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ASPET MEETING REPORT

Colloquium on Signaling and Molecular Structure in Pharmacology (La Jolla, California, March 11–12, 1997)

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A colloquium sponsored by the American Society for Pharmacology and Experimental Therapeutics (Molecular Pharmacology Division), The Burroughs-Wellcome Fund and the San Diego Supercomputer Center was held as a satellite meeting to Pharmacology '97 in San Diego on March 11 and 12, 1997. The Supercomputer Center in La Jolla provided the setting; 120 participants met in a large-screen auditorium to view structures in three dimensions, on-line projections and manipulations of molecular structures, and simulations of breathing motions and conformational changes in proteins.

The recent availability of structural information on interacting ligands and the targets of drug action (i.e., the working molecules of pharmacology) has added a new dimension to the pharmacological sciences. This extends not only to the signal transduction pathways mediated through receptors but also to target sites of such antimetabolites as the dihydrofolate reductases, to enzymes that catalyze the biosynthesis of such active molecules as the cyclooxygenases for prostaglandins, and to the enzyme families that control the disposition of drugs, such as cytochrome P450 and monoamine oxidase. Moreover, technological developments in X-ray crystallography and nuclear magnetic resonance spectrometry enable a far more rapid accumulation of data than was previously thought possible. Such information from the structural templates is augmented by computational techniques that can predict modes of molecular motion, conformational changes, ligand docking sites, and solvent influences on structure. Other techniques, such as fluorescence spectroscopy and electron spin resonance, can be used to monitor molecular motion, but only in limited time frames.

The goal of the meeting organizers was to capture this information and potential developments in a venue in which structure and function are intimately linked. To maintain a theme within the meeting, targets of signaling molecules and computational techniques were emphasized. Hence, investigators in various biological and physical sciences gave presentations that used the unique visualization facilities of the San Diego Supercomputer Center. In addition, several young investigators in the pharmacological sciences were invited to speak and present posters. It is likely that this group of young investigators and the twenty students at the Colloquium will ultimately be in the best position to realize the expanding potential of this new technology.

In the various talks, many speakers presented graphics in stereo from SGI workstations as the audience viewed the graphics in three dimensions on the large auditorium screen with Crystal Eyes glasses. In this format, the presenter has control over rotating the molecules to the optimal positions and zooming in to particular portions of the structure. Some people who were particularly accomplished with the workstations, such as Dr. Stephen Sprang, made the entire presentation from a workstation; others required an operator to assist with their expositions.

Although simultaneous viewing in three dimensions by the audience is an obvious advantage of this facility, other features of on-line visualization became apparent to the attendees. First, the capacity to view the entire molecule or complex and then to focus on a particular interface or element of the structure affords the audience a better perspective on structure. Second, the presenter can adjust the viewing frame and angles to the audience size and call up the structural detail in response to a question. Third, rapid switching between two or more conformations allows one to assess the critical structural differences. Those with experience could download coordinates and project structures in less time than is required to produce slides.

The Plenary lecture for the Colloquium, which also served as the Torald Sollmann Award lecture, was given by Dr. Alfred G. Gilman, of the University of Texas Southwestern Medical Center. This presentation provided an overview of G proteins and their linkage to seven membrane-spanning receptors and effectors. Dr. Gilman discussed the diversity of the pathways for G protein action and the distinct mechanisms for downstream signaling through their α and/or β,γ

ABBREVIATIONS: GAP, GTPase-activating protein; PLD, phospholipase D; RGS, regulator of G protein synthesis.

subunits. Particular emphasis was placed on a new family of proteins, the RGS family, the members of which act as negative regulators of G protein action. The RGS proteins effect inhibition by binding to activated protein α subunits and stimulating hydrolysis of their bound GTP. Stabilization of the transition state for GTP hydrolysis is suggested by the high affinity of an RGS-AlF_4^- complex of $G_{i\alpha}$. Other areas of emphasis included studies of adenylyl cyclase that delineate varied mechanisms for regulation of different isozymes and structural studies that define the two cytoplasmic domains of the enzyme as cooperative pieces in forming the active site and sites for regulation by forskolin and P-site inhibitors.

The lead presentation was given by Dr. Gebhard Schertler, from the MRC Laboratory of Molecular Biology in Cambridge, England, who discussed the structure of visual rhodopsin, a prototypical G protein-coupled receptor. He has used cryo-electron microscopy to determine the structure of the membrane-spanning rhodopsin core to a resolution of 7 Å in the X-Y plane. Densities of each of the seven helices are evident, suggesting both the locations of the second and third cytoplasmic loops that contact the G protein and the placement of helices around the retinal chromophore. Packing of the helical bundle in visual rhodopsin differs substantially from that of bacteriorhodopsin, another seven-span retinal-binding protein, confirming that details of the structures of G protein-coupled receptors cannot be modeled according to that of bacteriorhodopsin. Dr. Schertler also considered the potential and limitations of structural determinations by electron microscopy of two-dimensional crystals, the increasing uncertainty of the structure as one moves normal from the center of the bilayer, the potential for enhanced resolution from electron sources of higher voltage, and the possibilities for extending these studies to three-dimensional crystals.

Dr. Stephen Sprang, of the University of Texas-Southwestern, presenting directly from the computer terminal, led the participants through a comprehensive analysis of the conformations of the proteins and subunit interfaces involved in G protein activation. His crystallographic studies of a Gly203Ala mutation of $G_{i\alpha}$ provided a view of the transient GDP + P_i intermediate in GTP hydrolysis. The structure of the GTPase activating protein, RGS4, in a complex with the G_i protein shows that the RGS stabilizes the transition state for nucleotide hydrolysis, thus revealing the structural basis for the inhibitory activity of this family of proteins.

Dr. Heidi Hamm, of Northwestern University, analyzed the interactions between transducin, rhodopsin, and its effector, the cyclic GMP-dependent phosphodiesterase. Extensive studies of the interactions of the phosphodiesterase γ subunit with chimeras of $G_{t\alpha}$ and $G_{i\alpha}$ indicate at least two binding regions between the protein and define their differential interactions in the presence or absence of activator ligands for the G protein. These mechanistic insights were related to known structural motifs of transducin. Studies with peptides selected from a recombinant DNA-derived library were also used to define key residues in the carboxyl terminus of the G_t subunit for interaction with rhodopsin and to predict potential structural surfaces that may function in the coupling process between these proteins.

Dr. Henry Bourne, of the University of California-San Francisco, analyzed the structural and functional deficits found in several mutations in the α subunit of the stimulatory G protein (G_s) that give rise to pseudohypoparathyroidism Ia, a dominantly inherited endocrine disorder characterized by resistance to hormones that stimulate adenylyl cyclase. One of the mutations of $G_s\alpha$, Arg231His, affects the $\beta\gamma$ contact region and impairs receptor activation of $G_{s\alpha}$. This gives rise to large reductions in hormone stimulated cAMP formation. A second mutation in α_s, Arg201Cys, inhibits the GTPase activity that normally turns off G protein activation. The double mutant is compensatory exhibiting nearly normal activity. A third mutation, Ala366Ser, gives rise to both testitoxicosis and pseudohypoparathyroidism and results from thermal instability of G_s. Analysis of the positions of the mutations in relation to structure of the complex reveals unique roles of the mutations in preventing receptor evoked conformational changes of the G protein. The Arg 231 mutation is in the "switch 2" domain, where it interfaces with β,δ. The γ-phosphate of GTP changes the orientation of the "switch 2" helix to interact with a glutamate in the "switch 3" region.

Dr. Elliott Ross, also from the University of Texas Southwestern Medical Center, dealt with the protein-protein binding events that maintain rapid G protein signaling in cases in which deactivation is accelerated by GAPs. Several effectors, including phospholipase Cβ, are also GAPs for their own G protein activators. Maintenance of signal amplitude requires that a complex of receptor, G protein and effector remain stably associated throughout the GTPase cycle to allow rapid rebinding of GTP after GAP-stimulated deactivation. A corollary is that an effector's GAP activity enhances the G protein's selectivity for tight-binding receptors.

Dr. Richard Neubig, of the University of Michigan, described fluorescence spectroscopic studies of the activation and deactivation of heterotrimeric G proteins. A family of ribose-modified fluorescent nucleotide probes containing a methylanthaniloyl group are excited by energy transfer from tryptophans in the α subunit of G_o or G_i. The nonhydrolyzable methylanthaniloyl guanosine diphosphate, (β,γ methylene) phosphate was shown to be a partial activator of G_o and G_i whose fluorescence correlated with the degree of activation of the α subunit. Kinetic studies provided evidence for an unexpected equilibrium between two triphosphate-bound states (active and inactive). Such an equilibrium between G protein states represents a novel locus of regulation in G protein function. Deactivation of the $G_{i\alpha}$ subunit by RGS4, a regulator of G protein signaling, was studied by stopped-flow intrinsic fluorescence methods with GTPase rates up to 1 sec^{-1}. The fluorescence methods provide novel dynamic information about conformation of regulatory proteins not available from current structural approaches.

Dr. Palmer Taylor, of the University of California-San Diego, described the interaction of the peptide fasciculin with acetylcholinesterase as deduced from the crystal structure of the complex and site-specific mutagenesis. Fasciculin is a member of a large family of three-fingered toxins, including the α-toxins (which block nicotinic receptors), the muscarinic toxins (which are muscarinic agonists and antagonists), and calciseptine (which blocks Ca^{2+} channels). The tip of the largest loop (loop 2) in fasciculin seals the active center gorge at its mouth, which apparently precludes substrate access; the stability of the complex is buttressed by the two flanking loops. Despite the tight seal shown crystallographically, ligands can still enter the active center gorge, but they do so

far more slowly. An analysis of charge in relation to the kinetics reveals the likely entry route to be around the bound fasciculin rather than through a "back door" or other portal of entry in the acetylcholinesterase. The fasciculin-acetylcholinesterase complex serves as a model for understanding the mode of interaction by the three-fingered toxins with their respective target sites.

Dr. Paul Sternweis, of the University of Texas-Southwestern, discussed the regulation of mammalian PLD by phosphatidylinositol 4,5-bisphosphate, two classes of monomeric G proteins (ARF and RHO), and protein kinase C. Points of emphasis were the synergism observed among the various activators and the presence of kinase suggests a complex regulation of the phospholipase by convergence of multiple signaling pathways. The stimulation of PLD by the regulatory domain of protein kinase C_α (as opposed to the catalytic domain) identifies a novel, divergent mechanism for regulation by this enzyme. Several lines of evidence were also presented that suggest a role for PLD (via its regulation by ARF proteins) in intracellular vesicle traffic.

Dr. Wendell Lim, of the University of California-San Francisco, discussed small modular protein domains that recognize specific sequence elements in target proteins with particular reference to proline-rich sequences. A family of over 50 proteins contain a Src homology (SH3) domain that recognizes the consensus sequence PXXP. Remarkably, the affinity of the domain for the isolated peptide and surrounding region is low (K_D = 10–100 μM) compared with the intact protein. The proline-rich peptide adopts a polyproline helix that associates with aromatic regions on the binding surface of the SH3 domain. The family of SH3 domain proteins has members that bind in both orientations with respect to the helix turn. Variable loops on the SH3 domain restrict access to the pseudo symmetric polyproline helix and dictate the specificity of the interaction. Elements noncontiguous to the proline-rich region also participate in governing selectivity and affinity. Dr. Lim also dissected the elements that gave rise to the specificity in the proline-rich sequences through breaking of the pyrrolidine ring, which yielded modified peptides with methyl groups on the α-carbon, the amide nitrogen, or at both positions on the altered proline.

Dr. Greg Petsko, of Brandeis University, provided new insights into how structure and combinatorial chemistry could be used advantageously to guide drug design. He alluded to the importance of bound H_2O in crystallographic structures, pointing out that bound ligands must compete with 55 M H_2O. Often the positions of hydrophobic solvent molecules after crystallization in a binary solvent reveal potential sites of interaction for hydrophobic ligands. He and his colleagues have prepared combinatorial libraries, typically through opening of heterocycles to produce a matrix core resembling a rigid peptide. Screening for pharmacologic activity shows that compounds displaying similar pharmacologic characteristics will cluster in the two dimensional matrix of congeneric ligands. Hence, a selection process spontaneously emerges and the structural features of the cluster become the lead for further refinement in the selection of the most active compounds.

Dr. J. Andrew McCammon, of the University of California-San Diego, illustrated the current capabilities of computer simulations of protein dynamics and function by means of several examples. The diffusional encounter of enzyme and substrate molecules can be simulated by Brownian dynamics. For enzymes that operate at or near the diffusion-controlled limit, the analysis of such encounter trajectories provides information on the nature of the binding process, as well as predictions of the rate constant. The rate constants of some enzymes are increased by as much as 2 orders of magnitude by electrostatic interactions that steer the diffusing substrate toward productive collisions with the active site. This is true for certain acetylcholinesterase-ligand systems, for which the computed and experimental rate constants are in reasonable agreement for a range of variations in ionic strength, side chain charges, etc. Similar calculations have been used to study the transfer of intermediates from one active site to another in enzyme complexes. The Brownian dynamics simulations typically use simple, rigid models for the enzymes and continuum models for the solvent. Molecular dynamics simulations provide more detailed representations of the atomic motion in proteins and solvent but can require much more computer power. Very large-scale molecular dynamics simulations of acetylcholinesterase on a parallel computer at the San Diego Supercomputer Center suggest that breathing motions open a "front door" to the active site frequently enough to allow for diffusion-controlled reactions and that a variety of other "doors" to the active site may open to allow solvent to enter and leave the active site fairly readily.

Dr. Rebecca Wade, of the European Molecular Biology Laboratory in Heidelberg, described how computer simulations are being extended to study protein-protein and protein-membrane interactions. Her group has developed methods for calculating effective electrostatic charges in macromolecules that allow more accurate modeling of such encounters. These have been used in Brownian dynamics simulations of the diffusion-controlled binding of the proteins barnase and barstar. By recognizing that the initial committed step for complex formation involves the alignment of a subset of the intermolecular contacts observed in the crystal structure of the complex, it was possible to define encounter criteria that lead to good agreement of the computed and experimental rate constants for a variety of conditions. The continuum solvation models can also be used approximately to describe systems that include lipid bilayers as low-dielectric regions adjacent to the high-dielectric aqueous solvent. Such models have been used to suggest a mechanism by which the "lids" of lipase molecules may be induced to open, which allows access to the active site, as these enzymes encounter a bilayer.

Dr. Joe Noel and his colleagues from the Salk Institute crystallized each of the cytoplasmic catalytic domains (D1 and D2) of receptor-like protein tyrosine phosphatase alpha. In D1, the turn linking the first and second α helices inserts into the active site of a symmetrically related monomer, apparently blocking the conformational change needed to bind the phosphotyrosine residue of substrates localized on the inner leaflet of the plasma membrane. Three of the crystal forms with widely different solvent contents of the distal catalytic domain (D2) have been solved to 1.9+, 2.0+, and 3.0+ resolution using the D2 model. Although early in refinement, the amino terminal wedge observed in D1 is absent in D2. The latter crystal form organizes as a monomer. The most striking feature of the first two crystal forms of D2 is that the individual monomers dimerize, but in a manner very distinct from D1. The amino-terminal residues of each do-

main do not form a helix-turn-helix motif, but rather an extended β strand. Each strand forms a continuous four-stranded β sheet with the other monomer. Two strands from each monomer contribute to the β sheet. Most interestingly, the amino-terminal strand of each monomer carries out a domain exchange conformational switch, where the strand of one monomer is more closely associated with the opposite dimer. The structural differences between monomeric and dimeric D2 viewed in the context of the protein where D2 is linked to D1 would result in a dramatic change in the spatial organization of the D2 active sites with respect to the plasma membrane and membrane associated targets.

Dr. Susan Taylor, of the University of Califnornia-San Diego, discussed the cAMP-dependent protein kinase, a prototype for the large family of protein kinases. Structures of the catalytic subunit complexed with substrates and inhibitors define overall architecture and the configuration of the conserved residues at the active site cleft that contribute to nucleotide binding and phosphoryl transfer. In addition, these structures define open and closed conformations. By engineering unique cysteine residues into the catalytic subunit, Dr. Taylor and her colleagues developed a set of enzymes stoichiometrically labeled at a single cysteine. One such mutant, Asn326Cys, when labeled with acrylodan, serves as a sensor for nucleotide binding. Using stopped flow fluorescence, the rapid phosphoryl transfer step (500/sec) was readily distinguished from the slower, rate-limiting step (20/sec). This slow step correlates not only with product release but also with opening of the active site cleft, a prerequisite for release of ADP. The most mobile segment at the active site is the glycine rich loop between β strands 1 and 2. This loop lies over the phosphates of ATP, and in its fully closed and most stable conformation, interacts with the β and γ phosphates of ATP. The backbone amide of Ser53 is hydrogen bonded to the γ-phosphate of ATP; this seems to correlate with the transition state. Balanol, a natural product inhibitor, complexed with the catalytic subunit shows remarkable complementarity with the conserved residues in the small lobe; the extensive hydrogen bonding network accounts in part for the high affinity (K_i = 10 nM). The crystal structure of the regulatory subunit provides insights into the detailed features of the cAMP binding sites and the cooperative mechanism of cAMP binding leading to activation of the holoenzyme. Through site-specific mutagenesis and deletion analysis, structural requirements for cAMP binding were dissected from those necessary for high affinity binding to the catalytic subunit. These subsites define two distinct regions of cAMP binding site A: the docking site for cAMP and the docking surface for the catalytic subunit.

A subsequent presentation by Dr. David Johnson, of the University of California-Riverside, revealed the potential of fluorescence lifetimes and fluorescence anisotropy decay to investigate subunit dissociation of protein kinase A and molecular motion of particular domains within the enzyme. Dr. Johnson has employed site-specific mutagenesis to introduce sulfhydryl groups at particular locations on the kinase. These, in turn, are labeled with fluorophores. By measuring the decay of anisotropy, Dr. Johnson has been able to follow subunit dissociation as well as distinguish differences in segmental motion of protein kinase A. The collaborative effort between these two groups illustrates the value of having a crystallographic template and then coupling this information with techniques that measure segmental and global motion of the complex. The time frame for detection of molecular motion is in the nanosecond range, whereas computational molecular dynamics examines motion within short periods (~100 psec).

Dr. James Hurley, from the National Institutes of Health, described the then unpublished tertiary structure of half of the catalytic portion of adenylyl cyclase. Although the cyclase is a large, integral membrane protein, its catalytic region is composed of two homologous cytosolic domains that can be expressed as an active, soluble heterodimer. Hurley and colleagues forced homodimerization of one of these domains with the aid of forskolin, a cyclase-activating diterpene, which, as he showed, binds at the dimer interface. Although the homodimer has scant catalytic activity, its structure indicates the overall folding of each protomer, suggests the orientation of the protomers in the active heterodimer, and provides detailed information on the forskolin-binding site. The structure also predicts that the active site is formed by the dimer interface, in agreement with the results of previous mutageneis studies.

The final afternoon was devoted to advances in computational techniques and two workshops. Dr. Philip Bourne, of the San Diego Supercomputer Center, discussed protein database analysis. Although previous talks had focused on the enormous benefit of having individual protein structures for understanding biological function, Dr. Bourne's presentation highlighted what was possible by examining biological structures "en masse," a subject of particular importance given the exponential growth rate in number of structures. He described methodologies developed for addressing questions such as: (a) Is this fold unique? (b) What influences secondary structures more, residue propensities or patterns of polarity? (c) If it is polarity, what is the influence of exposure to solvent? (d) What happens to the propensities of residues for particular types of secondary structure if you change the favored regions of the Ramachandran plot?

Dr. Lynn Ten Eyck, of the San Diego Supercomputer Center, described a new approach to seeking free energy minima by direct computation of the statistical mechanical partition function. Through the use of fast Fourier transforms, possible docking interactions can be computed efficiently. The calculations account for electrostatic and van der Waals' interactions and estimate desolvation contributions to the free energy. Solvent is treated as a continuum and excluded space is determined from molecular volumes. The method does not allow for consideration of flexible molecules, but maps the interactions in sufficient detail that recognition complexes should be detectable in those cases where there is conformational change upon binding. Example calculations showed how the effects of electrostatic steering could be demonstrated in a plastocyanin-cytochrome c complex.

Dr. Jim Briggs, of the University of California-San Diego gave a brief survey of the use of electrostatic methods to compute binding energies. The methods discussed allow the inclusion of the effects of solvent and ionic strength, without the need for a representation of explicit solvent molecules. The talk included a description of the most relevant parameters involved in a finite-difference solution to the Poisson-Boltzmann equation, namely, internal and external dielectric constants, atomic charges and radii, surface representation, grid resolution, and others. These methods have been used to

estimate binding energies of ligand-macromolecule and protein-protein complexes. In addition, experimental free energies of solvation for small molecules may be reproduced. Although the binding energy computations are a bit too slow to be used in a high-throughput virtual screening effort, current efforts are directed at increasing the speed of these calculations by a factor of 10–1000.

Database and Ligand Docking Workshops:

Most of the workshop participants indicated that they had minimal or no experience with on-line databases as tools in their research. Surprisingly, a significant number of participants had not used a resource like Entrez for bibliographic/sequence/structure searching. An overview was given of several databases and then specific questions were taken from the floor. Where possible, these questions were then addressed through the databases that had been introduced. The goal was to indicate to participants what is and is not currently possible using on-line methods. Pointers to the databases that were examined at the colloquium can be found on the World Wide Web (*http://www.sdsc.edu/pb/edu/structdb.htm*). In the other workshop, emphasis was placed on discussing details of G protein, cyclase, kinase, and phosphatase structures. Workshop participants also considered the merits, ease of use, and accessibility of various docking and energy minimization programs.

To our knowledge, this may be the first occasion where a scientific society, a funding agency, and national supercomputer resource have developed a large-scale scientific program that combines structural, functional, and computational perspectives in a setting that enables the participants to view intimate detail of structures and assess the potential of computational techniques. In this sense, the colloquium was an experiment, but one that bears replication. Such a colloquium could be arranged where participants interact with a central meeting site from remote terminals and visualization facilities, but this carries the drawback of not visiting La Jolla in early March.

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