling RNA structures' subsequently led to the elucidation of the functions of post-transcriptional modifications in transfer RNAs (tRNAs), as described below.

When a small peptide, such as a biologically active peptide, is in equilibrium between the free and bound forms with phospholipid membranes, a transferred NOE [TRNOE), (*17*)] technique can be used to analyse the conformation of the peptide bound to the membranes. The technique was first applied to mastoparan-X, one of the mast cell-degranulating peptides in the venom of *Vespa xanthoptera* (*18*) and then to α-mating factor, a pheromone secreted by the α-type cell of *Saccharomyces cerevisiae* (*19*). The merit of the TRNOE method is that the NOEs, which provide structural information, of the membrane-bound peptide can be obtained by analysing the well-resolved signals of the free peptide. Figure 4 shows the conformation of membrane-bound α-mating factor, in which the N-terminal nine residues, Trp–His–Trp–Leu–Gln–Leu–Lys–Pro–Gly, are tightly bound to the membrane and the conformations of these residues are well determined. The C-terminal four residues,

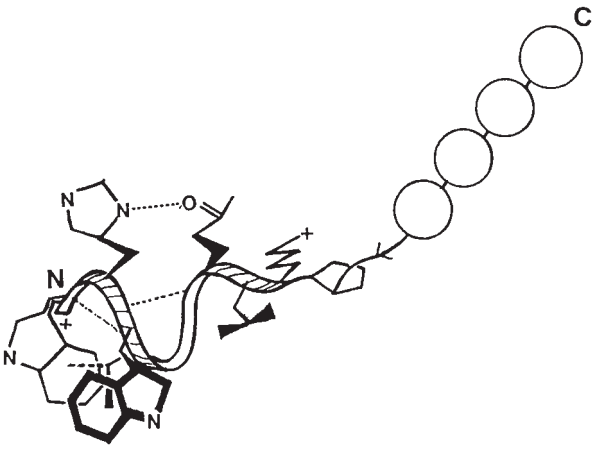

Fig. 4 Conformation of membrane-bound α-mating factor (*19*). Reprinted in part with permission from Wakamatsu *et al.* (*19*), Wiley-Blackwell.

Gln–Pro–Met–Tyr, are left free in the aqueous phase. Since the physiological activities of peptides are correlated with the conformations of membrane-bound molecules (*20*), the TRNOE analysis of biologically active peptides is beneficial for obtaining their structure–activity relationships. This line of study on the membrane-bound peptide conformations is currently advancing, particularly by solid-state NMR methods (*21*). On the other hand, the concept of the 'formation of the functional peptide conformation upon binding to its target' is being applied more generally now, for example, to the intrinsically-unfolded regions of large proteins that assume the functional conformation only when complexed with their partner proteins or nucleic acids.

As for small proteins, Prof. Miyazawa measured and analysed the 270-MHz ^{1}H NMR spectra of snake neurotoxins, such as erabutoxins a and b (Fig. 5) (*22*, *23*). Erabutoxins are neurotoxic proteins from sea snake venom and consist of 62 amino acids. The NMR signals from several residues were assigned and their structural characteristics were analysed. For example, it was unambiguously concluded that the imidazole ring of His7 is only protonated upon denaturation at pH 2.85, indicating that His7 is deeply buried in the interior of the protein. In this manner, Prof. Miyazawa powerfully demonstrated that the unambiguous assignment of signals to the amino acid

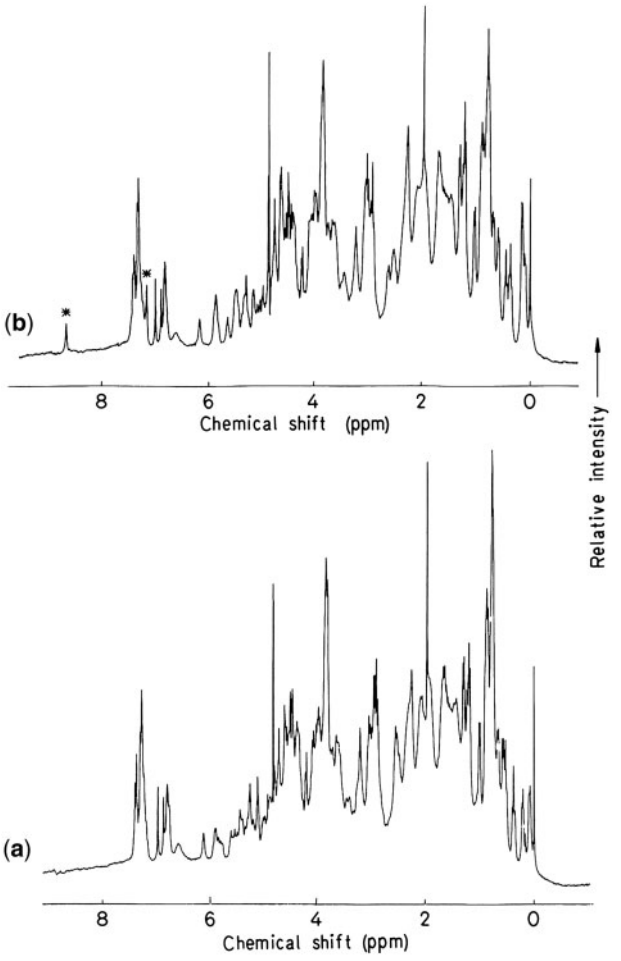

Fig. 5 The 270-MHz ^{1}H-NMR spectra of (a) erabutoxin and (b) erabutoxin b in D_2O solution at pH 5.2 and 296 K (*22*). These proteins consist of 62 amino acid residues. The protein concentration was 4 mM. Reprinted in part with permission from Inagaki *et al.* (*14*), Wiley-Blackwell.

residues in the primary sequence of the analyte protein is essential for understanding the structure and function of the protein. Since these pioneering discoveries by Prof. Miyazawa, the NMR methods have greatly advanced, *e.g.* multiple stable-isotope labeling techniques and two-, three- and four-dimensional NMR spectroscopy, so that the tertiary-structure determination of proteins in solution is now generally possible for relatively small proteins, from 50 to 300 residues, and still spanning towards larger ones. Moreover, the structures of functional RNA molecules can also be determined by NMR methods.

Professor Miyazawa also showed that the conformation of a small ligand bound to a protein can be analysed by NMR. In the case of an inhibitor bound to Ribonuclease (RNase) T_1 (*24*), the base orientation of the bound guanine nucleotides was successfully analysed from the NOE between the H8 and H1′ protons, together with the vicinal coupling constants between the H1′ and H2′ protons. The conformation of the guanosine moiety bound to RNase T_1 was found to be C3′-*endo-syn* for 2′-GMP and 3′-GMP and C3′-*endo-anti* for 5′-GMP and guanosine 3′,5′-bis(phosphate), suggesting that there is no specific binding site for the ribose moiety of the inhibitors. A similar analysis was performed to determine the conformation of the guanine nucleotide bound to the c-Ha-*ras* protein (*25*). These types of NMR analyses are now quite widely performed to determine the interactions of proteins with small compounds, such as inhibitors, substrates, cofactors and medicines, as well as the interactions of proteins with other biological macromolecules, such as proteins, nucleic acids and lipid membranes. The detailed structural information of the interaction, which can only be obtained by such NMR analyses, is generally useful from basic to applied studies in life science. It should be emphasized that Prof. Miyazawa's pioneering studies still serve as the bases for the frontier of life science research. For example, the lanthanide probe method is now being applied to study the transient and dynamic processes of protein–protein and protein–nucleic acid interactions (*26*, *27*).

Protein biosynthesis mechanisms and 'alloproteins'

Around 1980, Prof. Miyazawa started structural and functional studies on protein biosynthesis systems, including tRNA and aminoacyl-tRNA synthetase. These studies led to the *in vivo* production of an 'alloprotein', which contains unnatural amino acids, with the support of grants-in-aid for Distinguished Research (1985–88).

The codon recognition by tRNA is an important step for the fidelity in the translation of genetic information. A variety of post-transcriptionally modified nuclesides have been identified in tRNA molecules, and among them, modified uridines in the first position of the anticodon constitute one of the most diverse and complicated groups of naturally occurring, modified nucleosides. These uridine modifications were considered to be related to the properties of the tRNA in 'wobble' codon recognition through non-Watson–Crick base pairing (*28*), but there was no structural basis for the dynamic process of wobbling. Based on the above-mentioned conformational analysis of uridine nucleotides by the lanthanide probe method, the conformational characteristics of modified uridine residues in the first position of the anticodon were analysed (*29*). It was clearly demonstrated that derivatives of 5-methyl-2-thiouridine (xm^5s^2U) in the first position of the anticodon exclusively assume the C3′-*endo* form to recognize adenosine (but not uridine) in the third position of the codon. In contrast, derivatives of 5-hydroxyuridine (xo^5U) assume the C2′-*endo* form as well as the C3′-*endo* form to recognize adenosine, guanosine and uridine as the third letter of the codon (Fig. 6). Accordingly, the biological significance of the modifications of U to xm^5s^2U/xo^5U is in the regulation of the conformational rigidity/flexibility in the first position of the anticodon, to guarantee the correct and efficient translation of codons in protein biosynthesis. This is the first successful demonstration that the dynamic properties of biological molecules in

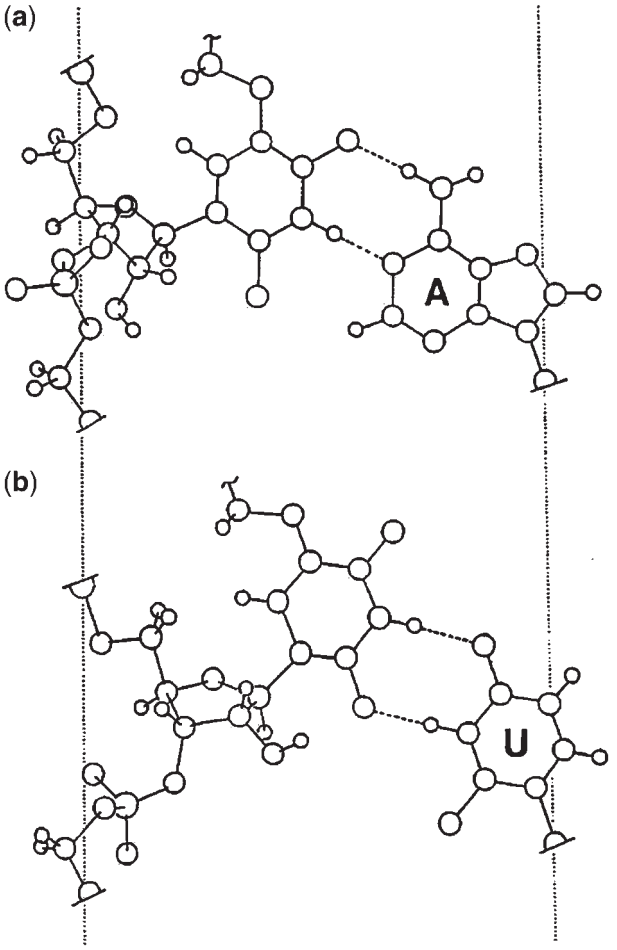

Fig. 6 Base pairs of xo^{5}U with adenosine (a) and uridine (b) as the third letter of the codons (*29*).

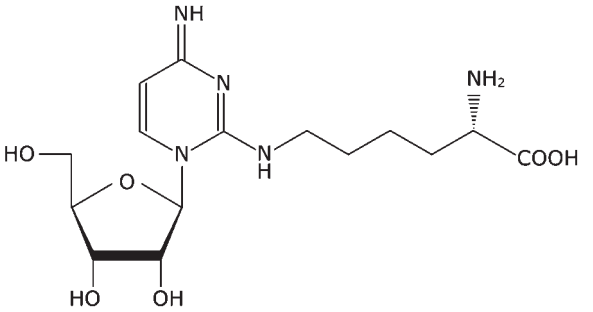

Fig. 7 Structure of the nucleoside N^+ (lysidine) in the neutral form.

conformational equilibrium are directly relevant to their biological roles. It is interesting that a similar 'rigid' 2-thiouridine derivative (2-thioribothymidine) is the major stabilizing factor in the tRNAs from an extremely thermophilic bacterium, *Thermus thermophilus* HB8 (*30*, *31*).

Professor Miyazawa revealed that the modification in the first position of the anticodon is required not only for the codon recognition but also for the tRNA identity, *i.e.* the specific recognition by the aminoacyl-tRNA synthetase. In *Escherichia coli*, the major isoleucine tRNA ($tRNA^{Ile}_{major}$) recognizes the AUU and AUC codons, and the minor isoleucine tRNA ($tRNA^{Ile}_{minor}$) recognizes the AUA codon only. The $tRNA^{Ile}_{minor}$ was purified from 10 kg of wet cells, and yielded 10 μg of the unknown modified nucleoside N^+, located in the first position of the anticodon. The chemical structure of N^+ was analysed by NMR and mass spectrometry, and was confirmed by chemical synthesis, as shown in Fig. 7 (*32*). N^+ was found to be a lysine-substituted cytidine, and was named lysidine, which must recognize adenosine, but not guanosine. Surprisingly, the unmodified $tRNA^{Ile}_{minor}$ bearing cytidine in the first position of the anticodon, which corresponds to the methionine codon AUG, showed methionine-accepting activity and reduced isoleucine-accepting activity, indicating that the codon and amino acid specificities of a tRNA are both converted by the lysidine modification (*33*). Recently, the mechanisms of the lysidine biosynthetic processes were revealed by X-ray crystallography (*34–36*).

In addition to tRNA recognition, aminoacyl-tRNA synthetases are also responsible for the strict discrimination of their cognate amino acid from others, which is required for the fidelity in the translation of genetic information. Isoleucyl-tRNA synthetase (IleRS) discriminates between L-isoleucine and L-valine with a very low error rate in protein biosynthesis, although the chemical structures of L-isoleucine and L-valine are similar to each other. Although IleRS is one of the largest among the 20 aminoacyl-tRNA synthetases (115 kDa), the TRNOE technique was applied with the hope of elucidating the conformations of amino acids interacting with *E. coli* IleRS (*37*). The TRNOE analysis revealed that the conformations of L-isoleucine and L-valine bound to IleRS are quite similar to each other, and that the hydrophobic interaction of L-isoleucine with the active site of IleRS is more significant than that of L-valine. Furthermore, the conformation of the non-protein amino acid furanomycin bound to IleRS was also analysed by TRNOE, which clarified the mechanism of furanomycin incorporation into proteins (*38*). This is an example of the usefulness of the TRNOE method for high-molecular mass systems.

In order to obtain more stable proteins for further studies, Prof. Miyazawa isolated several aminoacyl-tRNA synthetases, including IleRS and glutamyl-tRNA synthetase (GluRS), from the above-mentioned extreme thermophile, *T. thermophilus* HB8. The molecular mechanism underlying the strict specificity of *T. thermophilus* GluRS for glutamic acid tRNA ($tRNA^{Glu}$) was analysed (*39*). GluRS is one of the aminoacyl-tRNA synthetases that require the cognate tRNA for an apparently unrelated reaction, for the formation of an aminoacyl-adenylate from ATP and the cognate amino acid (the process of amino acid activation). The studies revealed that, in the absence of $tRNA^{Glu}$, GluRS binds not only the correct substrate, L-glutamate, but also incorrect amino acids, such as D-glutamate and L-aspartate. In contrast, GluRS recognizes only L-glutamate in the presence of its cognate $tRNA^{Glu}$. Thus, the cognate $tRNA^{Glu}$ acts as an allosteric effector for the specific activation of L-glutamate

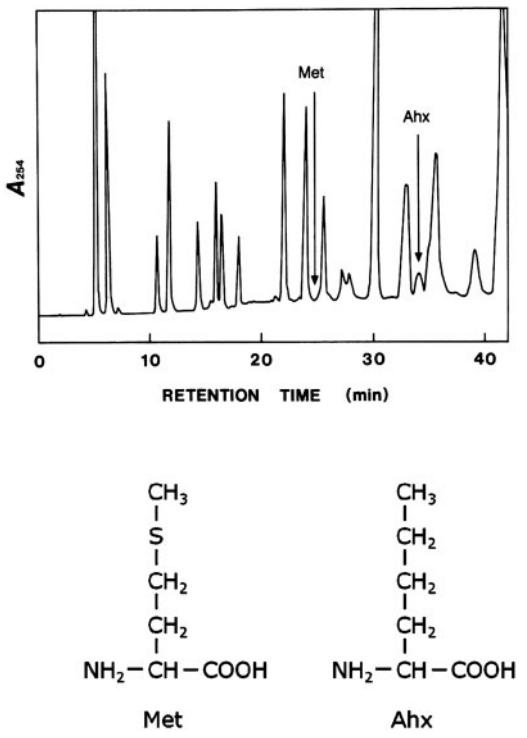

Fig. 8 Reverse-phase HPLC of PTC derivatives of amino acids from a hydrolysate of [Ahx^{21}]hEGF (*46*) and the chemical structures of Met and Ahx.

by GluRS. Subsequently, the crystal structures of *T. thermophilus* GluRS by itself and in complex with its substrates, ATP, glutamate and/or tRNA^{Glu}, revealed the structural basis for the tRNA-dependent glutamate activation by GluRS (*40–42*). Presently, a large number of studies, using many kinds of aminoacyl-tRNA synthetases from *T. thermophilus*, have established the structural basis for the amino acid and tRNA recognition mechanisms, mainly by X-ray crystallography (*43*).

The studies described above, as well as those on elongation factor Tu (EF-Tu) (*44*, *45*), paved the way towards the engineering of protein biosynthesis systems. Professor Miyazawa started the project to produce 'alloproteins' with unnatural amino acids, by using the protein biosynthesis system. As previously mentioned, *E. coli* IleRS recognizes the non-protein amino acid furanomycin. Furthermore, furanomycin-bound isoleucine tRNA (Fur-tRNA^{Ile}) can form a ternary complex with EF-Tu and GTP. Subsequently, the incorporation of furanomycin into a protein was accomplished by an *in vitro* translation system with an *E. coli* S30 extract (*38*). Thus, it was demonstrated that a protein carrying non-protein amino acids, an alloprotein, can be produced by the protein biosynthesis system. During his last few years at the University of Tokyo, Prof. Miyazawa promoted the alloprotein project, and eventually successfully produced human epidermal growth factor (hEGF) bearing a non-protein amino acid, L-2-aminohexanoic acid (Ahx) or L-norleucine, by using an *E. coli* secretion system (*46*). Figure 8 shows the amino acid composition analysis of the produced alloprotein. [Ahx^{21}]hEGF exhibited an activity comparable to that of the natural hEGF for the stimulation of cell proliferation as well as DNA synthesis. Moreover, [Ahx^{21}]hEGF is resistant to inactivation through the oxidation of the single methionine residue of hEGF.

After he retired from the University of Tokyo, Prof. Miyazawa continued the alloprotein project at Yokohama National University from 1988. It should be emphasized that Prof. Miyazawa is the pioneer who established alloprotein technology using non-natural amino acids in protein biosynthesis. Today, a large number of engineered aminoacyl-tRNA synthetases, which were created through random and structure-based approaches, are widely used to incorporate useful non-natural amino acids into specified positions of target proteins (*47–54*). The idea of using the non-natural properties of amino acids to drastically improve protein functions is becoming more and more realistic, and is regarded as 'superprotein' technology, as Prof. Miyazawa dreamed. Quite sadly, Prof. Miyazawa passed away suddenly in 1993, soon after he moved to the Protein Engineering Research Institute in 1991 to serve as its president.

Professor Miyazawa first used infrared spectroscopy, and primarily focused his efforts on the theoretical aspects of the field of physicochemistry. He then switched the methodology to NMR spectroscopy, and subsequently to biochemistry. He did not hesitate to change the direction of his research, and acted decisively to achieve his research goals. He always encouraged the laboratory members, from staff to students, to do everything possible to achieve their goals. Prof. Miyazawa's attitude strongly influenced all of his coworkers, and many of his former students are now active at the forefront of biochemical research, not only in structural biology but also a broad range of life sciences.

Conflict of interest
None declared.

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