

Notes of a protein crystallographer: my nights with ACTOR

Cele Abad-Zapatero

Department of Structural Biology, Protein
Crystallography Laboratory, Abbott
Laboratories, 100 Abbott Park Road,
Abbott Park, Illinois 60064-6098, USA

Correspondence e-mail: cele.abad@abbott.com

It would have appeared to be an impossible dream in the late sixties, when the field of protein crystallography first became established, just as the structures of myoglobin and hemoglobin were first unveiled. Even if somebody would have thought about it in the early seventies, when protein crystallography was 'coming of age' (Cold Spring Harbor Symposium, 1971), it would have still seemed utterly impossible or even a miracle. Yet, only 30 years later, here I am late at night putting a protein crystal in front of an extremely brilliant X-ray beam using a computer-controlled robot to mount, center and expose a protein crystal in front of the X-rays emanating from a third-generation synchrotron source. My diffraction data from this crystal will be collected in approximately 15 minutes, processed, as it is being collected, reduced and scaled a few minutes later. Things going well, the three dimensional structures of the protein and various ligand(s) within these crystals will be unveiled tomorrow and presented and discussed with chemists, biologists, pharmacologists rapidly. The detailed analysis will aid and direct the synthesis of new compounds for several drug-design projects. In a year or so, after several iterations of the process, the resulting optimized compound with the most suitable pharmacokinetic properties could be selected for clinical testing. After rigorous clinical studies, it might become one of those rare chemical entities with a therapeutic effect on a specific patient population.

Am I talking science fiction? Certainly not. This is the current state of macromolecular crystallography in certain industrial laboratories and other general user facilities at synchrotrons, and this *modus operandi* will soon spread to a sizeable portion of the laboratories around the world. How did this all happen? Who were the people involved in these amazing technological advances? What are the implications of these developments for the future of the field?

There was a sequence of modest, somewhat unrelated changes in the way that the macromolecular crystals were handled that has turned out to be of tremendous importance. A little bit of historical background is necessary to put this in perspective. Besides having read about it in the textbooks, the new generation of protein crystallographers may never have mounted a protein crystal in a glass or quartz capillary, the way it was done in the past. This was the critical observation by Bernal and Crowfoot in their 1934 *Nature* paper (Bernal & Crowfoot, 1934). Protein crystals needed to be in equilibrium with their 'mother liquor' in order to retain their internal order and to diffract X-rays, and reveal their atomic order. This translated into an elaborate crystal-mounting protocol. The crystals were 'sucked in' with a syringe or by an elaborated suction apparatus that was completely personal to the crystallographer, containing a mouthpiece, rubber or plastic tubing and a quartz capillary at the end. Sealing wax was carefully laid out on both ends of the quartz capillary prior to the suction step. For some precious seconds the crystal was seen through the microscope in the spot plate and then disappeared into the capillary, floating within a minute amount of mother liquor. The crystal was separated from the mother liquor by various tricks (*i.e.* further suction, drying the surrounding liquid with filter paper *etc.*) and eventually the crystal and mother liquor were sealed and exposed to the X-rays at room temperature. X-ray sources of the time began with sealed X-ray tubes and developed with different types of rotating anodes.

I briefly review the process for two reasons. First, it was tedious and elaborate, and second it was also eminently personal. The tools were individual and the different steps had a unique personal character that varied widely among different crystallographers. I have never seen Max Perutz mounting a hemoglobin crystal but I am quite certain that he did it somewhat differently from the way Dorothy Hodgkin, David Blow or even 'Sage' (*i.e.* J. D. Bernal) mounted the crystals of their favorite proteins. Probably they all had

their own set of utensils and used them in a unique way to break off the crystals from a cluster or to manipulate and control them prior to the final mounting step. There was also the 'supreme moment' when that precious crystal disappeared from the center of the circular drop viewed through the microscope, and it was hoped that it would reappear in the capillary, immersed in a small amount of liquid. In one of those inexplicable connections of the human brain, at least mine, I have often associated all these events with the losing battle of the bull in the bullring. The bullfighter using his cape and other instruments to move the bull around in the ring and followed by the 'supreme' moment of the toreador in the middle of the ring, when after having had a deadly dance with the bull in the circle, faces the bull eye-to-eye for the ultimate sacrificial moment.

All this agony was prevented when the first description of the cryoloop mounting method was presented by Teng (Teng, 1990), using small wire loops to immerse the crystal in a free-standing liquid film. There had been earlier insights and developments in the area of cryoprotection of protein crystals and data collection at subzero temperatures. Haas and Rossmann (Haas & Rossmann, 1970) used a combined solution of sucrose-ammonium sulfate to freeze crystals of lactate dehydrogenase in liquid nitrogen and collected three-dimensional data at 3.5 Å resolution. They were able to show that the rate of radiation decay decreased by tenfold and that the frozen crystals were isomorphous with the native unfrozen crystals. Further work by Petsko extended the method to other proteins using glycerol as cryoprotectant (Petsko, 1975). Hope later used methods for cryoprotection involving transferring the crystals to inert oil (or propane) with subsequent freezing in polyethylene glycol using a glass fiber (Hope, 1988) for mounting. For the extremely fragile crystals of ribosomes, Hope, Yonath and colleagues (Hope *et al.*, 1989) crafted minute glass spatulas to freeze (85 K) and mount the delicate crystals of ribosomal particles achieving three-dimensional data collection to 4.5 Å at synchrotron sources. The immersion of protein crystals in small wire loops, suggested by Teng (1990), was an amazingly simple and effective way of 'scooping' the crystal from the cryoprotectant solution to the X-rays bathed in a stream of liquid nitrogen. There is still some of that agonizing moment when crystal flies from the cryoprotectant drop onto the goniometer head, cradled in the rayon loop, but the process is so much simpler and effective that it actually almost never fails (Watenpaugh, 1991).

These modest advances paved the way to the automatic mounting, centering and exposing that we see nowadays all handled by computer-controlled robots. The most significant steps of this development took place in our laboratory, at Abbott Laboratories, driven by our interest in rapid data collection to screen compounds by crystallography (Nienaber *et al.*, 2001). Not being an instrumentalist, I followed the developments from the sidelines but I did participate in the testing of the prototype version using crystals of the human protein tyrosine phosphatase 1B (PTP1B) in one of our first unassisted 24 h runs and the ensuing testing of the system.

It is useful to break into different steps the technical and engineering challenges that Jeff Olson, Ron Jones and Jeff Pan faced when the project was first discussed with the engineers at Abbott Laboratories by Jonathan Greer, Vicki Nienaber and Steve Muchmore around November of 1997. The following goals were set at the outset.

1. Fully automated solution for mounting and centering cryosamples onto the X-ray apparatus goniostat.
2. The crystal should always be maintained at cryo-temperatures and protected from ambient air and humidity.
3. Enable hands-off around-the-clock operation.
4. Capacity for at least 60 samples for an effective long-term run.
5. Fully automatic centering and handling.
6. No compromise in data quality.
7. Simple user interface.
8. Reliable and robust for continued unassisted operation.
9. (Extra credit) Safe recovery of crystal after data collection for subsequent re-analysis.
10. Timeline: spring 1998 delivery – later revised to August 1998.

According to Jeff Olson's own recollections. 'All this was a little intimidating as we had no prior background or experience with X-ray crystallography and some of us had never even worked with liquid nitrogen before'. Nonetheless, the project was started.

A multitude of concerns surfaced right away on the mechanical side that had to be solved. How would the crystals be stored in an automation-friendly manner? How would we protect the crystals from the ambient atmosphere during transfer to and from the goniometer head? How could we track the crystal during the centering routine? How could we reliably grasp the crystal holder while it was immersed in liquid nitrogen? Should we use a commercially available robotic manipulator arm or design our own 'task-specific' robot?

There were a few key insights during the design process that eventually permitted the team to design a working system. Steve Muchmore demonstrated how a cryoloop-mounted crystal could be maintained under a protective layer of liquid nitrogen throughout the transfer process. Taking a cue from these tools, it was decided that the safest way to protect the crystal during transfer was to totally surround it in a protective, high thermal mass, metal cocoon. The 'task specific' *versus* multipurpose commercial robot question was settled in favor of the multipurpose commercial solution.

There were also a series of debates about the best way to sense the position of the crystal with reference to the X-ray beam during the centering process. Eventually it was decided to exploit the high-quality video microscope already built into the Mar base. If an effective image processing algorithm could be devised, the manual centering process could be mimicked by the automation. The simplest and most reliable images were produced by backlighting the crystal with a diffuse light source. This meant that the crystal could not be distinguished from the mounting loop and so it was agreed that the size of the cryoloop would be matched as closely as possible to that of

the crystal. Later, optical techniques that could distinguish the crystal relative to the loop would be investigated.

All these concepts formed the basis of the prototype design that was first demonstrated in the Automation Engineering Laboratory at Abbott in July 1998. The first successful operation resulting in good diffraction data took place in the Protein Crystallography Laboratory around September of the same year and the first round-the-clock run with 18 crystals successfully analyzed over a 42 h run was in November 1998. The achievement was presented at the ACA Meeting in July 2000 (Olson *et al.*, 2000) and published in *Structure* soon thereafter (Muchmore *et al.*, 2000). The technology was licensed to Rigaku/MSU in May 2001 and the commercial implementation of ACTOR (Automated Crystal Transport Orientation and Retrieval) received an R&D 100 Award for 2002. Other engineering implementations of similar technology are now available at various synchrotron beamlines for protein crystallography (Cohen *et al.*, 2002; Karain *et al.*, 2002; Snell *et al.*, 2004; Pohl *et al.*, 2004).

Engineers can always go back and think about what could have been done better but we can leave those things aside. For Jeff and other people involved in the project, ACTOR was one of the most enjoyable and rewarding projects that they have participated in at Abbott. It demonstrated once again how a complex human task could be performed by a computer-controlled instrument in a reliable and effective manner. For our community at large, it is an amazing engineering achievement. After the initial growing pains it will expedite our data collection, increase the productivity of our facilities and will permit a faster solution of ever more complex structures.

The engineering achievement described above is only one of the developments in the field that is giving the sense that the old skills are not needed anymore. Some might argue that old-fashioned crystallographers are becoming redundant, obsolete or even plain unnecessary and superfluous. I would rather think that they have now been freed to utilize their hard-won skills in more imaginative and productive ways. All these thoughts come to me in one of those long nights at the APS (17-ID, IMCA-CAT) when our ACTOR was mounting, centering, exposing, collecting and dismounting crystals with predictable and reliable motions and gestures; like a reliable old friend. Almost like a rigid mime, a dancer without music, only accompanied by the sounds of levers and mechanical actuators and the huffing and puffing of the liquid nitrogen passing through valves and filling reservoirs. ACTOR will perform those required tasks obediently and flawlessly even performing an annealing cycle of the mounted crystal without me having to move from my chair outside the hut.

Like in any other technological advance, there were other gloomier nights when both ACTOR and I had to learn to talk to each other and to trust each other. In those earlier nights, I had to learn about his vocabulary, his moods and idiosyncrasies, pauses and unexpected interruptions. I also needed to observe his one and every move and document his flaws so that his behavior could be corrected in future encounters.

From what we learned from each other in those sleepless nights, ACTOR has now become a true friend.

I also had to learn to be more trustworthy of my new friend; to give him a vote of confidence. I soon learned that I could allow this complicated piece of machinery to get close to my valuable crystals stored frozen in our 'pucks'. That I could abandon into his anonymous mechanical hands those precious crystals that I would have otherwise carefully, even tenderly, mounted and aligned myself in a few minutes. Thus the questions arise. What will robot technology bring to macromolecular crystallography? What will be the implications of the widespread use of this technology in the field? What doors will this unexpected technology open? How will it affect the future generations of students?

Some ramifications are easy to predict: higher throughput of samples and structures; faster screening of crystal quality, which will be critical for some of the more difficult membrane protein structures; more efficient use of synchrotron beam time and the ability to tackle the massive number of structures of the upcoming structural genomics projects. But what will be the effect in more unpredictable areas? What will be the impact on the student population that perhaps in a generation or so will take automatic crystal manipulation for granted? I will not even dare to make any further pronouncements. However, I will say that these new tools should allow us to concentrate on what lies ahead: the development of novel experimental designs that would permit us further insights into the molecular workings of the cell. Experiments that will allow us to explore by X-ray diffraction, scattering, spectroscopy, fluorescence or any other means the order and structure of even larger and more complex macromolecular aggregates. In addition and in a foreseeable future, they could also open a window into the subtleties of the transient interactions of the different molecular components of the living organisms, into the cell's most ephemeral 'dissipative structures'. Time will tell and we will all have to thank ACTOR and its creators for it.

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