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Some notes on crystallizing fibrinogen and fibrin fragments

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Abstract

We have recently determined the structure of a native fibrinogen at 2.7-Å resolution. Not the least of the hurdles during the many years of this project was growing X-ray-grade crystals from suitably purified proteins. Small, synthetic peptides based on the parts of fibrinogen exposed by the action of thrombin contributed greatly to these experiments. In addition, trimethylamine oxide (TMAO) was found to improve the diffraction of fibrinogen crystals. The history of my interest in fibrinogen and its crystallization can be traced back in part to some early interactions with John Edsall.

Keywords: Fibrinogen; Fibrin; Crystal structures; Trimethylamine oxide

1. Introduction

In their invitation to contribute a manuscript for this special issue of *Biophysical Chemistry* honoring John Edsall, the editors indicated that authors were free to submit either 'recollections' or a report of ongoing research in their laboratories. I have chosen to combine both aspects, since both elements are joined in my mind.

I was a graduate student in the Department of Biological Chemistry at Harvard Medical School in the late 1950s. The subdivision in which I worked had until shortly before then been the Laboratory of Physical Chemistry, the dominion of E.J. Cohn and John T. Edsall. The fourth floor of Building C housed a unique laboratory by any standard, including a labyrinth of cold rooms set at the sub-zero temperatures used for the fractionation of plasma proteins by the Cohn cold-ethanol fractionation procedure [1]. By the time of my arrival in 1957, E.J. Cohn was deceased and John Edsall had transferred to the Biological Laboratories on the Cambridge campus across the river. Professor Edsall often returned to his old haunts to visit his former colleague J. Lawrence Oncley, however. Professor Oncley eventually became my PhD adviser, and because of that relationship, John Edsall was a member of my thesis committee. A major part of my thesis was published in the *Journal of Biological Chemistry* during his tenure as editor [2].

Abbreviations: TMAO, trimethylamine oxide; GPR, Gly–Pro–Arg derivatives; GPRPam, Gly–Pro–Arg–Pro amide; GHRPam, Gly–His–Arg–Pro amide

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In those days there was a good deal of interaction between biochemists on the two sides of the river, and most graduate students in the Department of Biological Chemistry took courses in Cambridge. I still have my notes from John Edsall's course in biophysical chemistry, the first lecture of which dealt with the purification of proteins, their solubility properties and their crystallization. I can still visualize him striding into the lecture hall in Mallinckrodt Hall with an old-fashioned egg basket filled with CPK models of the amino acids slung over his arm. During the course of one of the first lectures, he casually mentioned that he and Jeffries Wyman had just published a book that might prove helpful with some of the topics being covered [3].

My interest at the time was in the evolution of proteins in general, and blood clotting in particular. I was fascinated by the phenomenon of 'species specificity', as exemplified by the observation that thrombin from a given species invariably clotted fibrinogen from that species faster than it did fibrinogen from another, more distantly related species. Among the animals from which I isolated fibrinogens (and usually thrombins) were various mammals, chicken, dogfish and lamprey. In preparing the fibrinogens, I used a modification of a method described by John Edsall and colleagues [4].

The dogfish experiments are particularly relevant to the tale I want to tell here. Dogfish are elasmobranchs, a family of fishes known to contain upwards of 0.3 M urea in their blood plasma and other tissue fluids. This was interesting, in that it was also well known that urea denatured proteins. In fact, it had been demonstrated that as little as 0.3 M urea inhibited the conversion of human fibrinogen into fibrin [5]. The fact that dogfish plasma obviously clotted, I put down to a species adaptation.

The fibrinogen–fibrin problem continued to interest me through my postdoctoral years and, indeed, through most of my academic career. In 1972, I spent a sabbatical year in Oxford, a period in England plagued by national power strikes. At some point during the winter, all laboratories at the University were closed in order to conserve electricity. The only recourse was to take up permanent residence in the library, where I used the time to write a long and comprehensive review of fibrinogen and fibrin. I submitted the manuscript to *Advances in Protein Chemistry*, unaware that most articles in that series were by invitation. Nonetheless, the three editors, all former inhabitants of Building C at Harvard Medical School, graciously agreed to accept the review. John Edsall subsequently sent me two pages of suggestions on how to improve the manuscript, which he had read carefully while on vacation in Vermont.

The review [6] detailed much of what was known about fibrinogen structure at the time, and especially the many physico-chemical data about its size and shape. A molecular weight for human fibrinogen of approximately 340 000 was not in dispute, having been determined by a number of procedures, including ultracentrifugation and light scattering. But the shape of the protein was still a puzzle. Flow birefringence studies [7] had suggested a highly elongated molecule with an axial ratio as high as 10, and viscosity measurements gave even higher estimates. Edsall had earlier assessed all the relevant data and concluded that, although a highly swollen molecule might be as little as 500 Å in length, he himself felt it would be longer [8]. He emphasized that the choice of model greatly influenced the outcome.

Subsequent shadow-cast electron microscopy specimens had persuasively showed that the molecules were approximately 450 Å long [9], and, excepting some perversely different images obtained by negative staining, the general notion of a triglobular structure connected by 'coiled coils' became the most favored depiction [10]. Amino acid sequence data were just beginning to make their appearance. The subunit nature of the protein had been pinned down with some certainty, the 340-kDa protein being composed of three pairs of non-identical chains: $\alpha_2\beta_2\gamma_2$. The review posed the challenge of how the six polypeptide chains were arranged in the three globules.

The review also remarked pointedly that crystals suitable for X-ray diffraction had not yet been obtained. Indeed, in my own laboratory we had been trying to crystallize fibrinogen from a variety of species, never with any success. Then, that spring, the prospect for single-crystal X-ray crys-

tallography studies suddenly brightened when Tooney and Cohen [11] reported 'microcrystals' of a proteolyzed preparation of bovine fibrinogen. They had taken electron micrographs of those preparations and hoped to be able to reconstruct images by optical diffraction methods.

2. Help from synthetic peptides

By that time, a great deal was already known about the transformation of fibrinogen into fibrin. Thrombin catalyzed the release of two different terminal peptides—the fibrinopeptides A and B—from the α- and β-chains, respectively. The exposure of new sites was clearly what led to polymerization [12]. In the mid-1970s, one of my graduate students, Andrew Laudano, synthesized a series of small peptides corresponding to the newly exposed amino-terminal regions [13]. One set of these peptides began with the sequence Gly–Pro–Arg, the sequence observed at the newly exposed amino-terminus of the α-chain. The other set of peptides began with Gly–His–Arg, the sequence at the exposed site in the β-chain. Both sets of peptides bound to fibrinogen and to fragment D, an 85-kDa degradation product of the plasmin digestion of fibrinogen, but only the α-chain set inhibited polymerization into fibrin [13].

Quite apart from being remarkable anti-polymerants, the α-chain peptides, which were denoted 'A knobs', were to prove exceedingly useful in many other ways, including the quantitative determination and general location of binding sites [13] and, later, their exact mapping by photoaffinity labeling [14,15]. They were also the basis for an elegant affinity-purification procedure [16]. And they were effective in protecting a vulnerable region of the molecule from proteolysis [17], a property previously restricted to calcium ions. Finally, they greatly contributed to our crystallization efforts.

After the appearance of the report by Tooney and Cohen [11], we turned our own attention to various core fragments of fibrinogen, and in particular to fragment D. The crystallization attempts were sporadic, however, and the first real success did not occur until many years later [18]. One of the factors that helped the project was an abundance of pure fragment D left over from the photoaffinity labeling project [14,15].

Our fragment D preparations were always maintained in the presence of 1–5 mM calcium, it being common knowledge by then that calcium protected the carboxyl-terminal region of the γ-chain against proteolysis. We soon found, however, that the fragment D crystals grew much better when the calcium concentration was considerably higher than that, and we routinely employed 50–100 mM $CaCl_2$. Naturally, we also wanted to co-crystallize the synthetic peptides with fragment D in order to determine the structural details of their binding. Interestingly, the presence of the peptide drastically lowered the amount of calcium that could be used while preserving crystal formation.

In fact, it was shown that crystals of the GPRPam–fragment D complex are in a different space group and have very different unit cells, leading us to believe that a significant conformational change must occur when the surrogate knob is bound [18]. It was pointed out to us, however, that a change in mode of crystallization is hardly evidence for a conformational change, and we were driven to attempt a kind of experiment first executed by Felix Haurowitz in 1938 [19]. Haurowitz had carefully exposed reduced hemoglobin crystals to air and watched them shatter before his eyes, correctly attributing break-up of the crystal to a change in the structure of the hemoglobin itself. In our case, when crystals of native fragment D were transferred to the presence of GPRPam, they immediately began to crack; controls with GRPPam remained smooth and intact [20]. We took this as corroborative evidence of a conformational change upon ligand binding.

Meanwhile, the progress in Cohen's laboratory seemed agonizingly slow, testimony to the problems of working with a large, distended, multi-domained protein. By 1991, they had managed an 18-Å structure for their protease-modified bovine fibrinogen [21]. One of their major frustrations had been finding isomorphous heavy-metal derivatives for phasing. In contrast, our fragment D crystals yielded several useful derivatives upon soaking with various heavy metals, and by 1997 we had a 2.9-Å structure [22]. Even before then, a Seattle group, using the same crystallization

conditions we had previously described, reported the structure of the 30-kDa recombinant protein corresponding to the γ-chain carboxyl-terminal globular domain [23].

Fragment D offered an additional benefit, in that it could be prepared from fibrin that had been cross-linked by factor XIII. In this regard, we were able to crystallize the (cross-linked) double-D and observe the packing that occurs between the ends of fibrinogen molecules in a clot. The double-D was also crystallized in the presence or absence of both sets of synthetic knobs, GPRPam and GHRPam, in each case yielding a unique unit cell with a characteristic crystal packing [24]. The side-by-side packing presented a number of insights into how the later stages of polymerization may occur [25].

Because the 85-kDa fragment D occurs twice in a given molecule of fibrinogen, it amounts to approximately two-thirds of the mass of the proteolytically modified bovine fibrinogen (285 kDa) being studied by Cohen's group. As such, they were able to solve their structure by molecular replacement, using the fragment D as a search model [26]. Their approximately 4-Å-resolution structure was the first to encompass the full sweep of the molecule, lacking only the highly flexible αC domains and amino-terminal portions of the six chains. Consistent with earlier electron micrographs, the molecule was approximately 450 Å in length.

3. Chickens and TMAO

Still frustrated by our inability to crystallize native human or any other mammalian fibrinogen, we had turned to chickens. This was not just another frantic attempt to try another species. Rather, the choice was based on our belief that the main barrier to crystallization of the native protein was the presence of the highly mobile αC domains tethered to the main body of the molecule by a long series of 13-residue repeats. It was principally this region that was removed by partial proteolysis of the bovine protein [11]. As it happens, chicken fibrinogen lacks the repeats linking the αC domain to the parent body, and we thought their absence might be helpful. In fact, crystals were obtained within a few weeks of blood collection. Prodded by the success of the Cohen group [26], we quickly published a 5.5-Å structure of a native fibrinogen [27]. The structure was superimposable with that of the modified bovine fibrinogen; it also suffered from many of the same associated problems [26]. The crystals, which contain approximately 67% solvent, showed very anisotropic diffraction, which made it difficult to obtain a high-resolution structure.

Early in 2000, I attended the annual Lorne Conference on proteins in Australia, where I saw a poster on denaturation of proteins by urea and its counteraction by trimethylamine oxide (TMAO). It reminded me of my graduate student days and the concerns I had about urea and dogfish fibrinogen. I was well aware that in solving their osmoregulatory problems, these creatures not only maintain 0.3 M urea in their blood plasma, but also TMAO as another osmolyte as well. In fact, I had measured all the major plasma constituents in these fish as part of another project [28]. It had never occurred to me, however, that TMAO might provide a counteraction to urea, and it surprised me many years later when other workers showed that TMAO actually offsets the denaturing effect of urea in these (and many other) creatures [29]. The Lorne poster indicated that TMAO was currently a hot topic in the area of protein stabilization and folding, possibly exerting a stabilizing effect on proteins, even in the absence of unfolding agents such as urea. Was it possible, I wondered, that it might stabilize a floppy molecule such as fibrinogen and aid in its crystallization?

Immediately upon my return from Australia we set up crystals with TMAO, first as companion molecules to the poly(ethyleneglycol) (PEG) we were using as a crystallizing agent, and then without any PEG present. Within a few weeks we were routinely growing large, single crystals of native chicken fibrinogen with 0.5 M TMAO alone (Fig. 1).

The second barrier we had to overcome, and one that most crystallographers solve empirically, was finding suitable cryogenic conditions. We had been using glycerol with only occasional success, but, following the lead of the Cohen group [26], we switched to the other commonly used cryopro-

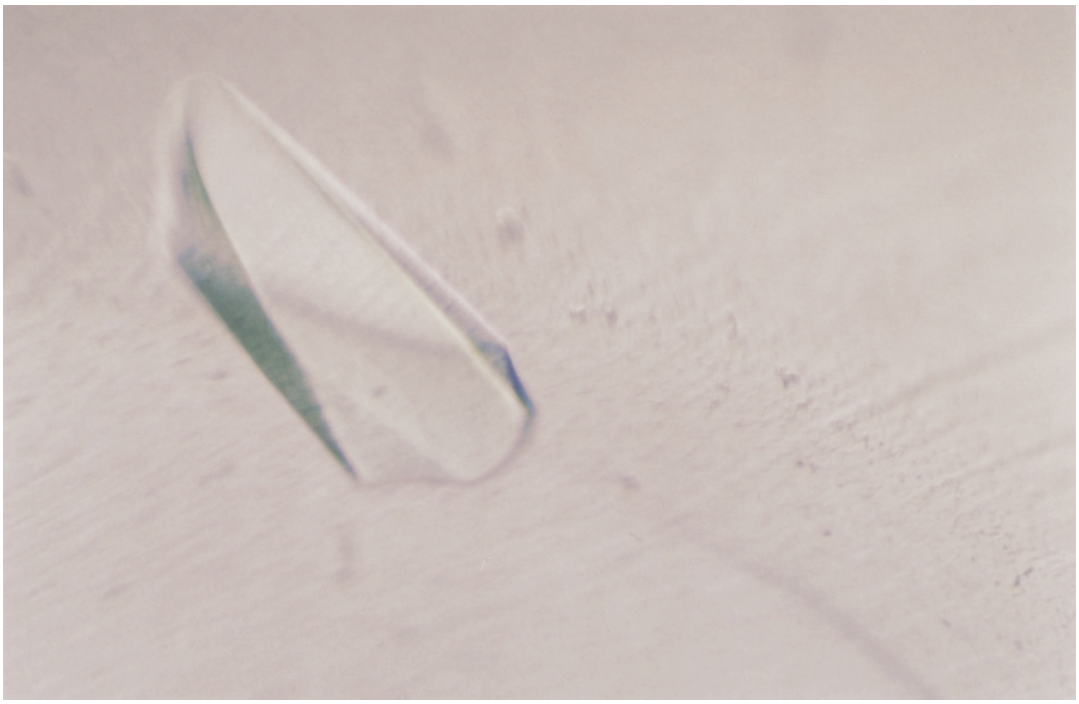

Fig. 1. Crystal of native chicken fibrinogen prepared by sitting-drop vapor diffusion with TMAO as a crystallizing agent; the crystal is approximately 0.6 mm in length. An equal volume of a chicken fibrinogen solution (6 mg/ml in 0.15 M NaCl, 0.05 M imidazole buffer, pH 7.0 containing 2 mM GPRPam and GHRPam) was mixed with an equal volume of a well solution containing 0.5 M TMAO, 2 mM $CaCl_2$ and 0.002 M sodium azide. (I am grateful to Leela Pandi for her help in growing this crystal and taking the photograph.)

tectant, methylpentanediol (MPD). Whether this was the real reason for the improvement we observed, or whether we had simply become more systematic in soaking procedures, I am not prepared to judge. Like so much else in crystallography, once you know the right conditions, you wonder why it took so long to find them.

In the end we generated TMAO-induced crystals that provided a 2.7-Å structure (Fig. 2) [30]. During the course of the study, we made numerous side-by-side comparisons with PEG-induced crystals from the same batches of protein at the same time, and never found PEG crystals that showed better diffraction than approximately 4 Å.

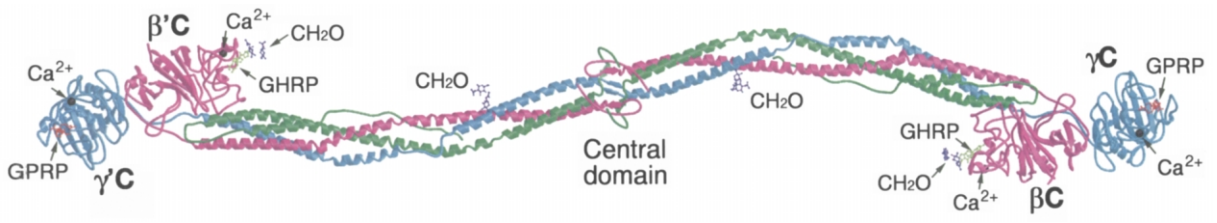

Fig. 2. Model of chicken fibrinogen at 2.7-Å resolution. The mobile αC domains and the flexible amino-terminal segments are not included in the model (from [30], with permission).

In the meantime, a review of the literature has shown that others have been using TMAO as a 'chemical chaperone' in folding studies [31], and a number of studies have attempted to elucidate how TMAO exerts its stabilizing action [32]. Whereas interactions with amino-acid side chains are indeed unfavorable, interactions with the peptide backbone are even more unfavorable, thereby pushing the equilibrium toward the folded state [33]. Whether or not TMAO will prove useful for other large and gangly proteins remains to be seen. In my view, however, it has already proved its worth, even if chicken fibrinogen alone turns out to be uniquely suited to its beneficial action.

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References

[1] J.T. Edsall, The plasma proteins and their fractionation, Adv. Protein Chem. 3 (1947) 383–479.
[2] R.F. Doolittle, J.L. Oncley, D.M. Surgenor, Species differences in the interaction of thrombin and fibrinogen, J. Biol. Chem. 237 (1962) 3123–3127.
[3] J.T. Edsall, J. Wyman, Biophysical Chemistry, vol. 1, Academic Press, New York, 1958.
[4] P.R. Morrison, J.T. Edsall, S.G. Miller, Preparation and properties of serum and plasma proteins. XVIII. The separation of purified fibrinogen from fraction I of human plasma, J. Am. Chem. Soc. 70 (1948) 3103–3108.
[5] S. Shulman, The conversion of fibrinogen to fibrin. IV. Reversible inhibition of the reaction, Arch. Biochem. 30 (1951) 353–3371.
[6] R.F. Doolittle, Structural aspects of the fibrinogen-to-fibrin conversion, Adv. Protein Chem. 27 (1973) 1–109.
[7] J.T. Edsall, J.F. Foster, H. Scheinberg, Studies on double refraction of flow. III. Human fibrinogen and fraction I of human plasma, J. Am. Chem. Soc. 69 (1947) 2731–2788.
[8] J.T. Edsall, Configuration of certain protein molecules. An inquiry concerning the present status of our knowledge, J. Polym. Sci. 12 (1954) 253–280.
[9] C.E. Hall, H.S. Slayter, The fibrinogen molecule: its size, shape and mode of polymerization, J. Biophys. Biochem. Cytol. 5 (1959) 11–17.
[10] C. Cohen, Invited discussion at symposium on protein structure, J. Polym. Sci. 49 (1961) (1960) 144–145.
[11] N.M. Tooney, C. Cohen, Microcrystals of a modified fibrinogen, Nature 234 (1972) 23–25.
[12] J.D. Ferry, The mechanism of polymerization of fibrin, Proc. Natl. Acad. Sci. USA 38 (1952) 566–569.
[13] A.P. Laudano, R.F. Doolittle, Studies on synthetic peptides that bind to fibrinogen and prevent fibrin polymerization. Structural requirements, number of binding sites, and species differences, Biochemistry 19 (1980) 1013–1019.
[14] A. Shimizu, G. Nagel, R.F. Doolittle, Photoaffinity labeling of the primary fibrin polymerization site: isolation and characterization of a labeled cyanogen bromide fragment corresponding to γ-chain residues 337–379, Proc. Natl. Acad. Sci. USA 89 (1992) 2287–2892.
[15] K. Yamazumi, R.F. Doolittle, Photoaffinity labeling of the primary fibrin polymerization site: localization of the label to γ-chain Tyr-363, Proc. Natl. Acad. Sci. USA 89 (1992) 2893–2896.
[16] C. Kuyas, A. Haeberli, P. Walder, P.W. Straub, Isolation of human fibrinogen and its derivatives by affinity chromatography on Gly–Pro–Arg–Pro–Lys Fractogel, Thromb. Haemostasis 63 (1990) 439–444.
[17] K. Yamazumi, R.F. Doolittle, The synthetic peptide Gly–Pro–Arg–Pro–amide limits plasmic digestion of fibrinogen in the same fashion as calcium ion, Protein Sci. 1 (1992) 1719–1720.
[18] S.J. Everse, H. Pelletier, R.F. Doolittle, Crystallization of fragment D from human fibrinogen, Protein Sci. 4 (1995) 1013–1016.
[19] F. Haurowitz, Das Gleichgewicht zwischen Hämoglobin und Sauerstoff, Hoppe-Seyler Z. Physiol. Chem. 254 (1938) 266–274.
[20] R.F. Doolittle, S.J. Everse, G. Spraggon, Human fibrinogen: anticipating a three-dimensional structure, FASEB J. 10 (1996) 1464–1470.
[21] S.P.S. Rao, D. Poojary, B.W. Elliot, L.A. Melanson, B. Oriel, C. Cohen, Fibrinogen structure in projection at 18 Å resolution. electron density by co-ordinated cryo-electron microscopy and X-ray crystallography, J. Mol. Biol. 222 (1991) 89–98.
[22] G. Spraggon, S.J. Everse, R.F. Doolittle, Crystal structures of fragment D from human fibrinogen and its cross-linked counterpart from fibrin, Nature 389 (1997) 455–462.
[23] V.C. Yee, K.P. Pratt, H.C. Cote, et al., Crystal structure of a 30-kDa C-terminal fragment from the γ-chain of human fibrinogen, Structure 5 (1997) 125–138.
[24] S.J. Everse, G. Spraggon, L. Veerapandian, R.F. Doolittle, Conformational changes in fragments D double-D from human fibrin(ogen) upon binding the peptide ligand Gly–His–Arg–Pro–amide, Biochemistry 38 (1999) 2941–2946.

[25] Z. Yang, I. Mochalkin, R.F. Doolittle, A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides, Proc. Natl. Acad. Sci. USA 97 (2000) 14156–14161.
[26] J.H. Brown, N. Volkmann, G. Jun, A.H. Henschen, C. Cohen, The crystal structure of modified bovine fibrinogen, Proc. Natl. Acad. Sci. USA 97 (2000) 85–90.
[27] Z. Yang, I. Mochalkin, L. Veerapandian, M. Riley, R.F. Doolittle, Crystal structure of a native chicken fibrinogen at 5.5 Å resolution, Proc. Natl. Acad. Sci. USA 97 (2000) 3907–3912.
[28] R.F. Doolittle, C. Thomas, W. Stone, Osmotic pressure and aqueous humor formation in dogfish, Science 137 (1960) 36–37.
[29] P.H. Yancey, G.N. Somero, Methylamine osmoregulatory solutes of elasmobranch fishes counteract urea inhibition of enzymes, J. Exp. Zool. 212 (1980) 205–213.
[30] Z. Yang, J.M. Kollman, L. Pandi, R.F. Doolittle, Crystal structure of a native chicken fibrinogen at 2.7 Å resolution, Biochemistry 40 (2001) 12515–12523.
[31] C.R. Brown, L.Q. Hog-Brown, J. Biwersi, A.S. Verkman, W.J. Welch, Chemical chaperones correct the mutant phenotype of the ΔF508 cystic fibrosis transmembrane conductance regulator protein, Cell Stress Chaperones 1 (1996) 117–125.
[32] T.Y. Lin, S.N. Timasheff, Why do some organisms use a urea–methylamine mixture as osmolytes?, Biochemistry 33 (1994) 12695–12701.
[33] I. Baskakov, D.W. Bolen, Forcing thermodynamically unfolded proteins to fold, J. Biol. Chem. 273 (1998) 4831–4834.