Adventures in cooperativity

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Abreviations: Hb – haemoglobin; MWC – Monod-Wyman-Changeux; KNF – Koshland, Nemethy, Filmer; PEG – polyethylene glycol; PKA – protein kinase A; ATP – adenosine triphosphate; AMppNp – adenylylimidophosphate

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ABSTRACT

Macromolecular X-ray crystallography has undergone a dramatic and astonishing transformation since its inception in mid 1950s, almost exclusively owing to the developments in three other fields: computer science; synchrotron radiation; and molecular biology. The process of structure solution from a single crystal, provided the quality of diffraction data is adequate, has been shortened from many years to hours, if not minutes. Yet, in spite of the exponential increase in the available structural information (~120,000 structures in the Protein Data Bank today), many fundamental problems continue to be the subject of scientific controversy. This article contains personal recollections of the author, pertaining to two research projects – conducted nearly four decades apart – both of which touch upon such long standing discussion of the Monod-Wyman-Changeux theory of cooperativity (or 'conformational selection') *vs* **the Koshland-Nemethy-Filmer theory of 'induced fit'. It is dedicated to Dr. Alexander Wlodawer on his 70th birthday, with best wishes of continuing success.**

INTRODUCTION

It is a pleasure and an honor to be a part of the celebration of Dr Alexander Wlodawer's birthday. I met Alex for the first time in August 1978. The event was the 11th IUCr Congress in Warsaw, the first 'serious' scientific conference I ever attended. A lot has happened in the 38 years that elapsed since, and I thought it might be entertaining on this occasion to share personal reflections on how far we have come in protein X-ray crystallography with respect to methodology, and how this progress is intertwined with the evolution of some fundamental concepts in structural biology. Alex has been at the forefront of many advances, and I hope that this article will bring back happy memories.

The story of how exactly I found myself at the IUCr Congress in 1978 is complicated. Suffice it to say, that just over a decade earlier, in 1967, a certain Mr. Dennis O. Overman, a 22 year old science teacher (and a Peace Corps volunteer) in the Community High School in Teheran, Iran, wrote the acronym 'DNA' on a blackboard and went on to explain to his 9th grade class what it meant. I was one of the students. The textbook we used had a precession photograph of the X-ray diffraction pattern of myoglobin, obtained by John Kendrew in the 1950s. Somehow, the notion that life processes can be investigated at the level of chemical molecules and atomic structures appealed to me. Two years later, on a shopping trip with my mother to a local supermarket on Takhte Jamshid Street (now Teleghani) I noticed a small paperback on a bookstand with something resembling DNA on the cover – this was the first paperback edition of James D. Watson's *The Double Helix*. I devoured it, and so at the age of 16 I knew what I wanted to do…

I returned to Poland in 1969, graduated from high school and went on to enroll in a Biology Master's program at the University of Lodz. The short and superficial encounter with the concept of protein structure was still on my mind. In 1975, my friend Marek Brzozowski (now a Professor at York University, U.K.) and I, had to choose courses for the upcoming year of our studies. Crystallography caught my eye, as I recalled *'The Double-Helix'*. Marek was fine with this suggestion. Unfortunately, the lectures by Prof Mieczyslaw Grabowski seemed totally disjointed from our expectations – he spoke of symmetry and space groups (totally alien vocabulary), while we had hoped for insights into protein structure. Disappointed, we stopped attending, but at the end of the semester I had to make an appointment to get a signature to earn the academic credit. This meeting with Prof Grabowski earned us jobs as assistants in the lab the following year: he was intrigued why two biology students were interested in a field that in his perspective was related to mineralogy, and this discussion led him to give us an opportunity to work on our own project. My first desk was in a room housing a small mineralogical museum, overlooking the entrance to the building,

which today houses a Guesthouse of the Lodz Jewish Faith Commune. The notion of pioneering protein crystallography in Poland in the 1970s, amidst strikes, food shortages, and generally tough life behind the Iron Curtain was – at best – insane, but we did not really understand what we were doing. Fortunately, no one tried to change our minds, and some good people even decided to help us. We were joined in our efforts in the Department by another student, Tadeusz Skarzynski (currently with Rigaku Corporation). We thought that trying to crystallize haemoglobin might be a good start. We really had no hypothesis for any project – we just thought we should try to crystallize some protein and play with the crystals.

HAEMOGLOBIN

Haemoglobin was the obvious choice: it was the only protein potentially available to us in large amounts (we could purify human Hb from out-of-date blood from the local Blood Bank, or go with a bucket to the local slaughterhouse and get tens of liters of fresh bovine blood). The Department of Biophysics at the University of Lodz (where we were students until 1977) had an active research program on the impact of ionizing radiation on erythrocytes and Hb. Aside from the fact that one could easily get good amounts of it, Hb was the canonical, nearly legendary protein, only the second one characterized by X-ray diffraction, mere fifteen years earlier. It offered a bridge between structural chemistry and the new, exciting field of molecular biology and biophysics. Shortly after receiving his Nobel Prize in Chemistry in 1962, Max Perutz published a low resolution study of the structure of horse deoxyHb, and its comparison with the earlier structures of human deoxyHb, and horse oxyHb (which was actually the oxidized form, metHb) [1]. The ultimate conclusion was that '*the structural transformation accompanying the reaction of haemoglobin with oxygen may now be considered as proven*.' In 1965, a paper by Monod, Wyman and Changeux described the theory of allosteric control, using a model of the T(tense) and R(relaxed) structural states of Hb, and argued that cooperative binding of oxygen can be explained assuming an equilibrium between the two, such that binding of oxygen shifts the equilibrium from the low affinity T-state to high affinity R-state [2]. No 'communication' between the subunits occurred in response to oxygen binding. A year later, Daniel Koshland,Jr. along with G. Nemethy and D. Filmer, published an alternative model, known as the 'sequential' model, according to which binding of oxygen in one subunit was 'communicated' to another and consequently increased the latter's affinity for the ligand [3]. Following several additional, higher resolution analyses of horse and human Hb in two different states of ligation [4-7], Perutz finally proposed a fairly detailed structural mechanism for cooperativity [8], drawing on both theoretical models. Specifically, he wrote:

"*Monod et al. assumed all the subunits in the quaternary T-state to be in the unreactive and those in the quaternary R-state in the reactive form, no matter whether they were liganded or not. In their discussion, they envisaged the possibility that this assumption might prove too simple and this is indeed the case. The haemoglobin subunits change their tertiary structure in response, not to the change in quaternary structure, but to the binding of ligand, as predicted by Koshland's sequential model.*"

The cascade of structural reorganization was inferred from the two end states, and the trigger was thought to be the iron atom, which changed its spin form. Here is Perutz's closing paragraph:

"*It is remarkable that there should be such an exceedingly complex, subtle and elegant instrument of respiratory transport, exploiting a difference in atomic radius of 13 per cent between the covalent and ionic forms of iron.*"

This was more or less the state of affairs when we embarked on our naïve efforts to start protein crystallography in Poland. After many failed attempts to reproduce any Hb crystals reported by Perutz, Marek Brzozowski found out a publication that had come out in 1975 in the Journal of Molecular Biology, reporting crystallization and a low resolution structure of deoxyhaemoglobin from polyethylene glycol (PEG) [9]. This was the original use of PEG in crystallization; the paper by Alex McPherson which made PEG a popular crystallization precipitant followed in 1976 [10]. Fortunately, PEG was available in our laboratory, but we had no glove box to crystallize Hb under nitrogen, so we mixed Hb solution with PEG 6000 in large glass test tubes and waited to see what happens. This was the so-called batch crystallization method, originally used in the 1930s, which required no special glassware and large amounts of material. In our earlier attempts we tried many approaches, including the Zeppezauer microdialysis cells [11]. We were completely unaware of the fact that several years earlier the sitting and hanging drop methods were introduced to reduce the size of the experiment [12]. One of the early users of the hanging drop protocol was Alex Wlodawer. His 1975 paper describing this strategy in the crystallization of monellin [13] is still cited as the pioneering example of this technique.

Huge crystals grew in our test tubes within a day. But even more dramatic was the fact that the solution (and crystals) turned purple, a hallmark of deoxyHb, even though the solution was exposed to air. It took us months to discover that PEG dramatically reduces partial pressure of oxygen in aqueous solution, and Hb turns into deoxyHb. This took a while, but upon vigorous shaking of the test tube the solution was aerated and turned red again (the limited solubility of O_2 in PEG solutions was a fact that was established at the time [14], but without Google and PubMed we had no way of knowing this; in fact, I had found the reference provided here while preparing this account). Harvesting the crystals was another challenge: everything was done on a macro scale as the crystals were millimeters in length, and so we tried to pour out the contents of the test tube onto a Petri dish (having first shaken the tube harshly to dislodge the crystals), and manipulate the crystals with a dissecting needle. Unfortunately, once the solution with the crystals was poured out, it slowly turned red and the crystals began to liquefy (oxyhaemoglobin is more soluble than deoxyHb). After a few failed attempts, Tadeusz – in desperation – did something that had a profound impact on our future lives: he grabbed a flask with the 50% stock solution of PEG 6000

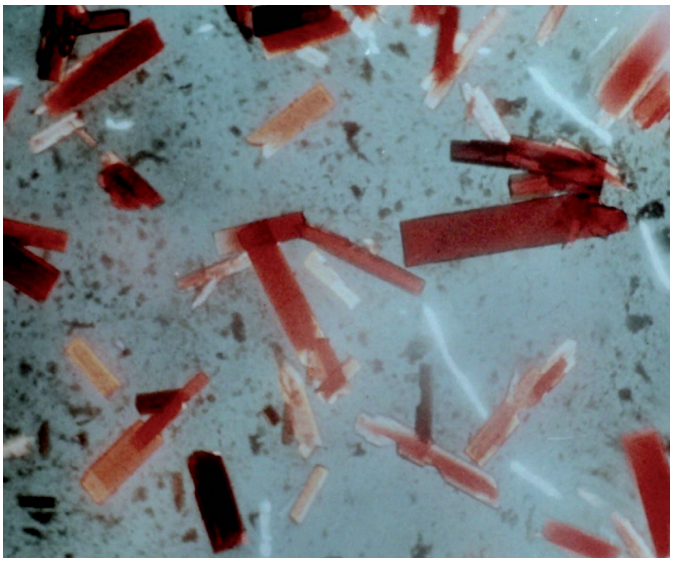

Figure 1. A micrograph of the Hb crystals fixed by the addition of concentrated PEG. Note the varying red and purple colors.

(twice the concentration of the crystallization solution), and poured it directly over the crystals. This prevented the crystals from liquefying and we now had stable crystals to work with (much later we found out that this procedure resulted in ~15% loss of solvent from the crystals, rearranging the packing and stabilizing the T-state; dehydration is now a standard post-crystallization protocol). The only issue was that they were not exactly purple, and some were outright red (Fig. 1). This was to be addressed later.

First, we needed to obtain diffraction. Prof. Grabowski insisted – against our muted opposition – that a crystal be dried and mounted on a pin with the use of Canadian balsam. Not surprisingly, there was no diffraction. We had read – of course – about the mounting technique of protein crystals using quartz capillaries, but we had none. This caused a major delay but we finally found a glassblower who could make some. The laboratory at the Department of Crystallography was equipped with a couple of ancient Micrometa sealed tube X-ray generators made in Czechoslovakia, which produced the weakest of beams from a Cu anode Ni-filter. There were a couple of old Weissenberg cylindrical film cameras installed (the term is probably alien to most readers – it was a contraption to record specific planes of reciprocal space for well diffracting, small molecule crystals, with the crystal rotating through an angular range). Because the crystal to film distance was only 28.6 mm, there was no chance we could use it for a protein sample. So we asked our technician (Mr. Marek Babski), to make a flat round film cassette, with a stand that could be screwed onto the platform on which the Weissenberg stood, some 10 cm away from the sample, thus replacing the cylinder with a flat film. This became known as the DBS attachment (after Derewenda, Brzozowski, Skarzynski). We were now able to expose the crystal, mounted in a glass capillary sitting on a goniometer head, while it rotated through a narrow range of ~2–3°, controlled by crude mechanical switches. We knew about the oscillation geometry from literature – it was used early on by Max Perutz. In this way, after a ~48 hr exposure – we finally saw diffraction. The next step involved analyzing these films on a light box, indexing by hand (with clear morphology and an orthorhombic symmetry we could align the crystal almost perfectly by eye), and evaluating the 2θ angles. We were able to feed this information (from 25 reflections) into a program that refined the unit cell parameters from powder diffraction data. It took several weeks to find out that within 'experimental error' – whatever that might have been – the crystals were isomorphous with those of deoxyHb described by Ward *et al* [9]. That was in 1976 – 40 years ago.

At this moment, for the first time, we were actually pursuing a 'hypothesis driven' project: what if the crystals were actually made up of some physiologically relevant form of oxyHb, but in a T-state? We had an idea that we should publish our 'accomplishment', but we needed a decent diffraction pattern, preferably using a precession camera. We did not have one, and we heard there were only two places in Poland where we could find them – one was Gdansk, 300 km from Lodz, on the Baltic coast. To cut the long story short, we visited Gdansk (this is how Zbigniew (Zbyszek) Dauter and I met), and under the cover of darkness we moved the Enraf-Nonius precession camera to the trunk of our friend's car evading security, and left the campus of the Gdansk Polytechnic in haste. A precession photograph of the *h0l* zone was indeed prepared soon after figure 2 and a paper describing crystallization of oxyHb passed the muster for *Journal of Biochemistry* [15]. It reported the crystallization of human oxyhaemoglobin.

BLAZING THE TRAIL

The IUCr Congress in Warsaw in 1978 created very rare opportunities. An event in a country behind the iron curtain was interesting, and many people were interested in making the trip. We used this to invite scientists that otherwise we would not be able to meet. Hillary Muirhead, who worked with Max Perutz on the liganded structure, accepted our invitation to visit Lodz before the Congress. This

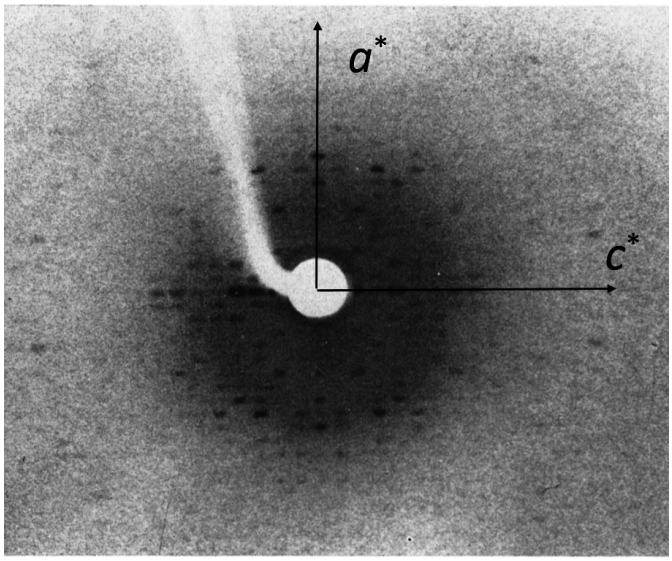

Figure 2. One of the first precession photograph of our suspected oxyhaemoglobin crystals, taken in Lodz with the 'borrowed' camera from Gdansk. The crystals belong to $P2_12_12$ space group symmetry with the unit cell a = 96.66, b = 97.88, c = 65.40 Å. The *h0l* zone is shown in the photograph.

was an exciting prelude to the Congress, although Hillary was not particularly enthused about our results. We went to Warsaw in August. We were finally part of global science – how wonderful. Alex Wlodawer was there, only two years after his paper that validated synchrotrons as a good source of X-rays for crystallography [16]. I have no recollection if the subject came up during the conference – I would not have understood the significance anyway. But we were very excited about the meeting with Simon Phillips from LMB in Cambridge, who had a poster with the X-ray structure of oxymyoglobin at 2.0 Å resolution (published earlier that year in Nature [17]) revealing for the first time the end-on bent stereochemistry of the Fe-O_2 bond. This will become important later.

After the Congress, Ted Baker from New Zealand visited Lodz, invited by our senior colleague, Mirek Cygler. Ted Baker had worked formerly with Dorothy Hodgkin and Guy Dodson on insulin in Oxford. We showed him our strange looking Hb crystals and he offered to ask Guy, who had recently established his own group in York University, if he could help us with further work.

Sometime later we received a letter and an invitation from Guy who managed to obtain financial support for a visit in the form of 200 GBP from the British Council, for a three-month visit to York. The aim was to bring our crystals and collect data on a 4-circle Hilger-Watts diffractometer that Dorothy Hodgkin gave Guy when he moved to York. I do not remember how it happened that I was chosen/selected to go – I guess my command of English had helped. When the time came, I mounted a dozen Hb crystals or so in quartz capillaries, sealed with plasticine, and set off for York (oh, I forgot about the lengthy process of obtaining a government service passport and an equally arduous task of procuring a UK visa).

I did stay three months in York, and this was only feasible as Guy and Eleanor offered me a room, although the logistics got complicated after window cleaners stole more than half of my entire stipend. Fortunately, Eleanor fed me, Guy gave me a bicycle, and it all worked out. I had marvelous time, and spent countless hours working on the Hilger-Watts, trying to collect 3.5 Å resolution data from my crystals. The protocol was to collect a 100 reflections and then 3 reference reflections to monitor decay. I recall that I was puzzled that the reference reflections did not decay uniformly: some intensities went up, while some went down. Guy and I hypothesized that specific structural changes are caused by radiation damage, but there was no way to find out as the 3.5 Å dataset took about a week to collect. Many years later Zbyszek Dauter and others demonstrated the essence of the process [18].

I recorded two sets of data, and with the help of Eleanor Dodson I managed to use the Molecular Replacement method to solve the structures. In a 'pioneering' way we showed that one could use only one half of the tetramer (i.e. the α/β dimer) as a search unit, and thereby determine the quaternary structure [19]. Eleanor Dodson and I calculated difference maps for one of the data sets and we did notice considerable noise around the α-heme group. Guy was very enthusiastic about this development, called Max Perutz at the LMB and arranged for all three of us to drive down in Cambridge. I recall that I was unable to sleep the night before the trip. It was only 12 years earlier that I had read *The Double Helix* and I remembered the famous photo of James D. Watson and Francis Crick in front of King's College Chapel in Cambridge; now I was heading there myself. Max turned out to be very nice to me and looked at our maps with excitement. I remember his words clearly: 'Perhaps you have what all of us having been looking for all these years!'. He was alluding to the elusive transient form of Hb that was oxygenated, but retained the T-state. Sometime after this visit to LMB, but before I returned to Poland, Guy and I had a conversation in which he stressed that higher resolution data were needed to resolve the dilemma of the noisy maps. Guy had deep understanding and appreciation of crystallographic techniques, much of which was learned from Dorothy Hodgkin, and he did not trust off-the-cuff interpretations of blobs in low resolution difference electron density: he wanted a refined atomic model. But how could this be done? Guy asked: 'Would you like to go to Paris and collect X-ray data using synchrotron radiation?' The year was 1979. Guy had heard about the possibility of using synchrotron radiation from Dorothy Hodgkin, who herself had only second hand information. Ken Holmes had published his paper on the use of synchrotron radiation in biological research, based on his work at DESY in Hamburg, in Nature in 1971, [20-22] but crystallographers did not pay much attention until few years later. In 1975 experiments were carried out using the Stanford synchrotron and the results were encouraging [16]; it is in those experiments that Alex Wlodawer played a crucial role. Hard to believe – but this was a controversial issue at the time. (This is another example of an achievement never given due credit – only 69 (sic!) citations). Another place were such work was ongoing was Laboratoire pour l'Utilisation de Rayonnement Electromagnetique (LURE), outside of Paris [23]. Anyway, I did not know what a synchrotron was – so I said, yes, I would like to go to Paris.

But that was to be in the future; on my return to Poland in 1979 I recall packing tens of kilos of computer printer output into my suitcase. Somehow I pleaded and no charges were levied by the Polish Airlines. Once back, I set to write a series of FORTRAN 77 programs to calculate three dimensional Fourier maps. This required punching cards for each reflection, and then several days of reading them and moving the data onto magnetic tapes (we have not had any disc memory on the ODRA 1305 computer, an ICL clone with 64K memory. The electron density maps were generated on a line printer and Marek painfully contoured them by eye using a marker (Fig. 3). He analyzed both sets of data and his conclusions (as translated by me from his Polish PhD thesis) read:

"The analyses of the electron density maps calculated in this study and comparison with those of Derewenda, prompted me to hypothesize that the iron Fe^{2+} atoms in the α1 and α2 haem groups in the K1 form are liganded, and most likely bound to molecular oxygen O_2. The β_1 and β_2 haem groups in the K1 form, as well as both α-haems and both β-haems in the K2 form are not bound to a ligand."

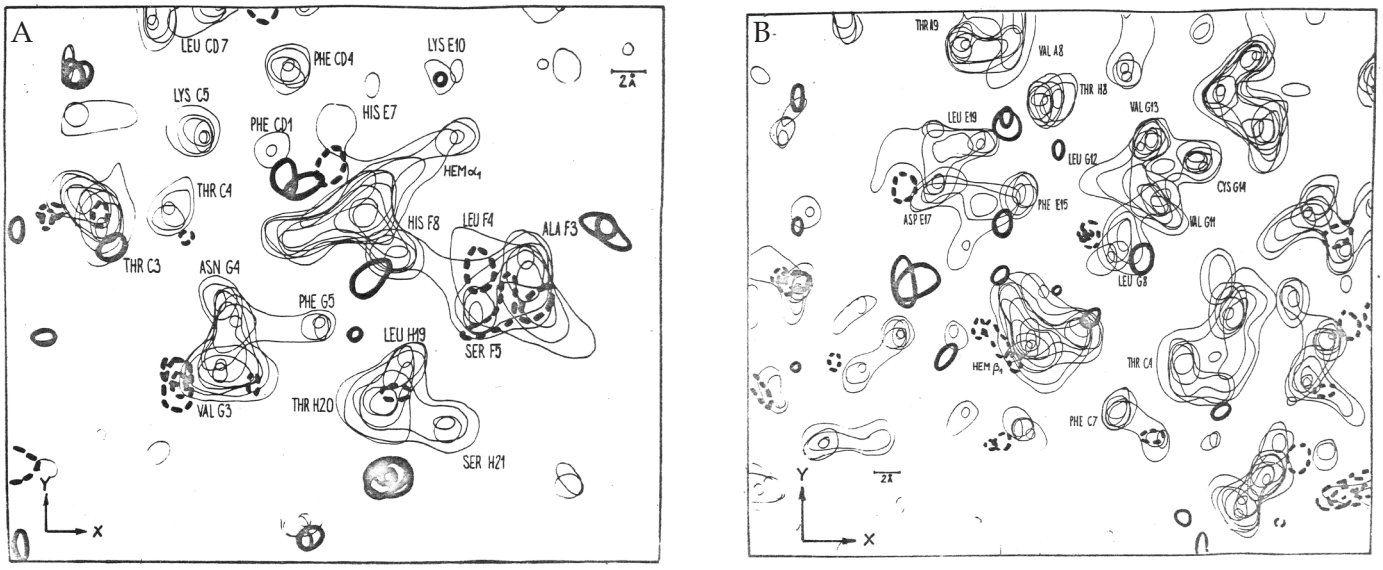

Figure 3. Hand-contoured $2F_{(obs)} - F_{(calc)}$ electron density maps, with overlaid difference maps, around the (A) α1-haem and (B) β1-haem. The thin lines are contours of the $2F_{(obs)} - F_{(calc)}$ map; thick lines are positive contours of the $F_{(obs)} - F_{(calc)}$ map and thick broken lines are negative contours. Taken from Marek Brzozowski's PhD thesis, Lodz University, 1980.

These proved to be prescient words. In the meantime, on Guy's suggestion, I applied to EMBO for a travel fellowship and with Max's support I got it. Guy knew Roger Fourme, the French pioneer of the use of synchrotron, made a phone call and arranged for me to go to Paris in 1980. I first had to come to York, and grow crystals. However, they were all purple and I was very upset. (One of the crystals I grew in the batch mode in a small test tube was ~1.5 cm long and ~ 1 cm in the other two dimensions; I regret not taking a picture and not contemplating a neutron diffraction study). Guy encouraged me that even if I only get deoxyHb data – it will be a step in the right direction. I mounted a dozen or so crystals in capillaries, put them in my jacket breast pocket and off I went: a train to London, coach to Dover, a ferry to Calais, a train to Paris. From Paris I called Yves Mauguen, another of Guy's many friends and acquaintances, and eventually got to LURE. The first generation synchrotron was used in the so-called parasitic mode only twice a week – Sundays and Wednesdays. I collected data using photographic film and the Arndt-Wonnacott rotation camera [24] – a fantastic instrument made by Enraf-Nonius. Each of the eight cassettes was packed with three films, with aluminum foil in between, to extend the limited dynamic range of a single film. The outermost one (i.e. the one closest to the surface of the cassette) was used to measure only weak reflections, while the innermost one hopefully had adequately attenuated strong reflections so the intensities were below saturation levels. Each exposure took 45 minutes (sic!) so the entire carousel took nearly seven hours. And that was only about 20 deg of data. I developed the first films on site – and nearly fainted.. Diffraction extended to almost 2.0 Å resolution!! Best ever for a Hb crystal (Later that day I found out that Max Perutz and Judd Fermi, the son of Enrico Fermi, had been collecting data at LURE from crystals of human deoxyHb; that structure – refined to 1.74 Å resolution – would get published in 1984 [25]). I soon gave up developing the films, and just packed all for processing in York. I shared some of the time with another visitor – Paul B. Sigler came from Chicago to collect data from hexagonal crystals of 5-3-ketosteroid isomerase, with a *c* axis of more than 500 Å [26]. He showed me holes burned through by X-rays in the hexagonal rods (flash freezing had not been around yet – we collected data from crystals chilled to 4° C in capillaries). Perhaps the most memorable time of my work at LURE was the 'live' alignment of the beam while inside the hutch, using a fluorescent screen (really). The interlock system was designed so that there was no way to leave behind someone inside the hutch without the key. Paul Sigler and I used a simple trick: because the hutch had no ceiling, one of us would stay inside, throw the keys over the wall, allowing the other to activate the interlocks. Paul was one of the most intelligent scientists I ever had the privilege of knowing. Consequently, I was invariably the one left inside with the fluorescent screen to do the dirty job…

Ten days later, with a suitcase full of boxes with exposed film, I retraced my steps, but not without a problem. I was detained on the ferry on my way to Dover, at Her Majesty's pleasure, and kept in solitary confinement, once in port, for several hours. My visa, it turned out, did not allow for re-entry. I do not know why I was let go. The rest of the trip was uneventful. Guy subsequently interceded on my behalf with the local MP, and in due course I received an apology from the Foreign Secretary Lord Carrington on Her Majesty's Government stationary.

The first thing to do upon return in York was to develop the films. I did that over a period of three weeks, several hours a day, in the darkroom in the Department of Physics. I am color blind (red-green, common type) and so turning on the red lights in the darkroom is a purely symbolic gesture to me. I chose to forego, and stayed in complete darkness each day. My work was flawless. (Sometime later I learned about a student who under similar circumstances 'developed' and 'fixed' the aluminum foil, while throwing away the film. Then turned on the light.) With all films developed, we faced another challenge. There was only one

place at the time where microdensitometry of the oscillation X-ray photographs was possible – Imperial College in London, the laboratory of David Blow, where Allan Wonnacott had written the MOSFLM package to process the rotation photographs. There was a Joyce-Loebl Scandig-3 rotating drum densitometer hooked up to a Data General NOVA-3 minicomputer. Guy Dodson made a phone call, and off I went on yet another trip. Somehow I found accommodation in the East End, a room in a house owned by a very talkative Polish lady, nearly an hour's ride by underground from South Kensington, the nearest tube station to Imperial College. I was allowed to use the equipment during the night only, starting at 8 pm. So my day would start around 4 pm, I would board the underground at 6 pm, show up at 7 pm in the lab, get ready and start my work promptly at 8 pm, then work through the night until 8 am. Another hour, and I was in bed. It took exactly one hour to process a pack of three films offline, because the NOVA-3 memory was unable to deal with a full image. The whole job took about three weeks. Finally, I boarded a train to York with several magnetic tapes.

At the time, the Hb dataset was the most complex computational challenge that was faced in York. Merging and scaling the data was a huge task, which required more than one hour, maximum CPU time allowed on the mainframe DEC-10. The next chapter in this odyssey was the refinement of the structure, starting with the solution obtained earlier using Molecular Replacement and the 3.5 Å data. Guy and Eleanor wrote in the May 1981 Daresbury CCP4 newsletter:

"The DEC 10 gagged more than once while the 195,000 observations were being handled but we were saved by the Christmas lull. We were glad that in the data processing we held on to all the observations even though those beyond 2.3 Å seemed very weak. The crystallographic refinement clearly benefitted from these high angle terms which were found to be usefully accurate. The refinement itself was a pretty large problem for the DEC 10 – there were 5,000 atoms and 37,000 terms. It took 55 minutes per cycle usually calculated between 3:00 and 6.30 a.m. For us, used to puny (E.J.D.) insulin these calculations had a new dimension, but, once a fundamental error had been spotted (well done John Campbell) convergence was smooth. The starting positions (Fermi's deoxy haemoglobin coordinates properly oriented in the PEG Hb cell) had an agreement factor R of 42%; after some 6 cycles this fell to 28% for a geometrically correct structure."

We took advantage of the relatively new scheme, which was first used for actinidin by Ted Baker [27]: a Fast Fourier Transform (FFT) based on the Cooley-Tukey algorithm, with intermittent structure regularization, i.e. restoration of proper stereochemistry. The two steps were done independently, so the arduous process consisted on ten steps forward and then nine backwards, with respect to the crystallographic R factor. Again, with Hb being a large problem (the entire tetramer was in the asymmetric unit), one cycle of the FFT took nearly exactly one hour. This would have been a very lonely and thankless task, had it not been for the fact that I now had a close buddy in York – Zbyszek Dauter arrived for his postdoc with Prof. Michael Wolfson, one of the pioneers of direct methods in phasing, and a Professor of Physics. Zbyszek's work had nothing to do with my project, but we quickly became inseparable, with Zbyszek getting intellectually dragged into the protein work.

Sometime in early 1981 we had a breakthrough – sort of. The University of York upgraded its mainframe DEC-10 computer to a new operating system – GALAXY. That was cool – except that the system had a flaw: when the scratch disc (there was only one – sic!) was full, the operating system stalled. If one was working interactively from the teletype terminal, and if there was a possibility to delete one's own files from scratch, the system would restart, but only for the job linked to that terminal. Zbyszek and I wrote a 'jiffy' that kept dumping garbage onto the scratch disc, until the latter was full. We would wait until midnight, when the time limit for interactive jobs was set to one hour, and then unleash our 'virus', which quickly brought the York University computer to a screeching halt. We would then delete the goofy files, and comfortably start a refinement job, with the mainframe DEC-10 serving only us. This took nearly three months – and no one noticed (no one else worked at so strange hours). The downside was that once again all the work was accomplished during the night. To facilitate this lifestyle and make further refinement possible (the R factor steadily moved by about one per cent each week) I moved in with Zbyszek to his 'apartment' in Wentworth College (technically I had a room with Guy and Eleanor). The only problem was the cleaning lady, who visited the apartment each Friday at an uncomfortable time around noon. To assist her, each Friday morning instead of going to sleep in Zbyszek's apartment, we drove around 9 am in his Polish Fiat 500 to the Dodson's residence at 101 East Parade, and we would go to sleep together in a large bed in my room. Guy and Eleanor drove in the opposite direction in the morning, and we would pass each other on the way. Although this was quite an unusual behavior, they never asked why the two of us were regularly to be found in their house together in bed each Friday morning…

The refinement kept going, but another hurdle had to be taken. It was very clear that the atomic model with an R-factor around 30% (R-free was yet to be proposed years later by Axel Brunger [28]) must be plagued by many problems, the least of which was lack of ordered water molecules that were expected to be visible at this resolution. Unfortunately, computer graphics was in infancy. It was around that time that the first Evans and Sutherland graphics system arrived at LMB in Cambridge and Ian Tickle with Alwyn Jones adapted FRODO written originally by Jones in 1978 [29]. We had to find another path - a flatbed plotter capable of plotting electron density maps on transparencies. Alas, there was only one such instrument at the University of York: in the laboratory of Prof. Venables, in the Department of Psychology. So Guy made yet another phone call – this time not a long distance one. We were told that we could use the Hewlett-Packard plotter on two conditions: that we bring our own pens and transparencies, and that we use the equipment between Friday night and Monday morning only (what else could be new…). I never met Prof. Venables, but I am very grateful to him. Each section of the $2F_{obs}–F_{calc}$ electron density map, representing 0.7 Å slice, took exactly

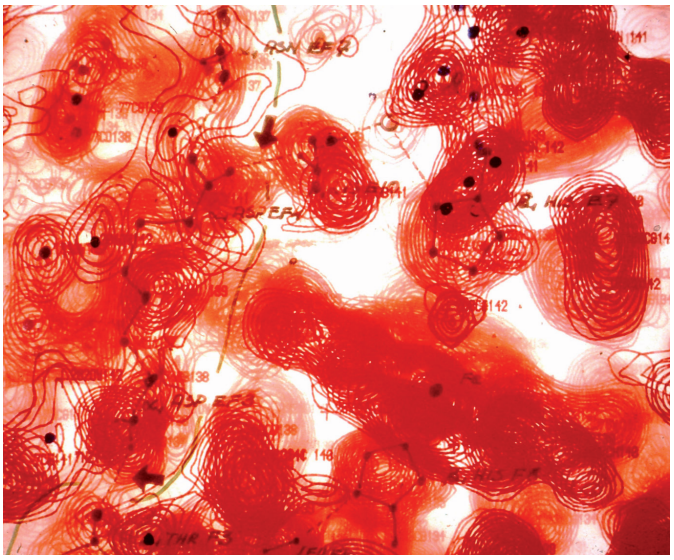

Figure 4. The 2.1 Å resolution $2F_{(obs)} - F_{(calc)}$ electron density map of the semioxy-Hb. Five transparencies are stacked in this photograph, showing sections through the α1-haem. The contouring was done using the Hewlett-Packard flat-bed plotter as described in the text. The oxygen peak is visible above the Fe^{2+} atom. The black dots denote new atom position which had to be measured by a ruler. The black line to the left of center with arrows represents the boundary of a tight crystal contact between the α1 and β1 subunits in adjacent tetramers.

45 min (there was something magical about the 45–60 minute timeframe: one frame of data, one package scanned, one refinement cycle, etc). It took precisely between Friday 8 pm and Monday 8 am to get the whole tetramer done. Zbyszek and I took 8 hour shifts: our intellectual contribution to this process was every 45 min putting in a new transparency and to monitor the fading colors of the pens, to replace them if necessary. Otherwise it was just the return button on the keyboard. I was intimately familiar with the projection, having done the 3.5 Å structure, and I was there on a Saturday morning, at 3 am, when the black pen started circling around contours through an elongated, sausage shape of one of the haems – the α_1-heme. Closer and closer to the center, the electron-dense peak of the ferrous ion grew in size, and finally the map was cutting through the iron itself. Then – what? An extension began to develop on the distal side of the iron. Modest on the first section, then growing into a distinct bent shape of diatomic oxygen attached end-on to the iron (Fig. 4). Exactly as seen in the oxymyoglobin structure determined by Simon Phillips (see above). Within a few minutes the β-haems showed up – empty. I could not believe my eyes. Evidently, somewhere on the ferry across the English Channel, the deoxyHb crystals in the capillaries slowly got partially oxygenated as air seeped through the seal… I took the transparencies, got into Zbyszek's small FIAT (I had no driver's license), drove to Guy at 5:00 am, barged into the bedroom, woke him and Eleanor up and shared my joy. There it was – we had the elusive intermediate!

It took a little longer to finish the refinement. I had to measure the coordinates of all atoms and the waters using a plastic ruler from the transparencies, and then type it all in. I clearly remember that the Hb model had 4,556 non-hydrogen atoms. We managed yet another round of refinement using this new model.

In the spring of 1981 I went back to Poland to defend my PhD, and then returned to York in the fall to continue the work, bringing Tadeusz Skarzynski with me. Martial law was declared in Poland in December of 1981, and things got complicated. (A few days earlier I had a terrible car accident, head-on collision, and was nursed by Mirka Dauter back to health). But eventually we managed to write up a paper for Nature and submitted on June 13, 1983. It was published in January of 1984 [30]. (I am embarrassed to this day that the paper was not co-authored by several people who made such a significant contribution – most of all Zbyszek). Scientifically, we felt it was a great result that showed how the sequential model of KNF could be applied to haemoglobin, complementing in many ways the MWC theory. The structure revealed that the α-haems got oxygenated even in the T-state, and the intermediate was undergoing structural changes, triggered by the shift in the Fe^{2+} position, as a result of the induced fit mechanism. The oxygenation of the α-subunits propagated changes into the adjacent β-subunits – as predicted by the KNF theory - which in turn shifted the equilibrium towards the R state. Much of it was expected from the theory Max Perutz put forward in 1970, but it was nice to see the cascade of small rearrangements at high resolution. The haemoglobin project continued in York, and I was involved in one more study involving the structures of deoxyHb and MetHb in the T-state [31]. Frankly – I thought we crossed the *t*'s and dotted the *i*'s, reconciled MWC with KNF, as Max Perutz had envisaged. I was wrong (not the last time), but I did not know that…

So I moved on. Fortunately, Guy had other ideas for me and we soon started exploring the nebulous mechanism of interfacial activation in triacylglycerol lipases, funded by the enzyme division of the NOVO-Nordisk company. I was joined in this endeavor in York by Marek Brzozowski, while Zbyszek Dauter moved from Physics to Guy's group and concentrated for a while on insulin. After I left York in Dec of 1990, to take up a position at the University of Alberta, in Edmonton, Canada, I did not keep up with the Hb field. Nor did I follow the details of the ongoing discussion around the MWC and KNF theories.

40 YEARS LATER: NEW TOOLS – SAME QUESTIONS

Fast forward nearly 40 years. Another place (University of Virginia, Charlottesville, USA), another project. This time, in yet another sharp turn of my career, I became interested in the discovery by a colleague (Dr. Deborah Lannigan) that an alkaloid (dubbed SL0101) isolated from an Amazon plant, *Foresteronia refracta*, is a selective inhibitor of the p90 ribosomal S6 protein kinase (RSK) [32]. The RSK kinases (there are four isoforms of them, RSK1-4) are serine/threonine protein kinases, with the RSK1 and RSK2 isoforms being the most ubiquitous in human tissues and organs [33, 34]. They have an unusual molecular architecture, likely a result of gene fusion, in that they harbor not one, but two catalytic kinase domains: the N-terminal (NTKD) classified as an AGC family member, and the C-terminal domain (CTKD) belonging to the family of the Ca^{2+}-calmodulin activated kinases. RSK kinases are regulated in a complicated way by other upstream kinases, so that phosphorylation by

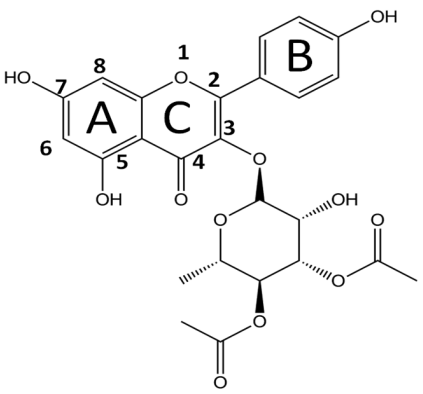

Figure 5. The chemical structure of the SL0101 inhibitor.

ERK activates the C-terminal domain, which in turn catalyzes autophosphorylation on Ser386, enabling the PDK1 kinase to dock, phosphorylate the N-terminal domain within the activation (T) loop. This finally confers catalytic activity on downstream substrates. The RSK kinases have recently come under close scrutiny, because of their apparent role in upregulating tumor growth in a range of cancers [35]. Also, mutations in the RSK2 isoform are associated with a debilitating, developmental Coffin-Lowry syndrome [36].

The key, fascinating question raised by Deb Lannigan's discovery, was how does a plant alkaloid with a rather mundane chemistry show specificity towards one particular kinase? This problem had important implications for drug discovery, because kinases are among the most important drug targets. The chemistry of SL0101 gave no clues. It is kaempferol-3-*O*-(3″,4″-di-*O*-acetyl-*a*-L-rhamnopyranoside), (Fig. 5). Although the compound was identified in a rare Amazon plant – it is actually a common plant alkaloid found in a whole range of plant species. Kaempferol, the key component of the compound is a common flavonol, and because of the similarity of its chromen core heterocyclic moiety to purines, it is known – like other flavonols – to bind promiscuously to ATP-sites in various enzymes, including kinases, and act as a non-specific inhibitor. In fact, there is abundant literature supporting the notion that this biological activity of flavonols is a great benefit of plant-rich diets [37]. Most flavonols are produced in plants as glycosides, and SL0101 happens to have an unusual monosaccharide – rhamnose – alien to an animal system. Finally, rhamnose is acetylated on two of its hydroxyls, another common chemical modification carried out by plant cells. So I thought we should try to crystallize the complex.

The N-terminal kinase domain of RSK2 can be easily expressed in *E. coli*; we followed the routine path of trying to soak the inhibitor into crystals of the apo-protein, which we grew easily – to no avail. One day, Darkhan Utepbergenov set up a crystallization screen of a sample of RSK2 NTKD that he previously froze for future use, and to his surprise yellowish crystals quickly appeared. He then remembered (echoes of our serendipitous discovery of partial oxygenation of Hb) that he had the protein mixed with SL0101 before freezing. What followed, lacked any of the drama of my seven year long struggle with the Hb structure. We flash-froze the crystals, put them in a dewar, mailed it to the Advanced Photon Source, Argonne National Laboratory, where it was set up for use at the SER-CAT beamline. Using remote access and working from our Charlottesville laboratory we promptly collected several datasets, each within no more than 20–30 minutes. After a few days, our disc has arrived and we copied the digital frames onto a PC running Linux, with the HKL-3000 package installed [38]. Indexing and processing of the data took about 10–15 minutes. The quality of the data was excellent at 1.5 Å resolution (rare for a kinase complex, as they tend to be dynamic and crystal quality is often poor). We simply 'dumped' the dataset into the BALBES server [39], along with the amino acid sequence file. This is a brilliant automated Molecular Replacement server, designed at the very University of York, U.K., where my Hb saga took place, which in an ingenious way screens the PDB and finds the best fitting pieces based on amino acid sequence similarity, that together make up a model to be used by the Molecular Replacement program. We also flagged the option to have ARP/wARP attempt to rebuild/build and refine the model [40]. Lo and behold, the next morning we found that the whole process was completed with a model of the RSK2 NTKD refined to an R-factor of ~0.20 (if I remember correctly). It took another ten minutes to visualize the ATP-binding pocket using COOT [41], with striking difference density revealing the bound Sl0101 molecule [42]. ***An endeavor that three decades earlier took seven years of sleepless nights, endless travel and considerable pain – was now distilled into several hours of microprocessor time, without the need for much human intervention***. All the romanticism was sadly out of crystallography….

But something was not right: when we overlapped this structure on a canonical structure of PKA with AMppNp (a non-hydrolyzable analogue of ATP) – it was clear that the RSK2 NTKD looks very different. In fact – it looked like no other kinase domain in the PDB. This was unexpected.

Few words of explanation are needed. The protein Ser/Thr kinase domain is made up of two lobes: the N-lobe, a dynamic subdomain largely made up of β-strands, and a sturdy C-terminal lobe, which α-helical in nature [43]. The ATP-binding site is between the two. Kinases (there are more than 500 in the human genome) constitute an important group of drug targets, and the key barrier is designing inhibitors that are selective, given that all kinases have essentially the same ATP-binding site. One of the possible paths is to design inhibitors that bind to inactive conformations, unique to a specific kinase (so called type II inhibitors) [44]. The amazingly successful drug Gleevec (imatinib mesylate), has been shown to bind to the Abl kinase in that way, and induced fit mechanism has been invoked [45]. However, the complex of RSK2 NTKD with SL0101 suggested a conformational change of much greater magnitude and I was very intrigued by this observation. Compared to a canonical kinase structure, the N-lobe was folded into a distinctly different topology and then twisted with respect to the C-lobe, while burying and sequestering the SL0101 molecule (Fig. 6). The obvious explanation was that SL0101 either binds to a generic structure as an ATP-competitor, or a structure in which the N-lobe is dynamic, but then induces an unusual conformational change which results in a stable

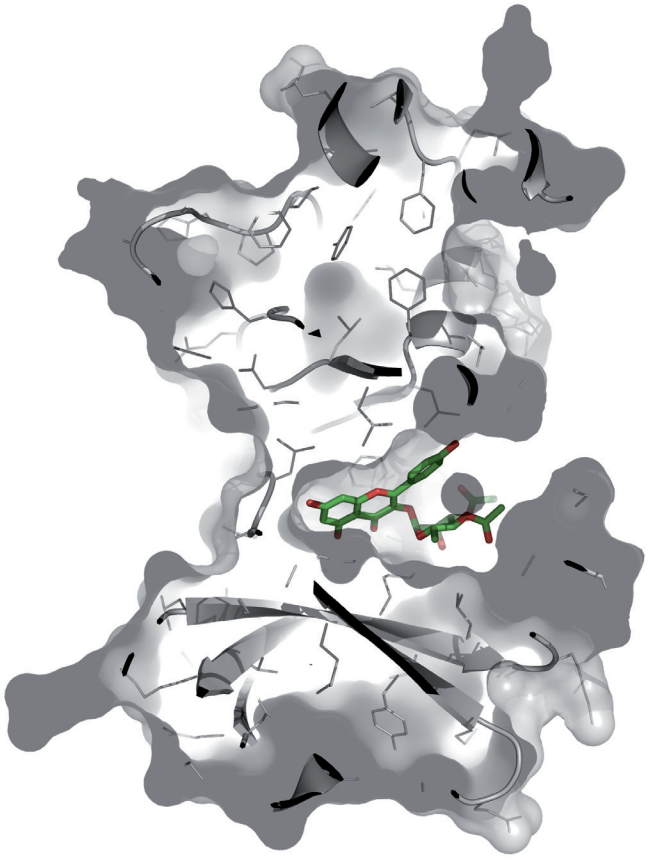

Figure 6. The structure of the complex of SL0101 with the N-terminal kinase domain of RSK2 (3UBD). The figure shows a slice through the center of the molecule, with the protein structure shown in a cartoon format, with the molecular surface shown in a semi-transparent mode. The SL0101 molecule is shown using sticks colored according to atom type.

species that we observe in the crystal structure [42,46]. In other words – we are seeing evidence of 'induced fit', just like we saw with the semioxyHb structure.

INDUCED FIT *VS* CONFORMATIONAL SELECTION

I was rather surprised that our discovery of the structure of the SL0101 complex was received with only lukewarm enthusiasm. In fact, I tried to obtain funding to continue the work, but the reviewers did not think that 'induced fit' was worth the investment. Nevertheless, a review of the literature showed that the effect has been frequently invoked as a mechanism of inhibitor binding to kinases, sometimes alone and sometimes in combination with conformational selection. For example, it was shown for tyrosine kinases, that large scale motions generate a range of conformations, some capable of binding type II inhibitors, and then inducing further conformational change [47,48]. A recent elaborate reassessment of the binding of Gleevec™ (Imatinib) to c-Src kinase, using X-ray crystallography, dynamics simulations, NMR and Plasmon Surface Resonance concluded with considerable emphasis that "*both conformational selection and induced fit play a role in the binding mechanism, reconciling opposing views held in the literature*" [49]. There is a broader significance of this issue in the field of drug discovery. In 2006, the concept of drug-target residence time was introduced and widely accepted [50-52]. This shifted the focus from just affinity (i.e. dissociation constant, K_D) to the lifetime of the drug on its target, defined by both *on* and *off* rates. The kinetic models of high-affinity interactions very often invoke induced fit, through a two-step-mechanism. The dissociation trajectory must by necessity proceed through a retrograde induced fit that involves steric and kinetic barriers that can be exploited to increase the residence time of the drug on the target. With all that said, there is significant dichotomy in literature, with a dominating view that 'conformational selection', i.e. the MWC model, is prevalent in protein-ligand interactions. To quote Changeux and Edelstein [53] – "*following 50 years of debate and experimentation, the vast majority of data support the conformational selection (Monod-Wyman-Changeux) scheme of signal transduction*". This is not just a matter of opinion, but a well-documented position. One of the diagnostics used in the past was the rate of approach to equilibrium $k_{(obs)}$. When ligand binding-dissociation events are fast compared to conformational transitions, $k_{(obs)}$ was assumed to decrease, or increase, with the ligand concentration, respectively for conformational selection or induced fit. More recently, Di Cera and colleagues have shown, however, that this assumption is not generally valid [54-56]. Instead, conformational selection is associated with a wide range of kinetic properties, with $k_{(obs)}$ decreasing or increasing with ligand concentration depending on the relative magnitude of the rate of ligand dissociation, $k_{(off)}$, and the rate of conformational isomerization. So while a decrease in $k_{(obs)}$ with ligand concentration is *always* associated with conformational selection, an increase is not an unequivocal evidence of induced fit. The conclusion is that conformational selection is far more common than current literature suggests.

So how is the Hb mechanism of cooperativity faring these days? Was it all settled long ago as I had naively thought? Not really… As it turns out, shortly after I left York, an interesting discovery took place: a third quaternary structure (denoted R2) was observed in carbonmonoxyHb crystals grown from PEG 6000 [57]. Amazingly, the conformational differences between R and R2 were found to be as pronounced as those between R and T. A similar architecture was observed for the carbonmonoxy form of the mutant Ypsilanti (β99 Asp→Tyr) [58]. Rather than being an intermediate in the T→R transition, the R2 structure was shown to be beyond the R state [59]. Also, investigations into oxygen binding of Hb in our T-state crystals from PEG showed absence of cooperativity [60, 61]. Moreover, our X-ray data came under scrutiny, because the α-subunits in these studies showed only twice the affinity of the β-subunits, while in our structure we saw O_2 on the α-hemes only. The problem here, I think, is that our crystals were 'fixed' using the concentrated PEG, and this procedure resulted in irreproducible extraction of solvent from the interstitial spaces and tightening of the crystal contacts. So the affinity measurements were most likely not representative of the exact crystal forms we studied. In the meantime, the York group continued the Hb project for a while and solved the structure of a fully oxygenated T-state Hb [62], and then a structure of a T-state cyanide complex, in which the Fe-His(F8) bond was ruptured in the α-subunits [63].

Based to a large extent on the work in York with the T-state structures, Max Perutz and Guy Dodson collaborated on what might have seemed like the definitive description of the Hb cooperativity mechanism [64]. In the abstract, Max Perutz referred to his 1970 proposal:

"*Almost every feature of this mechanism has been disputed, but evidence that has come to light more than 25 years later now shows it to have been substantially correct.*"

In this last review on Hb that Max Perutz wrote (he passed away in 2002), he reconciled various experimental data into the refined model which he referred to as 'Moland', for its hybrid nature bridging Monod with Koshland. He was not surprised by the emergence of the R2 structure. In fact, he expected its existence. Back in the nineteen fifties, Perutz studied Hb at various stages of swelling and shrinkage, and using difference Patterson maps he identified a derivative with an Hg-Hg distance of 27 Å, consistent with the R2 structure. But the story did not stop there. In 2005, Safo and Abraham reported crystal structure of two more novel R-state structures of human carbonmonoxyHb: one named RR2, as it appeared to be an intermediate between R and R2, and one called R3 as it seemed to represent yet another 'end state' [65]. All of this was now pointing to the possibility of a dynamic range of R-related structures in solution, and possibly to a conformational selection event during crystallization: only some conformations are selected under crystallization conditions during the nucleation/growth phenomena. Finally, the development of an *E. coli* expression system for human Hb [66], along with advances in heteronuclear NMR, made it possible to study the quaternary structure of Hb in solution [67]. Not surprisingly, the structure was found to be an intermediate between the R and R2 structures, and the more recent incorporation of data using stereospecifically assigned methyl groups and residual dipolar couplings (RDCs) [68] refined it to be somewhat closer to R. Finally, the resolution of the crystallographic data for the deoxy, oxy, and carbonmonoxy forms has been extended to 1.25 Å [69].

Any attempt to review recent literature on Hb is doomed to failure for lack of space. Suffice it to say that between 2005 and 2015 more than 6,500 papers were published on Hb, with several new models for cooperativity put forward [70,71]. Two publications caught my eye. Henry *et al.* [72] state:

"*The demonstration of non-cooperative binding to the T quaternary structure in the crystals, and in the gel, settled a long standing controversy by eliminating the sequential model of Koshland, Nemethy, and Filmer from further consideration. (…) We show that only the tertiary two-state model can provide a near quantitative explanation of the single-crystal and gel experimental results*."

And Yuan *et a*l. [70] argue:

"*The multitude of structures and recent results obtained by other methods clearly show that the classical two structure MWC/Perutz description for hemoglobin allostery as presented in biochemistry, biophysics, and molecular biology textbooks cannot account for Hb function in details and needs revision*."

It is remarkable, that after more than half a century of technological progress in structural biology, countless crystallographic structures and a multitude of solution studies, we are still debating the very essence of the nature of the biophysical phenomena that we set out to explain originally. It has been an exciting 40 years, and I hope that Alex Wlodawer shares this sentiment. All the very best!

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Przygody z kooperatywnością

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Słowa kluczowe: krystalografia, struktura białek, kooperatywność, hemoglobina, kinazy białkowe

STRESZCZENIE

Metodologia krystalografii makrocząsteczek biologicznych uległa dramatycznej transformacji od czasów pionierskich badań w tej dziedzinie w latach 1950., głównie w wyniku odkryć i postępów w trzech innych dziedzinach nauki: technik obliczeniowych, promieniowania synchrotronowego i biologii molekularnej. Proces rozwiązania struktury (tzn. wyznaczenia pozycji atomowych), jeśli dysponujemy kryształami o odpowiedniej zdolności dyfrakcyjnej, został skrócony w wielu przypadkach z wielu lat do kilku godzin. Niemniej, pomimo olbrzymiej ilości struktur atomowych jakie poznano w ciągu ostatnich kilku dekad (120 000 struktur zdeponowanych w Banku Struktur Białkowych, PDB), wiele podstawowych problemów biologicznych w dalszym ciągu pozostaje przedmiotem gorących dyskusji naukowych. Niniejszy artykuł jest oparty na wspomnieniach autora dotyczących udziału w dwóch tematach naukowych – realizowanych w odstępie prawie czterdziestu lat – z których obydwa dotyczyły zjawisk opisywanych rywalizującymi teoriami, jakie zaproponowano jeszcze w latach 60. ubiegłego wieku, autorstwa zespołów Monod-Wyman-Changeux i Koshland-Nemethy-Filmer. Artykuł ten jest dedykowany Dr Alexandrowi Wlodawerowi, jednemu z pionierów biologii strukturalnej, który obchodzi w 2016 roku 70-lecie urodzin, z najlepszymi życzeniami dalszej owocnej pracy.