PL

Mechanism-based Drug Design in the Coward Research Group: From Felicia Wu to the Present

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This lecture, in memory of Felicia Ying-Hsieuh Wu, will include representative examples of mechanism-based drug discovery efforts that have been pursued by us since the early 1970s when Felicia was a postdoctoral scientist in our research group. Initial research on the mechanism of methyl transfer catalyzed by catechol O-methyltransferase (COMT, EC 2.1.1.6) included Felicia's research on the development of a continuous spectral assay (1) and steady-state kinetics analysis of this reaction (2). Subsequent determination of the stereochemical course of both COMT-catalyzed methyl transfer and spermidine synthase (putrescine aminopropyltransferase, PAPT, EC 2.5.1.16), established the fact that these reactions proceed with inversion of configuration at carbon via a classic S_N2 transition state. Based on these mechanistic data, "bisubstrate analogs" were synthesized and determined to be potent and specific inhibitors of several alkyltransferases. Crystal structures of the inhibitors bound to the target enzymes have led to further insight on the nature of the active site and identification of specific amino acids involved in catalysis.

Similar mechanism-based approaches have led to the synthesis of phosphorus-based pseudopeptides as potent and specific inhibitors of two ATP-dependent ligases, folylpolyglutamate synthetase (FPGS, EC 6.3.2.17) and glutathionylspermidine synthetase (GspS, EC 6.3.1.8). In collaboration with Drs. Chun-Hung Lin and Andrew Wang at the Academia Sinica, Taipei, we have used a phosphinic acid inhibitor of GspS and protein crystallography to further elucidate details on enzyme catalysis by this unusual bifunctional synthetase/amidase protein. Ongoing research includes mechanistic enzymology and inhibitor synthesis for a cysteine peptidase, γ-glutamyl hydrolase (EC 3.4.19.9), an ATP-dependent ligase, dihydrofolate synthetase (EC 6.3.2.12), and glycosyltransferases that use N-acetylglucosamine-based donors or acceptors e.g., *N*,*N*'-diacetylchitobiosylepyrophosphoryldolchol synthase, EC 2.4.1.141).

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PL01

Molecular Dynamics Studied by Single Molecule Detection

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Biomolecules assemble to form molecular machines such as molecular motors, cell signal processors, DNA transcription processors and protein synthesizers to fulfill their functions. The reactions and behaviors of molecular machines respond to their surroundings with great flexibility. This flexibility is essential for biological organisms and biological systems. The underlying mechanism of molecular machines is not as simple as that expected from an analogy with man-made machines. Since molecular machines are only nanometers in size and have a flexible structure, they are very prone to thermal agitation. Furthermore, the input energy level is not much different from that of average thermal energy, k_BT. Molecular machines can use this thermal noise with a high efficiency of energy conversion for their functions. This is in sharp contrast to man-made machines that operate at energies much higher than thermal noise. In recent years, single molecule detection (SMD) and nano-technologies have rapidly been expanding to include a wide range of life science applications. The dynamic properties of biomolecules and the unique operations of molecular machines, which were previously hidden in averaged ensemble measurements, are now being unveiled. The aim of our research is to approach the engineering principle of adaptive biological systems by uncovering the unique operation of biological molecular machines. Here, I review our SMD experiments designed to investigate molecular motors, enzyme reactions, protein dynamics and cell signaling, and discuss how thermal fluctuations (noise) play a positive role in the unique operation of biological molecular machines allowing for flexible and adaptive biological systems.

Reference: http://www.phys1.med.osaka-u.ac.jp/

PL02

Astroglial Inwardly Rectifying Kir4.1 channel in brain and sensory organ

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An inwardly rectifying K^+ channel Kir4.1 is specifically expressed in glial cells and sensory epithelial cells, and plays key roles in physiological functions of these cells. In glial cells such as brain astrocytes and retinal Müller cells, Kir4.1 constitutes two types of K^+ channels, i.e., Kir4.1-homomer and Kir4.1/5.1-heteromer(1-4). They are differentially distributed in specific membrane macro-domains such as perisynaptic processes and end feet, and involved in "K^+-buffering" that is the uni-directional K^+-transport from perisynaptic region to vascular site via glial cells. The Kir4.1-containing channels occur together with water channel AQP4 on glial membrane, suggesting that these channels are functionally coupled and mediate concomitant transport of K^+ and water. Our pharmacological assays demonstrated that particular antidepressants blocked Kir4.1-current, which would be implicated in their therapeutic and/or adverse actions(5,6). We also found that, on astroglial membrane, Kir4.1 and AQP4 existed at the micro-compartments called "detergent-resistant membrane micro-domains (DRMs)(7)". Depletion of membrane cholesterol by MβCD resulted in loss of Kir4.1-association with DRM and its channel activity but affected neither the distribution nor function of AQP4. Immunolabeling showed that Kir4.1- and AQP4-DRMs were not co-localized but occasionally in close proximity, which may be the machinery involved in K^+-driven water-transport. Astroglial membrane would therefore harbor at least two distinct micro-compartments, MβCD-sensitive and MβCD-resistant DRMs, which respectively control localization and function of Kir4.1 and AQP4. Finally, in cochlea of inner ear, Kir4.1-homomer is abundantly expressed in epithelial tissue termed "stria vascularis". Our *in vivo* electrophysiological experiments revealed that Kir4.1 promoted K^+-transport via stria vascularis and played the central role in formation of highly positive potential of ~+80 mV in endolymph, a unique extracellular fluid filling cochlea(8,9). Because this potential is known to dramatically amplify the sensitivity of cochlea to sounds, Kir4.1 is indispensable for audition. These observations further imply that dysfunction of Kir4.1 could associate with neuronal and sensory disorders and this channel could be a potential target of new drugs for these diseases.

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PL03

Substrate specificity of phosphodiesterases, structural insight into Viagra function, and structure-based discovery of drugs for treatment of inflammatory diseases and erectile dysfunction

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Cyclic nucleotide phosphodiesterases (PDEs) are key enzymes controlling cellular concentrations of the second messengers cAMP and cGMP (Bender and Beavo, 2006; Ke and Wang, 2007). Human genome encodes 21 PDE genes that are categorized into 11 families and express about 100 isoforms of PDE proteins. PDE molecules contain a conserved catalytic domain, but possess different substrate specificity and inhibitor selectivity. Family selective PDE inhibitors have been widely studied as therapeutics for treatment of various human diseases. The best known example of this drug class is the PDE5 inhibitor sildenafil (Viagra) that has been used for treatment of male erectile dysfunction (Rotella et al., 2002) and pulmonary hypertension (Galie et al., 2005).

This talk will focus on the structural implications on the substrate specificity and inhibitor selectivity of PDE families and structure-based discovery of PDE4 and PDE5 selective inhibitors for treatment of inflammatory diseases and erectile dysfunction. The structures of PDE10 and PDE4 suggest that the substrates cAMP and cGMP are recognized by different orientations and interactions (Wang et al., 2007) and therefore provide direct evidence against the widely circulated mechanism of “glutamine switch” (Zhang et al., 2004). PDE5 shows multiple conformations of the H-loop upon binding of the inhibitors. The different conformations of vardenafil (Levitra) and sildenafil (Viagra) form the structural basis for their distinct physiological behaviors. The structure-based design has led to a discovery of a novel type of PDE4 inhibitors. The best PDE4 inhibitor suppresses the TNFα release with at least 20-fold more potency than cilomilast that is a GSK drug-lead in phase III clinic trial for treatment of chronic obstructive pulmonary disease. Our structure-based design also led to finding of new PDE5 inhibitors, the best of which is 4-fold more potent than sildenafil in enzymatic activity.

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PL04

Proteomic Approach to Discover Biomarkers and Therapeutic Targets

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The proteomic analysis of plasma and tissues in patients has been a major approach to determining biomarkers essential for early disease diagnoses and drug discoveries. Recently, we identified 40 ovarian cancer-associated proteins by 2-D difference gel electrophoresis and MS/MS (1), and iTRAQ and MS/MS. Among them, many proteins including annexin IV were regulated at transcriptional level in the cancer cells. The annexin IV gene has a transcription regulatory region containing a similar sequence to the NF-kB binding motif. When the expression of the genes encoding these proteins was suppressed with the siRNAs, the proliferation of the cancer cells was reduced at different levels depending on the protein. On the other hand, we identified proteins such as stat3 of which phosphorylation is stimulated in the ovarian cancer cells, by immunoaffinity chromatography, and subsequently by iTRAQ and MS/MS, suggesting that some of the cancer-associated proteins are regulated at post-translational level. Finally, we investigated if we can detect the ovarian cancer-associated proteins in the plasma. The dynamic concentration range of the plasma proteins is extremely wide. To analyze low abundant proteins efficiently, it is necessary to deplete the abundant proteins prior to MS analysis. Recently, we developed a novel “abundant protein depletion device (hollow fiber membrane)” that is linked to 3D-LC-MS/MS system (2). By this system, we can identify nearly 3,000 low abundant proteins in the plasma. However, among the ovarian cancer-associated proteins identified in the cancer tissues and cultured cells, we detected only annexin IV in the plasma of the patients by our system, suggesting that we need to develop a novel detection system for the biomarker candidates in the plasma.

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PL05

Predictions, Functions, Protein Interaction Networks, Drug Discovery, and Alternative Splicing

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Many proteins lack specific 3-D structure and yet carry out function. Starting in 1996, we began to explore the prediction of structured and disordered regions from amino acid sequence. Disorder predictions by us and others suggest that a large fraction of eukaryotic proteins contain significant-sized regions of disorder. Recently we developed a bioinformatics approach to assign the functions in Swiss-Prot to structure (order) or to disorder. Of 710 common functions, 310 correlate with structure and 238 with disorder, but the repertoire for disorder is broader; structured functions are mostly enzymatic. Protein interaction networks involve one protein binding to multiple partners. Disorder is commonly used for this purpose. In four recently reported examples, a promising drug molecule binds to a structured protein and thereby blocks its interaction with a disordered partner. Study of these examples has led to a new approach to drug discovery. Alternative splicing is very common in multicellular eukaryotes but perhaps nonexistent in single-cell eukaryotes. The RNA removed by alternative splicing is found to code for intrinsic disorder significantly more often than for structure. Given that signaling segments in regions of disorder are formed from small numbers of contiguous amino acids, and given that many disordered regions have been shown to contain many signaling and regulatory segments in tandem, alternative splicing within regions of disorder provides a mechanism for bringing about regulatory and signaling diversity. We propose that alternative splicing plus intrinsic disorder provided a means to “try out” alternative regulatory pathways, thus facilitating the evolution of multicellular organisms.

PL06

Specific interactions for *ab initio* folding of proteins

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Proteins fold into specific three-dimensional structures by specific, orientation-dependent interactions between amino-acid residues. The most well-known specific interaction is hydrogen-bonding. Little attention, however, has been paid to the orientation dependence of interactions between polar atoms that are not hydrogen bonded, despite evidence of their role in the formation of alpha helices and beta sheets. Moreover, the possible orientation dependence of interactions between polar and nonpolar atoms is ignored even though the hydrophobic effect is caused by the re-orientation of water molecules near a hydrophobic surface. Here we extract orientation-dependent interactions from protein structures by treating each polar atom as a dipole with a direction. This approach allows a consistent, integrated, and parameter-free treatment of both the orientation and distance dependence of interactions between all polar atoms and between polar and nonpolar atoms.

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PL07

Biological Mass Spectrometry for the Application of Disease Proteomics

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Proteomics deals with the large-scale determination of cellular function directly at the protein level. By the nature of the complexity of cellular proteomes, which contain thousands of proteins over a wide dynamic range of abundance, it is recommended to have a multidimensional type of analytical tools. Biological mass spectrometric analysis of human proteome such as plasma and brain is one of the important rapidly emerging techniques for biomarker discovery in diagnosis and therapeutic monitoring of many human diseases, where a high throughput characterization of proteins and peptides is necessary. For examp[le, because the brain is one of the most complex tissues of higher organisms, differing from other organs due to its many differentcell types, its structure at the cellular and tissue level, and the restricted regeneration capacity. Elucidating the protein complement of the brain is therefore at the upper limit of significant challenges to today's current technologies in proteome analysis. At the same time, the brain is of paramount interest in medical research and in pharmaceutical industry because of the widespread social impact of the more common neurological diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, prion diseases and stroke.

In human proteome analysis, tandem mass spectrometry is used to identify proteins using database search tools. Multidimensional protein separation technology and mass spectrometry work well together, and it is recommended that multidimensional separation precedes mass spectrometry to enhance the quality of the mass spectra data. Such separation has extended the dynamic range of human proteome analysis. The complexity and large dynamic range of protein concentrations found in human serum poses a serious analytical challenge in mapping the proteome. The implementation of multidimensional LC separations, on- and off-line coupled with electrospray and matrix-assisted laser desorption ionization mass spectrometry as a high throughput analytical strategy with high dynamic range, and high confidence identification of human proteins from plasma and brain is presented. As a resut from the human plasma proteome analysis, we could classify the proteins by three groups according to the molecular weight correlation value. For the human sample, these groups are characterized by different protein conditions. The protein filtering method could suggest a fast way to reach more confident protein list including the information on their disease characteristics such as cancer and diabetes for human sample.

In our attempt to analyze the human brain proteome, we applied multi-dimensional protein separation and identification techniques using a combination of sample fractionation, 1-D SDS-PAGE, and MS analysis. The complexity of human brain proteome requires multiple fractionation strategies to extend the range and total number of proteins identified. Proteins of the temporal lobe of human brain were fractionated into cytoplasmic and nucleoplasmic, membrane and other structural, and DNA-binding proteins. Each fraction was then separated by SDS-PAGE, and the resulting gel line was cut into approximately 50 bands. After trypsin digestion, the resulting peptides from each band were analyzed by RP-LC/ESI-MS/MS using an LTQ spectrometer. Ultimately, 1533 proteins could be detected from the human brain. We classified the identified proteins according to their distribution on cellular components. Our results show that the multiple separation strategy is effective for high-throughput characterization of proteins from complex proteomic We applied the band distribution analysis to the human brain proteome. Since we could separate the fraction of membrane and structural proteins and the fraction of soluble cytoplasmic and nucleoplasmic proteins from human brain tissue, we expected these fractions to have different characteristics that distinguished it from each other fraction. This approach was used to compare the fractions obtained using cell fractionation.