Protein Crystallography in Drug Discovery

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Abstract: Protein crystallography is the main technique used to obtain three-dimensional information for binary complexes involving protein and drugs. Once a protein target has its three-dimensional structure elucidated, the next natural step is the solving of the structure complexed either with its natural substrate, or any ligand or even an inhibitor. Such information is of pivotal importance to understand the structural basis for inhibition of an enzyme. The relevant features, for application of protein crystallography to drug discovery, are discussed in this review.

INTRODUCTION

Proteins accomplish their roles in the cell by interacting with other proteins and/or ligands. Their functional properties depend on the proteins three-dimensional structure. Protein structure can be determined experimentally by X-ray crystallography, NMR, spectroscopy, cryo-electron microscopy or alternatively, estimated by computational molecular modeling. X-ray crystallography continues to be standard method for high resolution protein structure determination and accounts for the vast majority of experimentally determined structures [1]. The marked increase in the number of protein structures being deposited in the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/) has arisen due to four reasons. First, development of methods for solving crystallographic structures during the 1960s [2]. Second, the increased availability and developments in high powered computing starting in the 70´s [3]; third, the availability of tunable wavelength synchrotron beamlines along with cryocooling technology for X-ray data measurement starting in 1991 [4] and, more recently, structural genomics approaches that have led to thousands of protein structures being determined following the completion of the human genome project [1].

Protein Crystallography

Then, X-ray crystallography is widely used technique for providing a three-dimensional representation of molecules in a crystal. Scientists have employed X-ray crystallography to determine the crystal structures of many molecules. 85.8% of the structures deposited in the PDB were obtained by this method [5]. However, this technique requires very specific equipment and highly specialized skills [5].

The progress of macromolecular crystallography in the past 70 years since the recording of the first diffraction pattern from a crystal of pepsin [7] has advanced in steps. Therefore, the only true revolution was achieved by Max Perutz when he solved the crystal structure of hemoglobin in the late 1950s. At the time, solution of an acentric small structure with less than 100 atoms was very difficult; horse hemoglobin has 2291 non-hydrogen atoms in the asymmetric unit. It required a very dedicated and patient researcher (Perutz) and a very involved laboratory chief to spend nearly 20 years pursuing such a marginally promising project. Today, in a time of strict planning of research goals and tight control of expenditure, such dedication would be unthinkable. The elucidation of the crystal structures of myoglobin [8] and hemoglobin [9] were certainly a revolution in structural science, rewarded by two Nobel prizes [8,9]. Since then, the progress of protein crystallography has followed an evolutionary rather than a revolutionary path. Many important advances have been achieved on the way, but most of the methods used by Perutz are still valid today and still useful, albeit often in modified versions [10]. Fig. (**1**) illustrates the main steps used to solve a protein structure by mean of X-ray crystallography.

Actually, the structural genomics initiatives are producing a vast number of novel protein structures that can provide important insights into protein structure and function. These data are all available due to requirements for rapid deposit of structures into the PDB [5]. The characterization of biological function from the newly determined protein structures is a challenge for structural genomics projects. Recent analysis indicates that 30 to 50% of deposited structural genomic structures are of unknown function [11] and that approximately 40% of the genes annotated in the sequence databanks lack annotation of biological function [1, 12,13].

The Pfam database is a manually curated database of protein families from sequenced genomes. As of July 2008, 33.5% of Pfam families (3464 of 10340) [5] contain a member with known structure, which allows the folds of all other members of the family to be inferred. They mapped each Pfam family to structural genomics targets and protein of known structure from the PDB, and they used the database deposition dates to identify the earliest structural repre-

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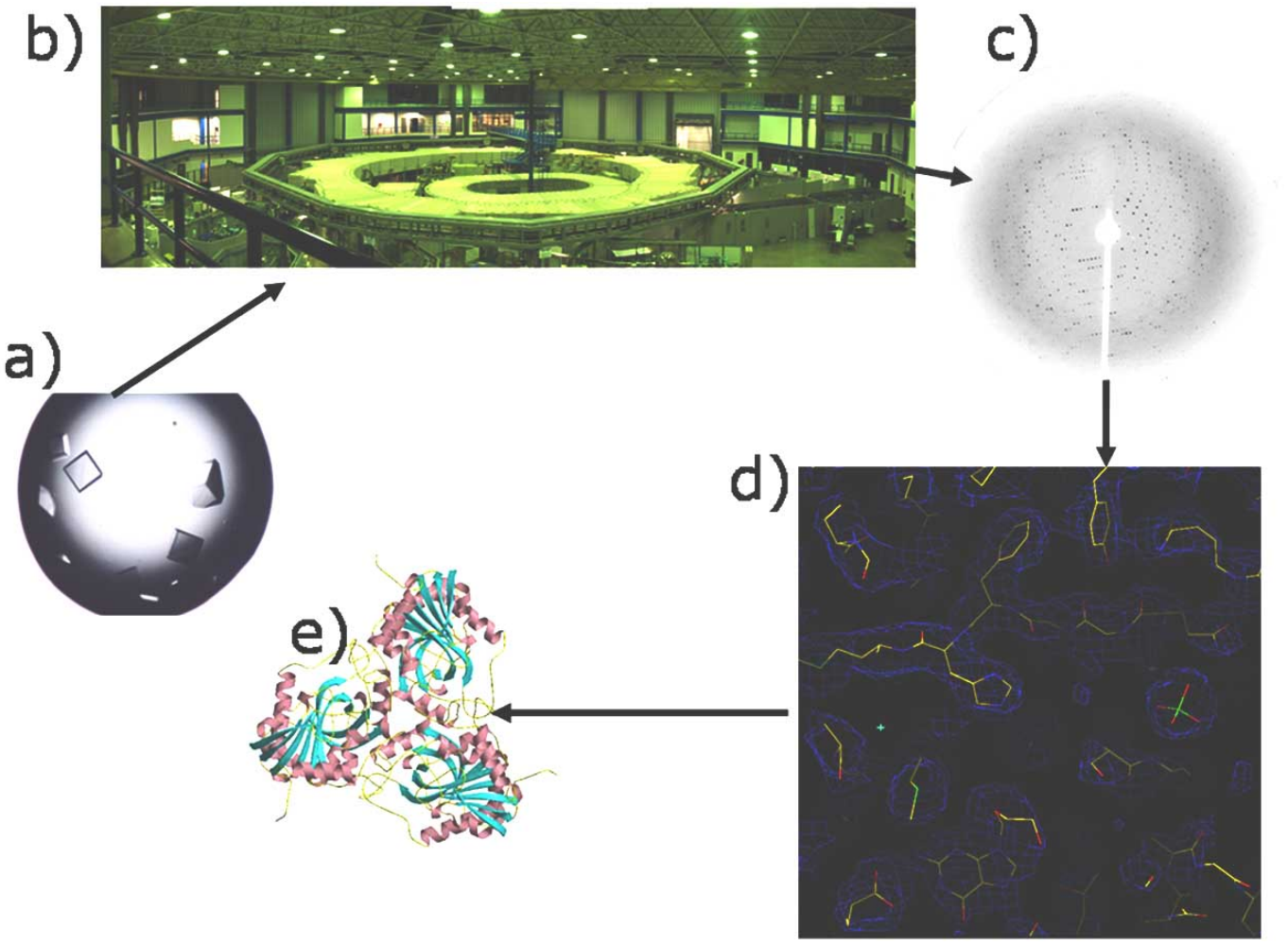

Fig. (1). Overall scheme used to solve a structure using protein crystallography. **a**) crystallization, **b**) X-ray diffraction data collections, **c**) data process, **d**) solving the phase problem and refinement, **e**) structure analysis.

sentative from each family. The rate of first structural characterization of families rose steadily throughout the 1990s but leveled off at around 20 new families per month since 1999, even as the total number of structures solved continues to increase [5,11].

Estimates suggest that there are between 1,000 and 5,000 distinct spatial arrangements of polypeptide chains found in nature [14]. The PDB contains three-dimensional structures of only about 1,200 distinct protein folds [5]. In eukaryotes, most genes encode proteins with multiple globular domains (the average domain size is 153 (±87) residues [15]), giving many larger proteins the appearance of beads on a string. Typically, a single 'bead' is responsible for carrying out a specialized biochemical task, such as phosphorylation of protein substrates by the kinase domain of the Src oncoprotein, [16].

Then, the rate of solution of first structures in a Pfam family by non-structural genomics structural biologists has decreased while Structural Genomics centers worldwide account for about half of new structurally characterized families, even though they contribute only about 20% of the new structures. Protein Structures Initiative centers account for about two-thirds of the worldwide Structural Genomics contribution. Only 5% of non-structural genomics reported since 2000 represent a new Pfam family, whereas the Protein Structures Initiative average was 20.4% [11].

Advances

The widespread use of robotics in protein crystallography has had a huge impact at every stage of the pipeline from protein cloning, over-expression, purification, crystallization, data collection, structure solution, refinement, validation and data management. All of which have become more or less automated with minimal human intervention necessary [1].

The field growth has the challenges as ever of crystal size and diffraction quality but now also with throughput issues. Bigger crystals for neutron structural studies are also needed and where they are reasonably perfect. In an interesting twist of this story, freezing of very large crystals of proteins suitable for high resolution neutron data collection is a recent breakthrough [17]. Neutron cryo-crystallography benefits the clarity of bound water structure, including the water deuteriums, and also opens up freeze-trap neutron protein structural studies [18].

Pechkova and Nicolini, (2004) [19] suggested a new technology defined as 'protein nanocrystallography' [20] because it uses nanotechnology to both produce (by nanobiofilm template) and characterize to the nano- and subnanoscale (by AFM, nanogravimetry and microfocused synchrotron radiation) diffracting and radiation-stable protein crystals of any dimension. This new technology has provided a route from art to science, through proteomics and crystallography, which can be used to determine the structures of proteins and protein complexes that have not yet been characterized [19]. The protein thin-film nanotemplate is created using Langmuir–Blodgett (LB) technology or modifications of it [21,22], and is subsequently deposited on a solid glass support, to be placed in the appropriate vapor-diffusion apparatus. This LB protein thin film assumes the role of the template for protein nucleation and crystal growth. During the screening procedure, the following parameters can be varied: protein monolayer surface pressure, precipitant nature and concentration, and number of protein thin-film monolayers. Temperature variation can be also used to control protein crystal nucleation and growth. For this method, the authors elected to use the traditional hanging drop vapor-diffusion method because requires small amounts of samples and permits variation of physical parameters during crystallization [23]. There are three major innovations highlighted in this method: 1. Diffracting and radiation-stable microcrystals have been obtained consistently for the first time by the nanotechnology-based method [20,24-27] for proteins that had not been crystallized previously, despite numerous efforts worldwide by standard methods. 2. Atomic resolution structure has been derived at the ESRF microfocus beamline from miniscule protein microcrystals with diameters of < 20 microns [27,28]. 3. The atomic resolution structures of proteins of central importance in life sciences have been determined [19,29-38].

Structural Genomics

The very significant investment made worldwide in more efficient structure determination is essential if the growth in structural data is to continue at traditional rates. The protein structure initiative was first suggested in 1998 at a time when the fall in the rate of structure determination was already occurring but it would not have been discernable [39].

Then, the evolution of the sample environment is quite recent. It is also the consequence of the emergence of the post-genomics era [40], with the development of systematic structure-solution projects (structural genomics projects). In order to face this rapidly growing demand for beam time, it is necessary to improve and facilitate sample handling [5].

In addition, the limit of protein crystal perfection evaluation has taken one into the territory of the silicon crystal level of quality [18]. Indeed that fact interested many protein crystal growers since proteins might be regarded as always totally floppy "balls of fluff" rather than capable of being orderly packable objects over the whole spatial coherence length of a crystal. Arai *et al*., (2004) [41] offered the opinion that crystal quality can be described by a single parameter: the resolution of diffraction. Textured patterns (with split spots in other words), but still diffracting to "decent resolution", tell us that more parameters are needed to describe one crystal from another than resolution alone (or a relative B factor too). Indeed most crystallographers now know that these various parameters are needed to define the various aspects of crystal quality that crop up. However single parameter evaluation [41] suggests is better suited to the high throughput area of structural genomics [18].

Protein Crystallography in Drug Discovery

As observed, the structural genomics affords are focused almost entirely on soluble proteins of unknown structure or function [42]. By contrast, most drug discovery programs are directed at a specific protein target of known function, which is often a membrane bound protein. It is estimated that >50% of all major drug targets are membrane proteins [43, 44] . As such, the protein targets that are chosen by structural genomics researchers are not necessarily of immediate value to drug discovery programs [45,46].

Biomedical researchers throughout the world are now busy establishing a new paradigm for human disease, one that implicates individual biological macromolecules. Instead of examining microbial invaders, the biomedical research community is studying the consequences of introducing foreign proteins (bacterial, fungal and viral virulence factors) into humans, the results of individual genetic lesions, as gain or loss of function and alterations in function, or the cumulative effects of multiple genetic factors contributing to diseases such as adult-onset diabetes mellitus, hypertension and so on [16].

A strategic investment made in high-throughput genome sequencing, a big science endeavour relatively new to biology, is also contributing to dramatic changes in our thinking. Using software packages, we can compare organisms at the level of whole genomes, gleaning important evolutionary insights and identifying clinically relevant differences between man and viral/bacterial/fungal pathogens. The availability of whole-genome sequences also creates the potential to develop massively parallel tools, such as arrays of immobilized DNA elements to study gene expression patterns, which will contribute to both – fundamental research and point-to-care diagnostics. Finally, newly characterized gene products themselves offer the promise of novel therapeutic agents, many of which will become protein pharmaceuticals [16].

The structure-based drug design is attractive because it promises to reduce the cost in time and money required to produce a drug and produce pharmaceuticals that are more selective and with fewer undesirable side effects. This will increase greatly as the technology for solving protein structures and designing ligands improves and becomes more widespread [47-49].

Additionally, the demand for protein crystallography in drug discovery is driven by the need to understand exactly how small molecules bind to their protein target. This information enables researchers to conduct medicinal chemistry projects in a more rational manner, taking advantage of structural information and applying it in structure-based approaches to making new compounds. However, structure-based drug design is complicated by the fact that computational screening methods often fail to accurately predict

ligand-binding modes to protein targets [53] and the binding of a ligand to its target can often result in large changes in protein conformation. Given the inadequacies of computational tools for predicting ligand-binding modes, there is a growing need for the crystal structure-determination of large numbers of ligand-protein complexes. To meet this demand within time scales that are reasonable for the process of crystal structure determination be automated and industrialized. The development of automated crystallography systems is poised to have an immediate and significant impact on the pharmaceutical industry by enabling protein ligand co-crystal structures to be solved with increasing speed [45].

Protein crystallography methods applied by research teams in the pharmaceutical industry to support the process of discovery of new medicines are not greatly different from those used by academic structural biology groups. However, owing to the specific aims of the pharmaceutical industry, the approaches and working practices are often quite distinct. This applies to both the determination of novel structures of drug targets and complexes of these targets with potential drugs. To make any significant impact on ongoing medicinal chemistry projects, crystal structures have to be delivered on time and must provide answers to specific questions [54].

Historically, protein crystallography has been fairly low throughput, which can sometimes limit practical use in drug design programs, but particularly over the past few years, the number of examples whereby crystallographic methods have been applied to small molecule design has risen. Two reasons for this trend are an increased focus on protein production and new crystallization facilities [55-58] and an increased use of new approaches, such as rational fragment assembly methods [59-62].

A simple common theme in structure-guided drug design examples, mostly from classic drug targets, is that atomic detail of binding sites helps generate new chemical ideas and can aid design of new small molecules and arrays. However, relatively more complex structure-guided concepts, such as virtual screening [63,64] and fragment assembly methods, are becoming increasing influential in compound identification and design [62].

Published examples of drug design have involved structural studies to varying degrees. Human immunodeficiency virus (HIV) protease has probably been the most intensely studied target, with hundreds of protease/inhibitor complexes solved and four drugs on the market [65]. Other successful cases of drug design include inhibitors of influenza virus neuraminidase [66] and thymidylate synthase inhibitors with anticancer effects [47,67,68]. The search for p38 kinase inhibitors offers an excellent historical perspective as to how technological changes that have taken place in the pharmacological industry over the last decade, have affected the ways in which new leads are discovered and advanced [69]. PKA, the prototypical serine/threoine protein kinase, and SRC, a tyrosine and the first identified oncoprotein, provide multiple examples of these various approaches to protein kinase crystallography for drug design [70].

Of the over 500 protein kinases identified in the human genome [71], structures of about 50 have been determined in some form [72], and a search in the PDB currently matches nearly 400 entries [5]. The large number of structures, both in terms of unique kinases, and also kinases in unique combinations with inhibitors, substrates, or co-factors, enables characterization of their functional features in an unprecedented way. Both the serine–threonine and tyrosine kinases are well represented among the structures [70].

Other examples are the protein tyrosine phosphatases play roles in many biological processes and are considered to be important targets for drug discovery. As inhibitor development has proven challenging, crystal structure-based design will be very helpful to advance inhibitor potency and selectivity. Successful application of protein crystallography to drug discovery heavily relies on high-quality crystal structures of the protein of interest complexed with pharmaceutically interesting ligands [73]. Protein tyrosine phosphorylation is a post-translational modification that regulates many important biological processes, including embryonic patterning, tissue growth and repair, metabolism and angiogenesis [74-79]. The balance of protein tyrosine phosphorylation is maintained in part by the opposing actions of protein tyrosine kinases and protein tyrosine phosphatases. Importantly, disruption of this balance has been implicated in a variety of disease processes such as cancer, atherosclerosis and diabetes, making both kinases and phosphatases prime candidates for drug discovery [73]. Reversible protein phosphorylation is a major control mechanism in eukaryotic cells and identification of selective kinase and phosphatase inhibitors is an area of intense current interest as a potencial cancer therapy [80-82]. Researchers have reported a novel class of cyclin-dependent kinase (CDK) inhibitors identified through structure-guided hybridization of known inhibitors [26,62,83,84].

Metabolic pathways are an attractive and well established target for the development of antibacterial agents. Structural characterization of enzymes that belong to microbial metabolic pathways is very important for structure-based drug design since some of these proteins may be present in the bacterial genome, but absent in humans [85-89]. The mechanism of action of these pathways are characterized by biochemical methods and supported by protein crystallography.

Statistical analysis from PDB shows that 32.3 and 11.5% of entries are bacterial and human structures, respectively, solved by natural source, against 37.2% and 30.5% from human and bacterial structures solved by engineering source, respectively. These numbers show the efforts to found strategies for discovery the structure and function of proteins in addition with intra and intermolecular interactions, potential target for drug discovery.

Examples of structure-guided drug design in other more "structurally challenging" target classes are transmembrane receptors, ions channels and multisubunit complexes such as the ribosome [46-52]. It is certain that for all classes of drug targets, visualization of binding mode *via* crystallography and rational chemical design will be a prominent tool [62].

The development of integrated technology platforms and database systems to automate all steps –from clone to final structure – is extremely important for realizing the full impact that high-throughput crystallography can have on the drug design process. The combination of crystallographically validated protein-ligand structures with increasingly sophis-

ticated computational chemistry tools offers an accelerated model to pursue a growing number of protein targets for small-molecule drug discovery [45].

Computational Chemistry Tools

The drug-design process is not linear. Once a target has been identified and its structure determined, the next step is to find a lead (a compound that binds to the target). Screening available libraries (large corporate collections of compounds from natural and/or synthetic sources) can do this. Computer programs can be used to search electronic databases of compounds for potential leads or design leads *de novo* using only the biomolecular structure as a template. Aknown substrate or inhibitor of the protein could also be used as a starting point. In addition to drug design, it is possible to use protein crystallography to examine how drugs are metabolized. For example, the structures of many of the enzymes involved in the metabolism of xenobiotics have been solved [47].

A number of programs exist for testing the mode of binding and the fitness of inhibition in the site of interest in the biomolecule. DOCK [90] is the most commonly used example. DOCK computes the surface of the active site of the protein and locates possible positions where ligand atoms may sit. It then tries to match the ligand atoms to those atom centers. Once a series of potential ligands have been produced by a search or design program, graphics computers can be used to visually inspect models of protein/ligand complexes. Other programs, such as GRID [91] position functional groups (called probes) on the protein surface using empirical potential energy functions to evaluate their interaction energy. The program does not attempt to connect fragments together to form an inhibitor, which is left to the imagination and skill of the scientist. LUDI [92] works in a similar way, except that it uses a mathematical description of hydrogen bond donors, acceptors and lipophilic points to retrieve matching functional groups or ligands from a database. Functional groups can be built up into ligands with this program [47].

FINAL REMARKS

Protein crystallography is an essential tool for the discovery and investigation of pharmacological interactions at the molecular level. It allows investigators to directly visualize the three-dimensional structures of proteins, including enzymes, receptors and hormones. Increasingly knowledge of these interactions is being used in the drug-discovery process. The desired drug could be an enzyme inhibitor or an agonist that mimics endogenous transmitters or hormones. Once the 3-D structure of a pharmacologically relevant target is known, computational processes can be used to search databases of compounds to identify ones that may interact strongly with the target. Lead compounds can be improved using the 3-D structure of the complex of the lead compound and its biological target [47].

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Received: November 1, 2008 Revised: November 15, 2008 Accepted: November 19, 2008