Protein Crystallography by Joint X-Ray and Neutron Diffraction

By 1984, the quest to understand the internal dynamics of protein molecules had created a pressing need for well-refined, high resolution data on protein structures. In a landmark contribution to the field of protein crystallography [1], Wlodawer, Walter, Huber, and Sjölin undertook an innovative joint application of x-ray and neutron diffraction methods to determine the structure of a new crystal form of bovine pancreatic trypsin inhibitor (BPTI). This work was the first to determine the atomic positions in a protein of this size to within the diameter of a hydrogen atom. By comparing structures from two crystalline forms of BPTI, the paper offered the first detailed analysis of how protein structure is affected by molecular packing. In addition, the paper provided the first detailed experimental picture, atom-by-atom, of hydrogen-deuterium exchange in a folded protein, revealing the existence of eleven "protected" amine hydrogens which exchanged at anomalously slow rates. This work also established that joint x-ray-neutron refinement could produce structural detail consistent with the then emerging technique of two-dimensional NMR protein crystallography and provided the baseline data for the further development of multi-dimensional NMR.

X-ray and neutron beams falling on a crystal are diffracted by its atomic constituents, and the periodic structure of the crystal causes the diffracted rays to be sharply defined. The directions and intensities of the diffracted rays are sensitively determined by the three-dimensional arrangement of the atoms and by specific aspects of their interactions with the incident radiation. Proteins do not occur as crystalline material in nature. However, it is possible to grow their structures artificially into crystals, which then can be studied by diffraction methods. BPTI previously had been prepared in a crystalline form (form I) that was not suitable for high resolution studies but, importantly, Walter and Huber had just succeeded in synthesizing BPTI into a new crystalline form (form II) that appeared to be more promising [2] for structural studies.

Protein crystallography raises many challenges. It is hard to make protein crystals of the size and quality needed for diffraction studies. Even with good data, the large number of atoms in proteins and their steric complexity make it difficult to determine such structures accurately at resolutions near 0.1 nm (1 Å), the diameter of a hydrogen atom. A major theoretical constraint also intrudes. Diffraction patterns depend only on pairwise relationships among atomic positions, and these are not unique attributes of structures. Protein crystallography does not yield a direct route from the experimental data to the atomic structure. The determination of atomic positions requires analytical procedures in which putative structures are refined against the data in stages of increasing detail, a computationally intensive process in which chemical and biological knowledge and intuition play significant parts.

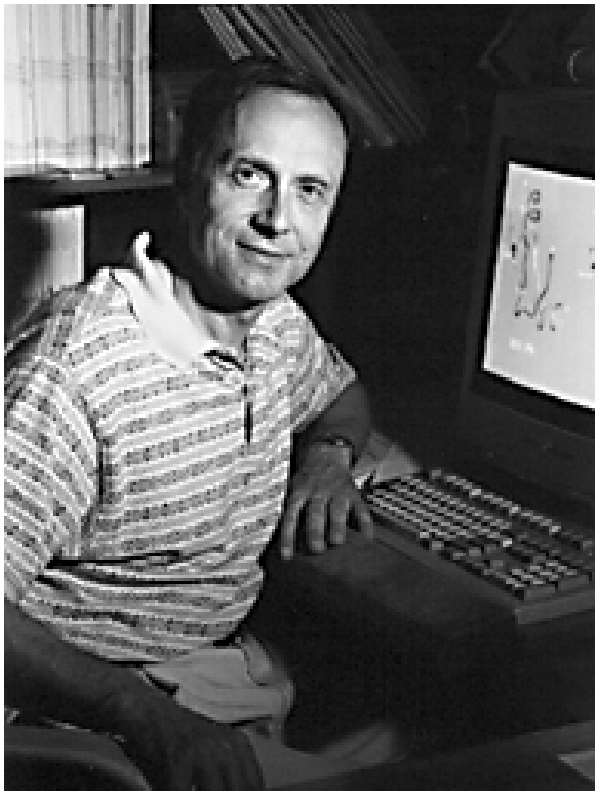

Fig. 1. Alexander Wlodawer, ca. 1999.

Bovine pancreatic trypsin inhibitor is one of the most thoroughly studied of all proteins, even though it is of minor biological moment. BPTI was interesting at the time of this work because it is a small protein (only 58 amino acids) with well-understood functionality. Its three-dimensional structure had already been determined to 2.5 Å resolution using x-ray diffraction and related techniques as early as 1970 by Robert Huber and coworkers [3] in a crystal form that became known as

form I. Subsequent studies had increased the x-ray resolution of the structure to 1.5 Å, and a large amount of work on BPTI structure and dynamics using various techniques had appeared in the 1970s and early 1980s. In fact, BPTI was the first protein for which detailed experimental information about the folding of the polypeptide chain became available. The quality of protein crystals was the major limitation to increasing the resolution of diffraction studies. In the early 1980s resolution was extended to an ångstrom or slightly less, but in proteins smaller than BPTI.

The work described in the 1984 paper was started when coauthor Lennart Sjölin, Wlodawer's postdoctoral worker at NBS (but then already on the faculty of the Chalmers Polytechnic in Goteburg, Sweden), learned that coauthors Robert Huber (who would win the 1988 Nobel Prize in Chemistry) and Jochen Walter at the Max-Planck-Institute for Biochemistry in Martinsried, Germany, had managed to grow a new crystal of BPTI, which they called form II. In Alex Wlodawer's words: "Since that molecule was at that time the 'hydrogen atom' of protein chemistry, and most physico-chemical techniques being developed were initially tested with it, we wanted very much to take neutron data on that protein. The previously available crystals, however, were tricky to grow and simply not good enough—they did not grow very large and did not diffract very well. So the new crystal form, growing easily, large, and with superb diffraction, was of clear interest. Lennart got Huber and me together on this project, and we were off and running." The authors also were alert to the scientific implications of having a new crystal form of BPTI to compare with the well-characterized form I, so that changes in protein structure could be correlated with the different molecular packings in the two forms.

However, Alex continues: "In the meantime, we found out that Wayne Hendrickson, one of the most prominent American crystallographers, also had these crystals, and we were afraid that he would go after the neutron data at Brookhaven." Wlodawer and Hendrickson, together, had already pioneered the simultaneous refinement of x-ray and neutron data in 1982 [4], showing that it can provide more information about protein structure than either type of data alone. Hydrogen locations, in particular, can be determined more precisely with neutrons since hydrogen is a strong neutron scatterer while being nearly invisible to x rays. "So there was also an element of competition as well," Alex allows. Wlodawer and Sjölin were in a good position to move quickly, since they had already published several papers on the joint refinement technique and were well armed with methodology. Moreover, neutron beam time and excellent instrumentation were available to them at the NBS Research Reactor, and Division 856 (the Reactor Radiation Division, now the NIST Center for Neutron Research) possessed a Vax 11/780, one of the most powerful minicomputers of the day, and an Evans & Sutherland display system, the most powerful tool available for three-dimensional visualizations of molecular structures.

Although Alex mentions the form II BPTI crystals as "growing easily," the 1984 paper describes an involved preparation protocol, including a two-month period of undisturbed growth from a seed crystal, followed by another three months for heavy water exchange for the neutron measurements. Heavy water greatly reduces background neutron scattering from the protein crystal. In addition, the exchange of deuterium atoms from the heavy water for hydrogen atoms in the structure provides additional information about the hydrogen locations in the protein. Curiously, the paper informs us that the heavy water used "was manufactured in 1983 by the facility in Rjukan, Norway." Alex explains: "The heavy water that we used came from the famous facility in Rjukan, Norway, and was produced in 1938. As many know, the Allies took great pains to destroy the place during WWII to prevent the Germans from having access to heavy water. So, in the page proofs, I had the bright idea that since half the authors were German, I should just insert the Rjukan bit (otherwise having no bearing on the contents of the paper). I did just that in the Materials and Methods section (p. 303), and then forgot about it. Last year, however, I told the story to somebody who looked up the paper and told me that the year of production of the sample was given as 1983, killing the joke. Clearly, an eager editor caught my 'obvious' error in transposing the numbers (how could anybody use a 1938 sample in 1984?) and 'corrected' me. So it goes."

The paper did not have an immediate impact, but after a year's gestation its influence grew very rapidly. Wlodawer attributes its long-range influence to several factors. The work resulted in one of the best-refined protein structures at that time, and so the paper has been extensively quoted as a baseline for the quality of protein structures. The work on hydrogen-deuterium exchange was influential, since interest in the kinetics of exchange persisted for some time. Also, its results bear on many fields of current interest, including protein folding and protein chemistry. Its achievement of a high resolution protein structure, especially the accurate hydrogen positions, was, and continues to be, influential in the field of NMR, which is also sensitive to hydrogen locations. Since BPTI had been chosen as a platform for the development of two-dimensional NMR in the early 1980s, the appearance of this paper was very well timed from a historical perspective. "I suspect the paper will be cited for quite a few years longer," Alex says.

The paper concludes with the words: "This paper is dedicated to Professor Paulina Wlodawer on the occasion of her seventieth birthday." Alex adds: "On a personal note, I am happy that this is the paper that will have its lifetime extended by inclusion in the NIST history. This was the only paper ever that I dedicated to my mother, who is a biochemist and provided major impetus for me becoming a scientist. Thus if one of my papers should be remembered, I am glad that this is the one."

Alexander Wlodawer, a native of Poland, received his Ph.D. from the University of California, Los Angeles, in 1974. Having completed his postdoctoral training at Stanford University, he joined the National Bureau of Standards in 1976, where he engaged in many studies of protein crystallography until 1987, when he moved to the ABL-Basic Research Program at the National Cancer Institute-Frederick Cancer Research and Development Center. From October 1998 to March 1999, he was on sabbatical as an elected Visiting Fellow of Sidney Sussex College, University of Cambridge. In 1999 Wlodawer was appointed the Associate Director of the Program in Structural Biology in the Division of Basic Sciences of the National Cancer Institute, where he is Chief of the Macromolecular Crystallography Laboratory and of the Protein Structure Section. He is a member of the American Crystallographic Association and the Protein Society and has been an elected officer in both organizations.

Robert Huber shared the 1988 Nobel Prize in Chemistry with Johann Deisenhofer and Hartmut Michel for "the determination of the three-dimensional structure of a photosynthetic reaction centre." He is in the Department for Protein Crystallography at the Max-Planck-Institut für Biochemie, Martinsried, Germany. Lennart Sjölin is an Associate Professor in the Department of Inorganic Chemistry, Protein Crystallography Group, at the Chalmers University of Technology in Goteborg Sweden.

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