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Abstract

I have enjoyed reading previous historical sketches that have appeared in Journal of Thrombosis and Haemostasis, and especially those by Ted Tuddenham on factor VIII and Bjorn Dahlback on activated protein C resistance. Like those authors, I have tried to capture some of the excitement-as well as the disappointments-that occurred along the way to a long-term goal.

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HISTORICAL SKETCH

Determining the crystal structure of fibrinogen

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Summary. I have enjoyed reading previous historical sketches that have appeared in *Journal of Thrombosis and Haemostasis*, and especially those by Ted Tuddenham on factor VIII and Bjorn Dahlback on activated protein C resistance. Like those authors, I have tried to capture some of the excitement—as well as the disappointments—that occurred along the way to a long-term goal.

Keywords: crystal structures, fibrin, fibrinogen, fragment D.

Introduction

In 1958, John Kendrew and colleagues reported the first crystal structure of a protein [1]. Not long after, Kendrew visited the laboratory in which I was a graduate student and gave an informal talk on the myoglobin structure and how that historic first had been achieved. It was very exciting, sufficiently so that one of my fellow graduate students immediately decided that the future lay with crystallography, and that was where he would now set his sites. For my part, the field seemed well beyond anything I could ever master. Mathematics aside, it seemed a massive endeavor involving big teams and open-ended resources. I clearly remember Kendrew telling us about personnel problems, especially keeping the large lay staff happy. In this regard, they needed a roomful of 'housewives' (his description; such were the times) whose tedious job involved visually judging the intensities of thousands of spots on hundreds of diffraction films.

Another obvious key to the future, and one more appealing to me, was amino acid sequencing. Nonetheless, I kept abreast of the doings of my fellow graduate student over the years and envied him belonging to that elite group that could determine three-dimensional structures.

Subsequently I went to Sweden as a postdoc in Birger Blomback's laboratory to learn the art of amino acid sequencing by the Edman method, particularly as it was applied to fibrinopeptides. As a result, I had the good fortune of attending the Nobel Prize festivities in 1962 when Kendrew and Max Perutz shared the prize in Chemistry for their structural studies. The excitement of that occasion may have encouraged a hidden desire to some day be a member of the crystallography community.

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Early efforts to crystallize fibrinogen

In 1965 I obtained a faculty position in the Chemistry Department at the recently formed University of California, San Diego (UCSD). One of my slightly more senior colleagues was Joe Kraut, a crystallographer who had spent a postdoctoral year in Cambridge working with Kendrew and Perutz. By this time, like others, I foresaw that it was only a matter of time before the complete amino acid sequence of fibrinogen would be known, and that the really difficult job would be finding its three-dimensional structure. Joe Kraut assured me that the field was advancing rapidly and that computers were replacing much of the labor-intensive aspects. A roomful of spot-evaluators was no longer needed! Even though fibrinogen was a big molecule—about 20 times bigger than Kendrew's myoglobin and 15 times bigger than the chymotrypsinogen molecule that Kraut was working on—he felt a structure could be dicovered. If I could get crystals, it was agreed, he would be happy to begin structural studies. My first notebook entry on the subject is dated 11 October 1969. It was an abortive attempt that did not go any place.

In 1972, Tooney and Cohen reported microcrystals of 'modified' bovine fibrinogen [2]. Although these preparations, similar to what others were calling fragment X, were to the eye not much different from precipitated fibrinogen, when examined by electron microscopy they were remarkably well ordered, to the extent that it was possible to perform optical diffraction studies on the electron micrographs themselves. The hope was that image reconstruction studies would eventually yield a low-resolution structure.

By chance, I was just finishing a long review of the structural aspects of fibrinogen and fibrin [3] and was able to include some of their beautiful pictures. Nonetheless, I wrote pessimistically, 'fibrinogen has never been crystallized in a form suitable for detailed X-ray diffraction studies', even as in my own laboratory we were trying in earnest to make that an untrue statement. During that period we undertook all sorts of strategies, some conventional, like using fibrinogen from different species, and

others more cockamamie, including attempts to orientate the protein in an electric field during the crystallization process.

Then, Cohen and Tooney reported a genuine crystal of the modified fibrinogen, thin plates that actually diffracted X-rays out to about 3 Å [4]. It seemed an end was in sight. During the late 1970s and early 1980s, the Cohen lab generated a string of elegant papers. Many of these dealt with the nature of the crystal packing and other aspects of the properties of the modified fibrinogen. It became apparent, however, that there is much more to getting the structure of a large and gangly protein like fibrinogen than having crystals. If it seemed to me that progress was agonizingly slow, it was my ignorance at the time of how difficult protein crystallography can be.

Meanwhile, in my own lab we abandoned any notion of crystallizing the native protein and instead took aim at the core fragments generated by plasmin, in particular fragment D. These were sporadic assaults that always took a back seat to other lab doings, although I still have numerous notebook pages filled with failed attempts.

The field advances

Arguably, the highpoint of the year 1982 for fibrinogen research was a New York Academy of Science meeting entitled 'Molecular Biology of Fibrinogen and Fibrin', largely organized by Mike Mosesson. At that meeting Carolyn Cohen reviewed 10 years' worth of crystallographic studies performed in her laboratory [5]. At the meeting, also, Rotraut Gollwitzer reported progress on obtaining fibrinogen crystals, showing beautiful needles of native human fibrinogen [6]. Not long after, the Gollwitzer lab published a report on X-ray diffraction by single crystals of 'modified' human fibrinogen [7], the material having been subjected to the same kind of limited proteolysis as had been used by the Cohen lab. There was the prospect that a little competition might accelerate progress.

Early in 1988 I visited the University of Arizona to present a routine seminar on fibrinogen, and in the aftermath I met a crystallographer named Ivan Rayment. Like Carolyn Cohen, Rayment was very interested in proteins with coiled coils, and he inquired whether we might collaborate on an X-ray study of fibrinogen. I told him about my longstanding if somewhat moribund arrangement with Joe Kraut, but I thought there would certainly be no problem in sending him some of our fibrinogen preparations. In particular, I told him that I thought fibrinogen from rats was the best candidate because it had smaller α-chains than either human or bovine.

However, Rayment then moved to the University of Wisconsin, and in the process he had to re-organize his research priorities. Nonetheless, we exchanged letters, and I was cautiously hopeful. In the spring of 1990 he sent me some photographs of large needles of human fibrinogen, using material he had purchased from Sigma. The material was quite degraded when examined on SDS gels, and my guess was that it was similar to the 'modified' preparations for which needle crystals had been got by others. I offered to send him my whole suite of materials, human and rat fibrinogens, as well as fragments D and E, but Ivan had a very full agenda at the time, and fibrinogen fell by the wayside. He went on to produce a series of superb structures of myosin and a host of other proteins and became a world-renowned figure in the field of structural biology.

More determined than ever, I began another broadside on my own, including efforts to crystallize fibrinogens, fragments D and E, and the complex derived from fibrin called D_2E. I was assisted in these efforts by my long-time research assistant, Marcia Riley.

At the same time we were struggling with a protracted study involving the photo-affinity labeling of fibrinogen and fragment D with synthetic peptides patterned on the knobs exposed by fibrinopeptide release. This study had been initiated early in the 1980s after my graduate student, Andy Laudano, had shown that such peptides could bind to fibrinogen and fragment D and prevent polymerization [8]. The affinity-labeling effort was an enormously difficult project, the successful culmination of which was almost entirely due to the heroic efforts of two consecutive postdocs from Japan, Akira Shimizu and Ken Yamazumi [9,10]. A secondary benefit of this Sisyphean project was that it required massive amounts of pure fragment D. This had forced us to follow in the footsteps of Cemal Kuyas, who, back in Switzerland after a 3-year stint as a postdoc in our lab, had constructed an affinity column based on the Gly-Pro-Arg-Pro we were using as a synthetic knob [11]. We made our own version of such a column, and it worked remarkably well [10], providing us with a virtually limitless supply of pure fragment D. With that, we intensified our effort to obtain fragment D in crystalline form.

During this period, I was joined by a new graduate student, Stephen Everse, who needed a thesis project. I explained to Stephen that going for crystals of fibrinogen or its derivatives was a high-risk endeavor, but the rewards would be great if successful. Because of the uncertainty of getting crystals, a back-up project would have to be carried on simultaneously to ensure that he would eventually get his PhD. He was more than willing, and during the next two years Stephen set thousands of drops containing fragment D in various environments. In fact, needles were gotten rather quickly, but needles are not suitable for diffraction and such preparations need to be coaxed into more prismatic arrangements before they are useful for X-ray crystallography. At one point we actually had some small plate-like crystals, but we were so inexperienced we did not realize how promising they were. I published a picture of some of these (Fig. 1) in a chapter in the third edition of Arthur Bloom's *Haemostasis and Thrombosis* [12] with the caption that 'unfortunately these were not of a quality to diffract X-rays'. Later we were to realize how close to being useful those crystals really were.

In 1991, I took a sabbatical leave in residence, and Stephen and I attended the graduate course in X-ray crystallography being taught by Joe Kraut. Ignoring the occasional snicker, I decided that I could not let the fact that I was almost three times the age of the average student deter me from sitting in the front row. Fortuitously, Stephen became friends with one of Kraut's graduate students, Mikey Sawaya. Mikey became our chief tutor on the practical aspects of crystallography.

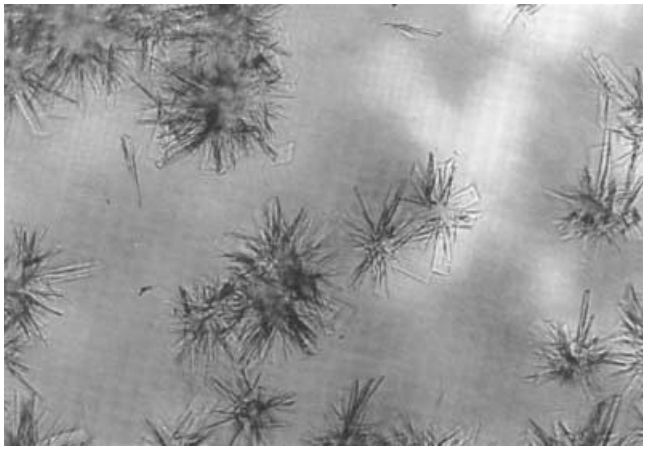

Fig. 1. Small unappreciated crystals of fragment D from human fibrinogen (from [12]).

By this time Cohen's laboratory had published a long paper on an 18-Å resolution structure [13]. Although 18 Å resolution is only a little better than high-resolution electron microscopy, what I was learning in Kraut's course made me appreciate the difficulty of the situation.

Real crystals

In an unusual development, one of my former postdocs, Takashi Takagi, upon hearing we were trying to do crystallography, sent me some protozoan hemoglobin to crystallize. After a few unsuccessful attempts, I consulted one of Kraut's postdocs, Hugette Pelletier, who was bragging that she was able to crystallize any protein. Within a month of my giving her some of that priceless hemoglobin, she had beautiful crystals. Stephen and I had a hurried consultation; should we give Hugette some of our fragment D? We decided it was worth a try. Within 6 weeks she had some crystals. When we saw them we were both elated and disappointed. The disappointment was that the crystals looked not much different from the ones we had photographed a year earlier. But Hugette was experienced and could see that it would be an easy matter to adjust conditions to where large prisms would appear. She showed that even the small ones she had got could diffract X-rays, however weakly. Indeed, after a few weeks Stephen was able to generate large single crystals (Fig. 2).

Fig. 2. Crystal of fragment D from human fibrinogen (from [14]).

There was yet another disappointment. Joe Kraut and Hugette were not interested in pursuing a structure of fibrinogen. Joe was nearing retirement and had his own closing agenda. He would, however, make available some of his facilities if we wanted to go it alone. In particular, he had an X-ray source with a film holder that was not much used. By this time crystallographers were using what is called an area detector that records the diffracted rays ('spots') electronically. Film was regarded as a primitive medium, even though densitometers had replaced the roomful of spot-evaluators. Nevertheless, we jumped at the offer. One of the most exciting moments that Stephen and I shared was when we cut up films of precession pictures of fragment D, put them into an old slide projector, projected the spots on a big piece of white paper taped to the wall, marked the spots, and then determined the unit cell dimensions with a meter stick and a plastic protractor. To this day it both astonishes and pleases me that we got the right answer.

Impressed by our industry, Kraut began to give us limited access to his area detector, and with that Stephen began collecting full datasets and even managed to obtain some heavy-metal derivatives. We also managed to get crystals of fragment D complexed with the synthetic 'knob' Gly-Pro-Arg-Pro-amide.

'Very early retirement'

July 1994 was a turning point in my life. The state of California was experiencing hard times financially, and the University of California was encouraging senior faculty to take 'very early retirements' (VERIP was the Very Early Retirement Incentive Plan). I myself had wavered. Although it meant relief from a heavy teaching load and a chance for full-time research, it also meant giving up any influence or authority one had within the university. But given the crystals and the encouraging diffraction data, I went for it. Like some other professors, I was promised that I could keep my lab so long as I could keep the National Institutes of Health funding that paid for it.

By this time, Stephen had been a graduate student for almost 6 years, and it seemed reasonable to publish the preliminary work so he could gain his degree. Naturally he wanted to stay

on as a postdoc so as to be involved with the final structure. Accordingly, we published a preliminary report in *Protein Science* in May 1995 [14]. As will become clear, although we may have thought we were staking a claim, the opposite may have been the case. Science thrives on competition.

In July 1995, we were joined by Glen Spraggon, who had just completed his DPhil at Oxford, where he had been well trained in crystallography. As soon as Glen had sized up our situation he starkly announced: 'We've got to get to a synchrotron'. Some of the derivative data we had collected in Kraut's lab were simply not strong enough for phasing. At a synchrotron, quite apart from the stronger beam, the wavelength can be tuned to optimum conditions for so-called anomalous data that aid greatly in phasing.

Synchrotron data

Our first synchrotron data were the result of some fortuitous circumstances. Glen Spraggon's DPhil advisor in Oxford, David Stuart, was anxious for Glen to visit in order to finish up a paper based on his dissertation work. If Glen could return to England for a bit, Stuart offered to give us a day of his synchrotron time at the nearby Daresbury facility to allow Glen and Stephen to collect data on our fragment D crystals. This was a difficult decision: should we invest in two low-cost air tickets for Glen and Stephen to fly all the way to England carrying a box of crystals to collect data in a single 24-h time slot? Budgetary concerns ignored, off they went, with me monitoring events by e-mail. My first e-mail message after they arrived was 'our day has been postponed'. I was more than a little nervous. But in the end it worked.

Back in San Diego with the new data, phasing proceeded rapidly and the first clear maps were obtained. The three of us were thrilled one day when the coiled coils suddenly cast themselves into prominence, four tubes of density being clearly evident. We were expecting only three, but now we could see that one of the chains—presumably α—reversed its course and headed back in the opposite direction. It was exciting.

Each day a little more of the structure became clearer. The three of us were glued to our adjacent computers, fitting residue by residue along the core sheets and coiled coils. The most difficult part was connecting all the many ends of the core strands. We were also able to get more and better data by visits to synchrotrons at Stanford and Brookhaven. In an another development, we were able to obtain crystals of D-dimer ('double-D') from material generated from plasmic and tryptic digestions of cross-linked fibrin.

Surprise competition

In April I received an e-mail from a friend on the East Coast: 'Do you know (surely you do) that Vivien Yee of U. Wash. has solved the gamma fibrinogen C terminal to 2.1 Å? It's listed as a prediction target for CASP2'. I was stunned. It had never occurred to me that we had competitors other than the Cohen group, although I had heard a rumor that in Germany Wolfram Bode, a coworker of the late Rotraut Gollwitzer, was pursuing fragment D. Vivien Yee had worked with David Teller at the University of Washington on the structure of factor XIII, and it was natural that they should now be collaborating with Earl Davie and colleagues on recombinant fragments of fibrinogen. Nonetheless, it took my breath away. The γ-chain domain, while only a third the size of fragment D, obviously contained the most interesting features, including the nature of the fundamental domainal fold for both the γ- and β-chains.

CASP is an acronym for Computer Assisted Structure Prediction, a periodically held challenge contest in which protein structure predictions are tested against newly determined but unpublished structures. The CASP listing about the γ-chain carboxy-terminal domain noted that the structure would first be made public at a meeting that summer.

The race is on

That summer, 1996, the International Union of Crystallographers was meeting in Seattle. We had put in an abstract on our emerging structure, and Stephen and Glen attended the meeting and manned a poster. The University of Washington group had garnered an oral presentation, during which Stephen and Glen sat down in front and tried to take notes to see how concordant our current structure was. The slides flew by too fast to be of much use, but they did not see anything that would lead us to think we did not have things traced pretty well at that point, loop connections notwithstanding. However, we knew a publication would be forthcoming shortly, and upon their return we worked all the harder. I was determined that we would get our structure solved on its own and without recourse to shortcuts such as molecular replacement with a smaller molecule whose structure was known.

In December I flew off to the American Society of Hematology (ASH) meeting being held in Orlando where I was able to show slides not only of the fragment D structure, but also that of double-D. Still no paper from Seattle. Then, in mid-January I had an e-mail from Dave Teller: their paper would be appearing the next day in *Structure*. He apologized, but they had decided that coordinates would not be released until April. I was delighted. I did not want their coordinates. We would climb to the top of the mountain on our own without a rope being thrown down from someone above!

Nonetheless, we scrutinized their *Structure* paper [15] carefully, and it was indeed helpful for verifying chain connectivities. We also noted that the Seattle group had employed exactly the same crystallization conditions that we had reported for fragment D in our 1995 paper [14], but there was no reference to it (Dave Teller later apologized for the oversight).

By April we had prepared two manuscripts: one long and one short. I telephoned to *Science* and asked if they were interested. They were non-committal but suggested I send the papers along for a preliminary evaluation. Almost two months went by before I telephoned again. *Science* wanted to know just what was so exciting about fibrinogen. I tried to tell them. After another call or two, they said they would consider a single short

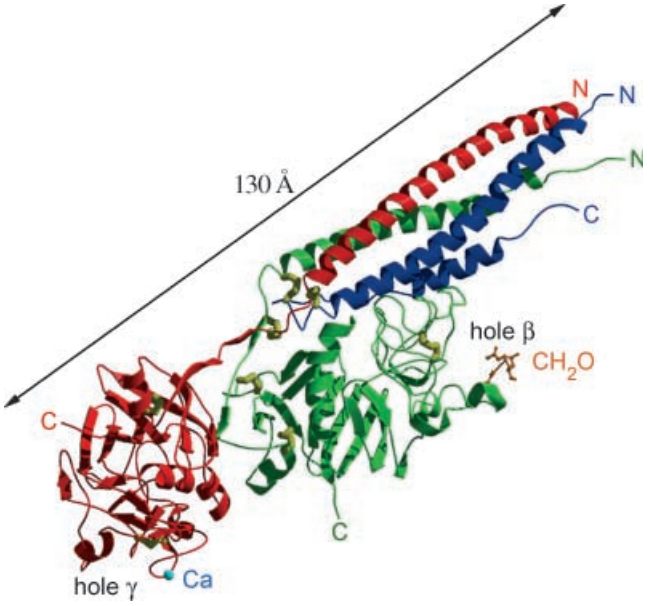

Fig. 3. Ribbon model of fragment D showing β and γ holes, bound calcium, carbohydrate attachment point, and residual coiled coil. Red, γ-chain; green, β-chain; blue, α-chain (from [17]).

paper. Send the manuscripts back, I told them; we were withdrawing. By this time the Seattle group had published a second paper, this one on a structure with the A-knob bound in its hole [16].

Much valuable time had been lost, and we now had to re-format for *Nature,* with whom I was now exchanging e-mails. In the end, *Nature* also suggested a single manuscript, but they favored the long version. When the article finally appeared [17], we all exhaled a long sigh of relief. The paper contained not only the structure of fragment D (Fig. 3), including the arrangement of all the principal domains and the relative orientation of the 'holes' into which 'knobs' must fit, but also that of double-D isolated from cross-linked fibrin. This structure showed in exquisite detail how the molecules must pack together during the formation of the protofibril (Fig. 4). Once we had the fragment D structure, it was a much smoother road to obtain related structures. Gerd Grieninger and colleagues at the N.Y. Blood Center sent us some recombinant material from fibrinogen-420, and Leela Veerapandian (now Pandi) was able to grow some absolutely beautiful crystals. Because the α_EC is about 45% identical with the βC and γC domains, the structure could be determined by molecular

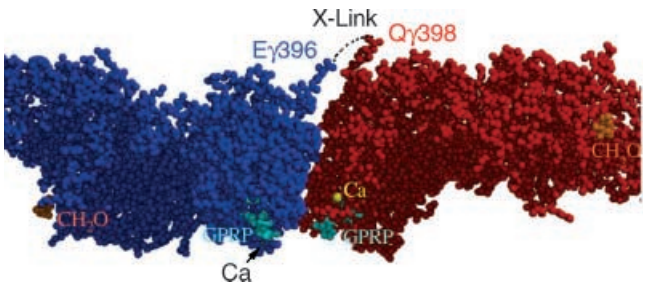

Fig. 4. End-to-end abutment at the D : D interface (from [17]).

replacement [18]. Similarly, we were able to solve a variety of fragment D and double-D structures in the presence or absence of the synthetic peptide 'knobs' and discovered some remarkable conformational changes that occur as a result of the knobs fitting into their holes [19,20].

Structure of a native fibrinogen

Even though the structures of fragments D and double-D turned out better than I had dared to hope, I still had the nagging obsession that we could get crystals of a genuine native fibrinogen. It was clear that the Cohen lab would soon be able to calculate a structure of their bovine 'modified fibrinogen' by the method of molecular replacement using our fragment D structure. Why should we not make one last try at a different native molecule? By this time the best choice seemed to me to be fibrinogen from chickens. In 1990, workers at the NY Blood Center had shown that chicken α-chains lack the 13-residue repeats that are found in varying numbers in many mammalian α-chains [21]. The 'free-swimming appendages' that are composed of the carboxyl domains of α-chains distal to the repeats had always seemed to me to be the most likely barrier to crystallization. Certainly these moieties were absent from the 'modified fibrinogens' that had been crystallized.

Finding chickens to bleed was not as easy as it was when I was a graduate student. Even though more than a billion chickens are slaughtered in the USA every year, gaining access to where the action occurs and having proper institutional permissions are major problems. By chance, I had an undergraduate student in my lab whose family had immigrated from Hong Kong. He told me about a small poultry shop in downtown San Diego, *Wing Lee Poultry*, where Chinese

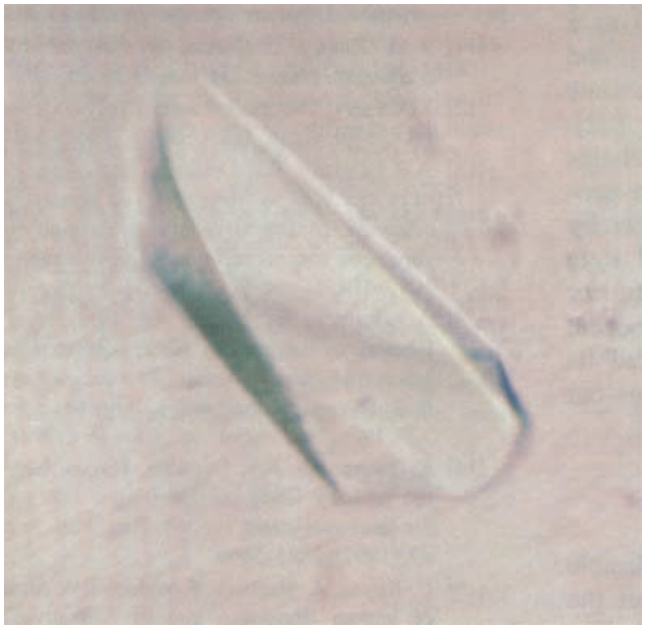

Fig. 5. Crystal of native fibrinogen from chicken blood. The crystal was grown with trimethylamine oxide (TMAO) as a precipitant (from [24]).

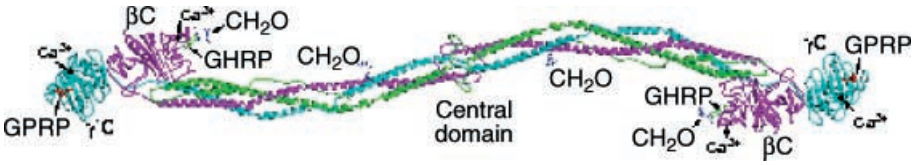

Fig. 6. Ribbon model of chicken fibrinogen at 2.7 Å resolution. (from [25]).

housewives bought the very fresh chickens to which they were accustomed. In March 1999, he and I went there one morning and were able to collect blood directly from 10 freshly killed chickens. Back in the lab, we made a batch of fibrinogen the next day. Extraordinarily, we had crystals within a month.

By this time, Stephen Everse had gone on to a faculty position at the University of Vermont, and Glen Spraggon had accepted a position with Novartis. By good fortune I was able to replace them with Zhe Yang from Beijing and Igor Mochalkin from Michigan State. Zhe and Igor tackled the chicken problem, and by the end of the year we were well along on a low-resolution structure. Meanwhile, the Cohen lab had managed a nominal 4-Å structure of their bovine modified fibrinogen; theirs was the first to show the full sweep of the 450 Å long molecule [22]. They very kindly sent us the backbone coordinates, and we were able to ascertain that the chicken molecule was virtually superposable on the bovine [23].

Still, I was not happy with the quality of the chicken crystals. Quite apart from their rather poor appearance, the diffraction was very uneven ('anisotropic'). We were never going to get a high-resolution structure from these crystals. How to get better ones? Another chance occurrence, which I have described in detail elsewhere [24] and will forego repeating here, led to a different set of crystallizing conditions. It began by the addition to our crystallizing solutions of a reagent, trimethylamine oxide (TMAO), thought to stabilize proteins against denaturation, but it turned out that the TMAO was itself the perfect crystallizing precipitant for chicken fibrinogen (Fig. 5). By the end of 2000 we had collected a complete dataset at 2.7 Å, after which we were able to build a great structure [24] (Fig. 6). For me, it was the beginning of the end of the long march. Meanwhile, Leonid Medved and Carolyn Cohen had combined forces and gotten an ultra-high resolution structure of fragment E [26].

Since then it has been a gentle downhill decline. We were able to determine structures of fragments D and double-D from lamprey [27,28], one of my old favorite organisms. These were important to us because we had done many experiments on the lamprey fibrinogen–fibrin system over the years, and most researchers had ignored them because they were from such a primitive vertebrate. The X-ray structures lent legitimacy to those experiments by showing how closely related the lamprey molecules are to mammalian ones.

Crystal structures have contributed greatly to the fibrinogen–fibrin field on many fronts, from the underlying basis of variant human molecules to the details of the associations that occur during polymerization, to the many different interactions that occur between fibrinogen and other proteins. That there has been scientific progress is undeniable, but I must confess that, over time, the real pleasure was in the day-to-day struggle, watching the structure slowly reveal itself. The excitement of first images flashing on a computer screen, the fascination of seeing new crystals appear, the simple satisfaction of watching key values ('R-factors') drop during refinement calculations: this is all heady stuff. There are days when I shake my head in wonder that all this really occurred.

Acknowledgements

I thank all the many students and others who worked in my laboratory over the last 39 years and participated in this work. In particular, I must single out Stephen Everse, Glen Spraggon and Zhe Yang for their immense contributions to the crystallographic studies. Until very recently, all our studies were supported by the U.S. National Institutes of Health.

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