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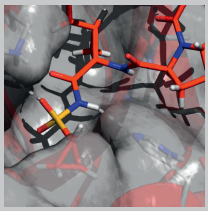

Medicinal Chemistry and Chemical Biology Highlights

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Adding New Dimensions to Protein Structures with Room-Temperature Serial Crystallography

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Abstract: Structure-based drug design (SBDD) uses experimentally determined structures of proteins to aid in the identification of hits and development of leads for protein targets. Improving the physiological relevance of protein structures that are routinely determined at cryogenic temperature will undoubtedly help to improve this design process. One way that this can be achieved is through serial crystallography; a method that can be used to determine the high-resolution structure of proteins at room temperature. Additionally, through the application of time-resolved serial crystallography, protein dynamics can be investigated – providing another dimension to protein structure determination. How this information can be used to guide SBDD is yet to be seen. This article gives a brief overview into serial crystallography, including the experimental challenges faced by researchers in the field and some recently published pharmacologically relevant proteins studied using this technique.

Keywords: Dynamics · Structure-based drug design · Serial crystallography· Time-resolved

Introduction

The vast majority of protein structures have been determined at cryogenic temperatures, far from the physiologically relevant temperatures at which these proteins function. Additionally, crystallography is only capable of providing the structure of a protein at one snapshot in time, averaged over all copies of the protein making up the protein crystal. Information on the inherent kinetics and flexibility associated with protein structures is therefore missing. To address these concerns, serial crystallography has been developed. In this technique, room temperature protein structures can be collected, with the option of introducing trigger systems to probe high resolution structural information on protein dynamics. Although still in its infancy, the number of protein structures produced by this technique has been steadily increasing (Fig. 1). However, the technique remains relatively niche and is yet to be adopted by the wider community.

How does Serial Crystallography differ from Conventional Crystallography?

To understand why the uptake of serial crystallography has been limited, it is important to understand what differences there are compared to a conventional crystallographic experiment. As opposed to conventional methods, where cryogenic temperatures are used to slow down radiation damage, room temperature data collection can result in the rapid onset of radiation damage. This

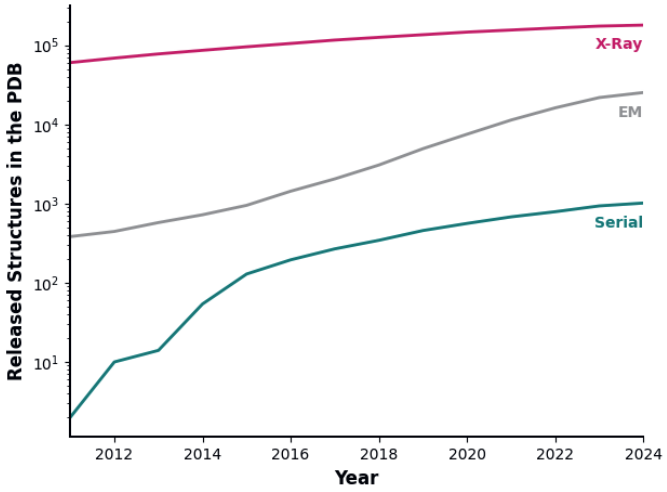

Fig. 1. Comparison of the cumulative number of structures released on the PDB over time for conventional cryo-crystallography (red), EM (grey) and serial crystallography (green).

damage causes a reduction in the diffraction power of crystals and structural changes within the protein itself.[1] To avoid these effects, crystals are continuously replaced in series, resulting in room temperature datasets collected from thousands of crystals with minimal radiation damage. The consequence of this strategy is that sample requirements for serial crystallography are much higher than conventional crystallography, by at least one order of magnitude. In addition, collecting data from crystals on this scale would not be possible without sample delivery systems to automatically replace the crystals in front of the X-ray beam. Initial methods to do this focused on using jets of the crystals in solution[2] or embedded in viscous media.[3] More recently, a lot of work has gone into the development of new sample delivery techniques, such as the solid support-based methods, that aim to make serial crystallography more accessible by lowering both the sample requirement and the skill level required to prepare and run samples.[4,5]

Recent efforts have highlighted the increased pharmacological relevance of room-temperature protein structures. One interesting example can be seen from experiments comparing structures of glutaminase C collected under cryogenic conditions and at room temperature using serial crystallography.[6] BPTES (bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulphide) and its analogues make up a class of allosteric inhibitors targeting glutaminase C, that have been investigated as potential anticancer drugs.[7,8] Despite having a wide range of potencies, similar binding poses have been observed in cryo-structures of glutaminase C with a selection of ligands in this class. A structural explanation for the difference in potency could potentially be provided by comparing the room temperature structures obtained with seri-

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al crystallography. This comparison revealed differences in the binding poses and flexibility of the ligand in the binding pocket that were not present in the cryo-structures (Fig. 2). Without these data, lead optimization efforts would likely be misguided. These results demonstrate the unique insights that serial experiments can provide – insights that would not be possible otherwise using traditional methods.

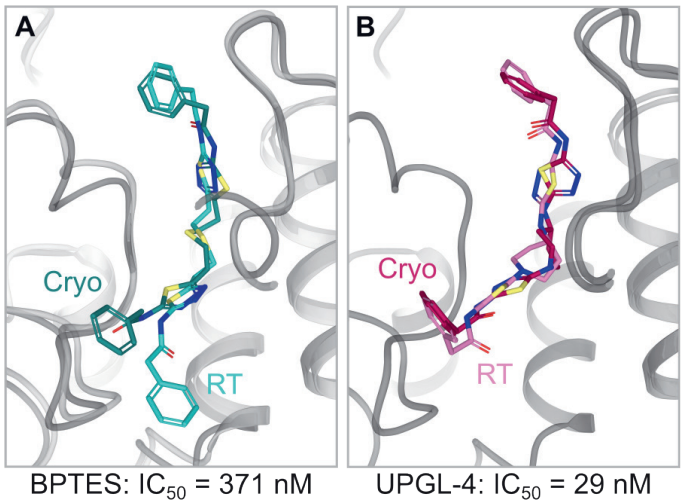

Fig. 2. BPTES and its analogue UPGL-4 in the glutaminase C binding pocket. Differences in the binding position of BPTES determined at room temperature (PDB: 7RGG, light blue) and cryogenic temperature (PDB: 4JKT, dark blue) can be seen. Whereas UPGL-4 displays similar binding positions at room temperature (PDB: 7REN, light pink) and cryogenic temperature (PDB: 5WJ6, dark pink).

Exploring Additional Dimension in Protein Structures with Time-Resolved Serial Crystallography

Aside from being able to collect room temperature data without radiation damage, one of the main benefits of serial crystallography is the ability to collect time-resolved data. Triggers are introduced to the crystal system to initiate a reaction prior to X-ray diffraction. Varying the time delay between the trigger (pump) and the X-ray pulse (probe) allows structural snapshots of the reaction process at different time points to be collected – helping to elucidate the protein dynamics involved.[9,10] One common suggestion for data from time-resolved serial crystallography, is that the protein dynamics information could be used to improve SBDD.[11] This could be through the identification of transient intermediates or cryptic pockets that could be targeted and may provide explanations for unexpected ligand association/ dissociation kinetics. So far, there have been no examples of this application, as the number of proteins that have been studied using this technique is still limited. Over time, as more examples become available, greater emphasis will also be placed on utilizing the dynamic data for more advanced applications besides SBDD, for example as training data for artificial intelligence models to predict protein-ligand complex structures.

Carrying out time-resolved serial crystallographic experiments has an added level of complexity on top of the challenges already present in serial crystallography. To begin with, an appropriate trigger is required so that reactions can be initiated synchronously to allow time-resolved data collection with a clear separation of states. The main trigger system used so far has been light. For photoactive proteins, such as rhodopsins,[12,13] photosystems[14] or photolyases,[15,16] light is an ideal trigger that allows time-resolved data to be collected for the full fs – s timescale accessible at X-ray free election lasers (XFELs) like SwissFEL. However, the number of endogenously photoactive proteins is limited, with only <0.5% of proteins having this capability.[17] To overcome this, either a synthetic photoswitch or alternative triggering system can be used. Synthetic photoswitches, such as photo-isomerisable affinity switches or photocaged compounds, enable the activity or pharmacology of a ligand to be changed depending on the illumination state. For example, using photoswitches, processes such as ligand dissociation and binding have been observed. These experiments have allowed key states to be observed, such as the transient binding state during the multi-step ligand binding pathway of fluoroacetate dehydrogenase,[18] loop movements involved in binding pocket accessibility for tubulin,[19] and the lid opening and apo state of the adenosine A2a receptor.[20] On longer timescales, the catalytic cycle of enzymes such as fluoroacetate dehydrogenase[18] and L1 metallo-β-lactamase[21] could also be observed. These studies allowed the fluctuations of the bound substrate in the active state and catalytic intermediates in an enzyme to be observed.

Using photoswitches comes with its own challenges. Careful spectroscopic characterization of the ligand photochemistry prior to undertaking a serial experiment is often required, to identify the illumination conditions and to ensure that a photoreaction takes place in the protein, which, especially in the confines of a crystal environment is not always guaranteed. Additionally, although photoswitches have been developed for a number of protein targets, this list is not extensive and may not be suitable for all proteins of interest. Using alternative trigger systems, such as temperature jump[22] or rapid mixing[23] can help to circumvent these problems and opens the technique to a much broader range of proteins. A careful consideration of the type of data wanted and the protein system to be studied is therefore needed to select an appropriate trigger. Depending on this, a decision can be made whether data collection at an XFEL is necessary, or synchrotron data collection is sufficient. Collecting data at synchrotrons, where there is a higher availability of beamtime, would also increase the throughput with which experiments can be carried out. The longer timeframe required to collect data are a limitation for serial crystallography. Following the production of crystals, a structure can be produced within a few weeks by conventional crystallography. Whereas for serial crystallography, this process can be extended longer.

Where Does the Future Lie for Serial Crystallography?

Serial crystallography and time-resolved serial crystallography provide exciting opportunities for research, allowing more physiologically relevant structures to be obtained. Results have the potential to enhance drug discovery by capturing dynamic conformational changes, enabling a deeper understanding of target flexibility and function. This approach presents an opportunity to advance structure-based drug design by accelerating lead optimisation and drug selectivity for more effective therapeutics. Due to the high sample requirement for these experiments – particularly for time-resolved experiments – it is important to demonstrate that these benefits outweigh the costs of carrying out the experiments. Improving methodology to make experiments easier and less costly to carry out, whilst demonstrating how the data produced from these experiments can be used will be crucial to cement the role of serial crystallography in the future.

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