Protein Crystallography

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"How I Chose Research on Proteases or, More Correctly, How it Chose Me"

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proteases · protein crystallography · structure elucidation · X-ray diffraction

It came to me, one day in 1968, by way of the person of Professor Ernst Auhagen (Figure 1), head of biochemical research at Bayer company. I had a small laboratory and office in Schillerstrasse, Munich at the Max-Planck-Institut für Eiweiß-und-Lederforschung, harboring two chairs, a desk, a Richards-box for protein model building and a hood.

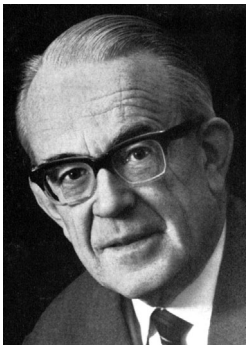

Figure 1. Ernst Auhagen.

It was difficult to squeeze by these voluminous objects, but Auhagen was a tall, slender man and managed to do so. He rummaged around in the pouch of his jacket and pulled out a test tube, where I observed glistening crystals at the bottom. A chemist examines with eyes and nose. Therefore I opened the tube, but almost suffocated from the intense stench of ammonia. The substance was suspended in concentrated ammonia solution from which it had been crystallized for final purification. It was Trasylol/Aprotinin, produced by Bayer, for application in heart surgery to reduce blood-loss, as Auhagen explained to me. It had been discovered and isolated from bovine lung as inhibitor of the kallikrein (a trypsin-like protease)-kininogen (a peptide hormone precursor) system of Kraut, Frey, and Werle, which is central in blood-pressure regulation and many other physiological processes. Auhagen asked me whether I would be interested in a crystallographic and structural study of Trasylol. I was.

Before I continue with Trasylol/Aprotinin, let me give some background and cast a glance some 10 years further back: I had studied chemistry at the Technische Hochschule Munich and was much attracted by minerals and crystals, which I collected as an amateur geologist during my frequent climbing excursions in the Bavarian and Austrian Alps. Why are they symmetrical, have regular shapes, sharp edges, shining faces, and intense colors? It was obvious that only by crystallography using X-rays one may be able to look inside and find an answer. I therefore asked Professor Walter Hoppe (see Figure 3) if I could join his research group at Günter Scheibe's Institut für physikalische Chemie und Elektrochemie at the Technische Hochschule Munich (THM, now TUM) in 1959 for experimental work and my diploma, followed by the dissertation.

The institute was located in the central building of the THM in Luisenstrasse and still heavily marked at that time by the bombs of the war. Heinz Gerischer was Professor of electrochemistry at the institute with his collaborator Gerhard Ertl. Our paths crossed frequently in the old edifice, but we did not take notice of each other. Our research subjects were indeed quite different: mine, visualizing the inside of crystals (and molecules), his, studying their surfaces. Both fields of research turned out to be interesting and rewarding, honored with Nobel prizes in the times to come.

Molecular X-ray crystallography was a neglected field in Germany at that time and Hoppe was highly respected for his design and construction of instruments, a mechanical Fourier synthesizer (electronic calculators for the computation of Fourier series were not available) and a single-crystal diffractometer (marketed later by Siemens), and for the development of methods for crystal-structure solution. His discovery of the "Faltmolekülmethode"[1] to determine orientation and position of molecules or molecular fragments of known structure in Patterson functions of an unknown crystal structure meant a revolution of modern protein crystallography as we now realize, because most protein structures are presently solved by "molecular replacement", which Ross-

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mann and Blow had independently discovered and developed in 1962,[2] based on closely related considerations. Hoppe's publication in 1957 was not immediately viewed as a landmark, perhaps because it was written in German (albeit so clearly and succinctly that readers unfamiliar with that language will grasp the idea easily), but also because the analysis of crystals of related molecules—the essence of the method—became important in protein crystallography only many years later, when the database of protein structures had grown to a sufficient size. In those early times Patterson functions, which represent the vector sets of all atoms in the crystal unit cell, and Faltmoleküle, the intra- or intermolecular vector sets of the search model, were drawn on transparent paper and rotated and shifted manually until both matched. This could only be done in two dimensions, of course.

As computers became available in the late fifties, I embarked on developing a program for the Faltmolekülmethode and was given the chance to apply it on a challenging project, that introduced me to a new field, biochemistry, and to famous biochemists, Peter Karlson (Figure 2) and Adolf

Figure 2. Peter Karlson.

Butenandt, at the Max-Planck-Institut für Biochemie, who had isolated the insect molting hormone ecdyson. Its molecular structure, even its molecular weight were unknown. Crystals were given to me and I recorded the first X-ray diffraction images, by which I determined the molecular mass and corrected earlier values, followed by the collection of a complete data set. I pondered on a model for the Faltmolekül construction and, after many trials, succeeded with a steroid moiety fitted to the Patterson map. By cycles of adding and removing atoms and altering stereochemistry, I was able to localize all substituents, including hydrogen atoms, and stereochemical centers defining ecdyson as 2β.3β.14α.22β_F.25-pentahydroxy-δ7-5β-cholestenon-6 in 1965.[3] Professor Wolfgang Steglich at the THM had helped me with the formidable systematic nomenclature problem, when we discussed viewing my hand-drawn molecular model at a meeting, memorable for both of us. Karlson was excited about my discovery, Herr Butenandt, President of the Max-Planck-Gesellschaft, was barely approachable for a doctoral student or post-doc at that time. However, he must have been quite satisfied to see in insects a hormone and molecular entity related to the sex hormones, he had discovered in humans thirty years earlier, work, that earned him the Nobel Prize in 1939. At that time ecdyson was the largest molecular structure to have been solved without making use of a heavy atom for phase determination. The determination of the precise structure enabled chemical synthesis by teams of Schering and Hoffmann-LaRoche competing with a Californian pharma company Syntix trusting that ecdyson may offer a novel strategy for the development of insecticides. For me, it meant a first encounter with pharma industry.

I learned later, that Max Perutz was aware of my achievement and had good words for me, which were helpful for my future career and, in particular, prompted Walter Hoppe to give me the option to establish a small protein crystallographic group. Together with Helmut Formanek, we, now in Schillerstrasse at the Max-Planck-Institut für Eiweiß- und Lederforschung, where Hoppe's research group had moved to, isolated a red protein from insect larvae called erythrocruorin. By classical heavy-atom isomorphous replacement we found, in 1967, initially at low resolution, extended later to atomic resolution,[4,5] that the polypeptide chain folds like myoglobin and hemoglobin analyzed by Perutz and Kendrew.

This discovery documented conservation of proteins across species, mammals and insects, that diverged several hundred million years ago. This is text book knowledge now, based on an overwhelming mass of data of gene and protein sequences, but at that time was a first observation and for me revisited what I had seen in an entirely different chemical class, in steroids, in ecdyson of insects and the human sex hormones.

With this work I had established my small protein crystallographic group in 1968, at a time when, worldwide, less than a handful existed, but it was now large enough to start a new project, Trasylol/Aprotinin. The crystals, Auhagen gave me, were well ordered and diffracted to high resolution (after some manipulation and exchange of mother liquor). Phase determination by isomorphous replacement went smoothly. I could readily interpret the resulting electron-density map on the basis of the amino acid sequence, available in two independently determined (and identical) versions, as kallikrein inactivator and as bovine pancreatic trypsin inhibitor (BPTI).[6]

It occurred to me, remembering the procedure in organic molecule crystallography, that, by adding other information on the geometry the amino acids and peptide bonds, phases may be improved and extended to higher resolution, where the isomorphous phases were poorly determined. But while we usually have atomic resolution and see individual atoms in small-molecule crystallography, this is not so in protein crystals. Bob Diamond in Cambridge, UK, had developed a computer program that maximized the overlap of molecular fragments with electron density by rotations and shifts, yet maintaining correct bonding geometries. We (two PhD students of physics, Wolfgang Steigemann and Johann Deisenhofer had joined my small group) had moved to the new Max-Planck-Institut für Biochemie in Martinsried, where in 1972 I had accepted an offer to become a member of the Max-Planck-Society and director at the institute (Figure 3). We thought to use this tool, but apply it in cycles by recalculation

Figure 3. Directors at the Max-Planck Institut für Biochemie, Martinsried (photo taken 1974) First row: (from left to right) Heinz Dannenberg, Adolf Butenandt, Feodor Lynen, Gerhard Ruhenstroth-Bauer. Second row: Robert Huber, Pehr Edman, Erich Wünsch, Gerhard Braunitzer, Peter Hans Hofschneider, Walter Hoppe, Kurt Hannig, Klaus Kühn, Wolfram Zillig. Butenandt and Hoppe are mentioned in the essay. Construction and opening of the Max-Planck-Institut für Biochemie in 1973 was a signal for the establishment of the Campus Martinsried, which has grown to one of the largest centers for research and technology in Europe.

of phases from the improved protein model. This worked beautifully and produced electron-density maps of hitherto unseen clarity and resolution.[7,8]

John Kendrew visited me at this time and remarked: "Such density I would have liked to produce for myoglobin, but we failed". I felt flattered, but many thought, that it was unscientific "hocus-pocus". It required some years until refinement of protein crystal structures became accepted by the scientific community. Today it is routine in the process of structure determination, of course, helped by greatly improved computational tools, both hardware and software. The availability of interactive graphics systems and the development of a method and program for use in protein crystallography and its first practical application (Frodo) by Alwyn Jones,[9] who had joined me as a post-doc, was a key step in this process. The existence of an accurate model of a relatively small protein was noticed by the scientific community and BPTI became a model protein for experimental and theoretical folding studies and molecular simulations, the "lab rat" of computational chemists (a term coined by Michael Levitt) worldwide. The Bayer Company generously provided material for other users, in particular for nuclear magnetic resonance (NMR) experiments, a field, in which researchers had just begun to explore its potential for protein structure analysis. The identification of the aromatic spin systems in the homonuclear one-dimensional ^{1}H NMR spectra and their dynamics was a remarkable achievement by Wüthrich, Wagner, Sykes, and others before the later advent of two-dimensional spectra and systematic sequential resonance assignments through Ernst's and Wüthrich's work. It led to the observation that aromatic residues, apparently tightly packed in the protein structure, undergo ring flips. A detailed analysis in the light of the molecular structure, jointly by my and Wüthrich's research groups in 1976, offered unequivocal evidence for large-amplitude, low-frequency concerted motions in the protein core, invisible for crystallography, because the non-equilibrium excited conformational states are not significantly populated:[10] this was a big surprise for many, probably most crystallographers, who were likely prejudiced by the well-defined electron density maps. I remember that Wüthrich and I had to convince the disbelieving Max Perutz in an open discussion in front of a large audience. We now know that many—probably all—proteins examined, display aromatic ring flips. Also in the regime of slow dynamic processes BPTI served as a paradigm by comparing amide hydrogen exchange measured with NMR techniques and with neutron crystallography, a method able to discern protons and deuterons, and finding excellent agreement.[42,43]

Later, in 1986, a joint project with Wüthrich's research group with the objective of an independent and simultaneous analysis of an amylase inhibitor, tendamistat, in crystals and solution, showed identical structures demonstrating very convincingly the power of NMR spectroscopy as a tool for protein structure determination.[11,12]

BPTI, not unexpectedly, led me now to its receptor, the prototypic serine protease trypsin. We wanted to find out how the two proteins interact. Functional studies had shown that the binding is extremely strong and the complex very long lived.

We embarked first on a model-building experiment together with David Blow, Cambridge, who had determined the structure of chymotrypsin, a close relative of trypsin and also a strong ligand of BPTI. Docking in 1971 was performed quite differently from now (with the help of computer graphics) and required physically bringing physical molecular models, assembled form Kendrew-type wire and screw model parts, in contact. I therefore stored my BPTI model in the car and drove to England. A first barrier was the UK border, where the customs officer asked what this clutter of wires and screws may mean. "A model of a protein", my answer. "What is it worth?", his next question. "Nothing", I said. "How much did it cost?" "Several ten thousands of pounds, if we add costs for work force, material, and investment". He stared at me and obviously classified me in the category of harmless morons and let me pass.

Docking in David's lab in Cambridge was hard work, as my model, albeit being smaller than chymotrypsin, was heavy to rotate and shift for optimal fitting. Back in Martinsried we continued with the experimental structure determination of the trypsin-BPTI complex.

The stoichiometric complex crystallized easily and isomorphous phasing went smoothly to produce an easily interpretable electron-density map. The model essentially confirmed David's and my docking model.[7,13]

Wolfram Bode, who had joined my department in 1972 determined the structure of bovine trypsin offering the possibility, with all three species, for which the complex and the free components were available, to map structural differences. Apart from a few different side-chain rotamers, particularly of a lysine which is inserted into the specificity pocket of the enzyme, no structural differences were ob-

served, an exemplary "lock and key" model of protein–protein interaction, the first seen.

Quite naturally, we now wanted to learn more about the mechanism by which the inactive precursor trypsinogen is converted into the active enzyme trypsin. This process involves cleavage of an N-terminal hexapeptide to generate a new N-terminal isoleucine, as the biochemists Hans Neurath and Earl Davie had unveiled in 1955. David Blow had proposed that the formation of an internal salt bridge by Ile16 and Asp194 as seen in chymotrypsin and identical in trypsin may be a key element in this process. We crystallized trypsinogen and determined phases in 1976, but encountered a serious problem with the interpretation of the electron-density map after refinement, because we saw no trace of significant density for three (or four, when considering the N-terminal segment), peptide segments, which form a well-defined coherent domain in trypsin. This domain we termed activation domain for obvious reasons. In trypsin it is the major interaction surface with BPTI and substrate.[14,15]

Meanwhile, with the help of Peter Colman and Alwyn Jones, postdoctoral fellows at the institute, we had, in 1976, collected more examples of large-scale disorder, in particular one from an antibody crystal structure, for which the entire Fc part of 50kD was invisible.[16] We had proven the validity of crystallographic refinement and the betterment of phases and crystal structures assuring us that the absence of electron density in trypsinogen truly reveals a molecular property of disorder. In contrast, the use of raw phases by our competitors working on that same structure led to weak and fragmented electron density, misleading one to trace and to interpret it with an ordered model.[17] In both the antibody and trypsinogen, the transitions between ordered and disordered segments are sharply discontinuous and centered in glycine-rich amino acid sequences.

With the complete system in our hands, we built on the observation that BPTI also forms a stable and crystallizable complex with trypsinogen. This species is isomorphous to the trypsin PTI complex and has an ordered activation domain in the absence of the Ile16 N-terminus, whose activating function can be carried out by exogenous Ile-Val di-peptide to form a ternary complex, that is structurally indistinguishable from the trypsin–BPTI complex. These structures and thermodynamic data defined an allosteric system of a single subunit protein, the first to be discovered and analyzed. Max Perutz invited me to present these findings at a Conference on Allostery in the UK, which pleased me enormously.

Disorder may be dynamic or static or a mix of both, discernible by low-temperature crystallography. The trypsinogen crystals tolerated a high percentage of methanol, and this allowed structure determinations at very low temperature without formation of water ice. No significant electron density of the activation domain appeared after cooling, as expected for predominantly static disorder. But the question remained: how many different conformers are required to reduce the electron density to insignificant levels? We therefore labeled, by reduction and insertion, a unique disulfide located in the activation domain with a heavy mercury atom, but again saw no trace of it, indicating extensive heterogeneity; however, it appeared as a huge peak of the expected height after conversion into the trypsin-like state by complex formation with BPTI.[18,19] Further work by Eberhard Neumann on this system using elegant temperature-jump techniques showed the allosteric coupling between substrate-like ligand and N-terminal peptide binding by an induced-fit mechanism.

What we had first seen and mechanistically explained in the trypsin system, the disorder–order transition to generate a substrate binding surface and a proper active site configuration, was found to be generally valid in the serine protease family. Specifically, Wolfram Bode and I suggested in 1976[20] that the insertion of an N-terminal segment may be a general mechanism of activating co-factors in the trypsin-like serine protease family, which we coined the molecular sexuality hypothesis. More than twenty years later, in 2003, it has indeed been discovered and shown in atomic detail with a bacterial activator of the clotting factor thrombin (by staphylocoagulase, together with our US colleague Paul Bock[21]).

In the complex, multi-domain HtrA/DegP proteases, with a trypsin-like catalytic domain, different cues trigger ordering of the activation domain and oligomerization, namely ligand binding to a PDZ domain as shown by Michael Ehrmann and Tim Clausen. Thus the mechanisms of biological regulation are versatile, utilizing in diversified ways the basic principle of serine protease activation through the creation of a proper ligand binding site by disorder–order transition of the activation domain[22,23].

BPTI was not unique for long. More small natural proteinaceous serine protease inhibitors were discovered and structurally characterized.[24] Most of them obeyed the same mechanism of tight binding to the target protease through preformed complementary surfaces, following the 'lock and key' paradigm Emil Fischer had proposed to explain enzyme specificity more than hundred years ago. Other protease families were explored and natural inhibitors found, which block substrate binding with high specificity and potency and interact, at least partially, in a substrate-like manner. Some of them are larger than their protease targets and employ extended *exo*-sites to enhance binding, as we saw in 1997 in an exemplary way in the tissue inhibitors of matrix metallo proteases, the TIMPs.[25] Natural inhibitors of the clotting enzyme thrombin, however, featured unconventional binding modes, very different from substrates, but at overlapping sites, as we saw in 1990 in hirudin,[26] the main clotting inhibitor in leeches. In other blood-sucking parasites, in ticks, we discovered in 1996 a thrombin inhibitor with two BPTI domains arranged in tandem that does not bind by its canonical surface loop as defined in the BPTI-trypsin complex, but uses its N-terminus instead.[27] Nature has many tricks.

Thus the structures and mechanisms of protease inhibition seemed to display largely recurrent features, diversified by variable facets, all quite interesting and rewarding to study, especially in view of their pharmacological importance.

The serpins (serine protease inhibitor), however, presented a breathtaking surprise, a spring-loaded molecule. The serpins are abundant in mammalian blood where they control serine protease activity and comprise a populated superfamily of proteins, larger in size than most of their targets. They are

found almost ubiquitously in the living world, some with non-inhibitory functions. We had chosen to work with α1-antitrypsin (α1-PI), an abundant blood component, we set up crystallizations of a stoichiometric complex of α1-PI with chymotrypsinogen, but saw in the electron density map of the crystal structure (published in 1984) no trace of the protease.[28,29]

Instead, we saw only the inhibitor molecule, and its known reactive site peptide bond had been cleaved, with the resultant new N- and C- termini positioned on opposite poles of the molecule separated by 70 Å, whereby the C-terminal segment had been integrated as the central (antiparallel) strand in the dominant architectural feature of the molecular structure, a five-stranded β-sheet. This was indeed a breathtaking surprise for two reasons. Firstly, when compared with cleaved canonical small serine protease inhibitors, whose termini remain in close proximity, poised for re-synthesis by their cognate protease under special conditions: the cleaved α1-PI is completely and irreversibly inactivated as an inhibitor. Secondly, when considering the apparent reorganization of the protein architecture: the expansion of a β-sheet by the separation of an existing (parallel) β strand pair and insertion of a new strand was, as we suggested, the minimal structural perturbation required, but was unprecedented in scale. The transformation is associated with a dramatic stabilization against thermal or detergent-induced unfolding.

It took six more years to prove this mechanism by determining crystal structures of plakalbumin (together with Tonie Wright[30]), the proteolyzed form of albumin, a non-inhibitory member of the serpin superfamily, that does not undergo the transformation seen in α1-PI, and of albumin analyzed by others.[31] The observation of an unsatisfied peptide binding site in intact serpins reminded me on our experiments with trypsinogen and the activating Ile–Val dipeptide and we were indeed able to show that a peptide mimicking the inserted C-terminal segment induced transformation to a molecule with properties of cleaved α1-PI.[32] We suggested that this may be at the core of the process of self-aggregation in "serpinopathies". The discovery of a latent, intact form of a serpin, the plasminogen activator inhibitor, with the characteristic secondary structure of the cleaved species by Elisabeth Goldsmith in Dallas, US, and the multitude of structural and functional studies, initiated by Robin Carrell and his research group in Cambridge, UK, has added essential detail to the general mechanism. It is clear, however, that the β-sheet expansion discovered in our initial studies is at the heart of the multifaceted structural and functional properties of the serpin superfamily.

With these reflections on my beginnings with protease research and a glimpse at my apprenticeship as protein crystallographer I will end, but not without adding that proteases and their regulation remained a focus of the work of my research group. The proteases studied became more complex and much larger, aided by the novel tools and methods available comprising all aspects of the workflow of protein crystallography, protein preparation, crystallization, structure determination, functional interpretation by biochemical and biological analysis, to some of which we had made useful contributions as mentioned earlier.

These encouraged us, often in collaboration with colleagues, to embark on work on the very large intracellular proteases, multi-subunit assemblages, such as the proteasome, the bacterial HslVU, tricorn, and DegP proteases.[22,23,33–36]

My research group had gradually become large, crowded with doctoral students who frequently came with well-defined ideas on their preferred research.

By them and through collaboration with academic and industrial colleagues and partners many new projects and biological systems were introduced to us, broadening our expertise and knowledge in structural biology and contributing to a basic understanding of structures and functions of a wealth of protein systems.

I shall mention as particularly noteworthy the study of proteins with a central role in photosynthesis, the light-collecting organelles, the phycobilisomes of cyanobacteria,[37–39] and the photosynthetic reaction center of a purple bacterium, the biological photocell, and a membrane protein,[40,41] work that contributed essentially to an understanding of the physics underlying the primary photosynthetic light reaction and charge separation and revealing basic principles of the structures of integral membrane proteins. It was honored with the Nobel prize in chemistry in 1988, which I shared with Deisenhofer and Michel (Figure 4).

Figure 4. Nobel Prize in Chemistry 1988, Hans Deisenhofer, Robert Huber, Hartmut Michel.

I was lucky to choose protein crystallography as the focus of my research activity at a time soon after its foundation by Perutz and Kendrew in Cambridge, England, and to escort it while it matured and to note its generally recognized, fundamental role in life sciences and our understanding of biological processes. The role of protein structures in medicine and crop-plant science and their use in discovery, design, and development of active substances for therapy and diagnosis and for plant protection fascinates me greatly, as most of the proteins we studied play indeed well-defined roles in health and disease of man and plants. My own beginnings in protein crystallography were with Bayer's protease inhibitor and drug Trasylol, as said earlier.

The development of valuable therapeutic drugs, however, exceeds the scope of an academic institution. I was open therefore for collaborations with industry, fruitful for both sides, as long as the different interests and missions and the legal constraints are adequately respected. A special and intimate contact was established with Boehringer, Mannheim, later Roche, by the foundation of a joint research group headed by Richard Engh and domiciled in my institute.

It was exceptionally fruitful and is documented in more than 40 joint publications on kinase and protease structure and function, that were in the focus of interest of both sides. To further extend translation of academic research, I founded together with collaborators and colleagues two companies, Proteros (http://www.proteros.de) and Suppremol (http://www.suppremol.com), both located in Martinsried, whose business plans were initially based on research methods and projects of my research group and have been developed successfully in the industrial setting.

I apologize to all unattributed colleagues and collaborators, many of them holding academic positions, without whose essential contributions and enthusiastic work most of the research projects mentioned, would not have been begun and successfully completed. They can be found, however, as authors in the original publications of my Department (http://www.biochem.mpg.de/xray/publications/index.html). I am most grateful for the expert help of Richard Engh and Matthias Bochtler with text and language of the manuscript.

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