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Review

The covalent binding story of the complement proteins C3 and C4 (I) 1972-1981

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ABSTRACT

Alex Law and Paul Levine recall their work to establish the covalent bond between C3 and target surfaces. It started with a naive experiment by analyzing the membrane polypeptides of sheep erythrocytes bound with ^{125}I-labelled C3. They found complexes with molecular weight higher than the individual C3 polypeptides. These complexes survived all conditions designed to disrupt non-covalent interactions. They then showed that the bond was an ester, with an active acyl group on C3 which reacted with a hydroxyl group on the acceptor molecule. With the discovery of an internal thioester by Jim Prahl, Jamila Janatova, Brian Tack and their colleagues, it became clear that the reaction was by an acyl transfer from the thioester of C3 to the target hydroxyl group. Later on they showed that C4 also bound covalently to target molecules. By establishing a fluid phase system to study the kinetics of the binding reactions of C3 and C4, Alex was able to continue the work in the MRC Immunochemistry Unit in Oxford from 1981, to eventually determine the chemical mechanism of the binding reaction. In order to give some sense of reality, this article is written as a narrative from Alex, who did the experiments.

Both Alex and Paul are retired. Pauls lives on Martha's Vineyard where he writes occasional articles on science for one of the Island's newspapers. Alex lives in Hong Kong and tries to make some sense of the local politics.

1. Paul Levine's lab in the Department of Biology, Harvard

I was studying in Caltech (1968–1972) towards a BS degree in Physics. During that period, there was an increasingly popular opinion that the next phase of scientific advances will be in biology. Looking back, I must be quite brave, confident, foolish, or a combination of all three, to only apply to Graduate Programmes in Biology to continue my academic pursuit. I ended up in the Department of Biology at Harvard, and in research, the laboratory of Professor Paul Levine. At the time, one of the big questions was on membrane proteins. How were they different from soluble proteins, and how did they end up as integral components of membranes? The first membrane protein described to have a hydrophobic segment to anchor it through the lipid bilayer was the erythrocyte glycophorin, which was published later in 1975 (Tomita and Machesi, 1975). Paul's lab was working on photosynthesis, including the characterization of the membrane protein components of the complex photosystems. Paul was astute to realize that he would need a simpler system to answer the fundamental nature of membrane proteins.

Paul read extensively, and the complement proteins caught his attention. The complement proteins are serum proteins. When activated, they would bind to the target cells. The standard assay system for the study of complement was the lysis of sheep erythrocytes (E). Nothing would happen if serum was added to a suspension of E. However, if E was treated with rabbit haemolysin, an antibody against a sheep cell surface antigen to become EA, it would be lysed in the serum. The proteins responsible for this lysis are the complement proteins. In a monumental effort, the nine components were not only identified, but each was purified. Furthermore, the sequence of activation of the nine components, and their deposition on the EA membranes were determined. For historical reasons, the sequence was C1, C4, C2, C3, C5, C6, C7, C8, and C9 (Nelson et al., 1966)[1] .

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[1] The complement system is complex. The components C1 to C9 were first characterized in full and the activation sequence is now known as the classical pathway, which is antibody dependent. Two other antibody independent pathways have been described, namely the alternative pathway and the lectin pathway. All three pathways converge on the activation of C3, followed by the activation of the terminal membrane attack complex formed by C5, C6, C7, C8 and C9. As complement activation must be contained there are many regulatory proteins in the blood plasma and on cell surfaces to ensure that activation is focused on target surfaces and only for a necessarily short duration of time.

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2. Purification of C3 in the Austen Lab

Paul's plan was to use this system as a general approach to understand how proteins can be incorporated into membranes. As I was the new student, I was given the project. Paul's lab was not working on complement. We needed help. There were two groups in the Harvard Medical School that worked on complement, led by Professors Frank Austen and Fred Rosen respectively. Paul went to talk to them. Both were supportive but they gave the same advice that we should work with one group or the other, but not both. Eventually, Paul decided on working with the Austen group.

After some discussions, we (Paul and I) decided that we should work with purified proteins, and we chose C3. C3 is the most abundant protein in the complement system. Furthermore, how C3 bound to E coated with antibody and the active fragments of C1, C4 and C2 had been described (Müller-Eberhard et al., 1966). When C3 was cleaved into C3a and C3b[2] by the surface-bound C4b2a enzyme complex, C3a was released into the fluid phase, but only about 5–10% of the C3b would bind to the cell surface. Those C3b found in the fluid phase would forever lose the capacity to bind to the cell surface. The binding site was described as "labile" as it only made a transient appearance to enable the deposit of C3b on the cell surface (Fig. 1).

I was to learn how to purify C3 from the Austen group. Shaun Ruddy was the first person I met. (In fact, I did not meet Professor Austen till much later). Shaun was very friendly but it was Mohamed Daha, a visiting fellow from the Netherlands, who would team up with me to purify C3. We became good friends. On the first day, we rolled up our sleeves to give some blood. (This type of practice has gradually phased out in laboratories. It is probably an absolute no-no today.) It was in the spring of 1974, and for two weeks I would take the shuttle bus from the Cambridge side (the Department of Biology was housed at 16 Divinity Avenue in a building guarded by two life-sized bronze rhinoceros) to the Boston side (where Harvard Medical School was) every day. At the end, we had 10 mg of C3 which I shared with Mohamed. I also learned how to make EAC14 cells[3] and prepare functionally pure C2. In those two weeks, I also met Doug Fearon who became our contact to the Austen group after Mohamed returned to the Netherlands. We later collaborated on the characterisation of iC3b, the next degradation product of C3b by factor I and its co-factors, as a very stable C3 fragment on the surface of erythrocytes (Law et al., 1979a).

3. The first experiment on C3

Our first experiment was quite simple-minded. We would prepare EAC1423 using ^{125}I-C3. The cells were lysed osmotically and membranes were obtained. The membranes were solubilized in SDS and the membrane proteins were analysed by SDS-PAGE (the procedure was well established in the Levine lab). The gel was dried and an autoradiograph was obtained (Fig. 2). C3 is composed of two disulphide-linked polypeptides, an α chain of 110 kDa and a β chain of 75 kDa. When activated, C3 is converted to C3a, which is released from the N-terminal of the α chain, and C3b with a shortened α chain of 100 kDa and an unchanged β chain. What else could we see in the gel other than the two chains of C3b? Yes, they were there, but in addition, we also saw a smear of radioactivity (which could only come from C3) above the C3bα chain. What was it?

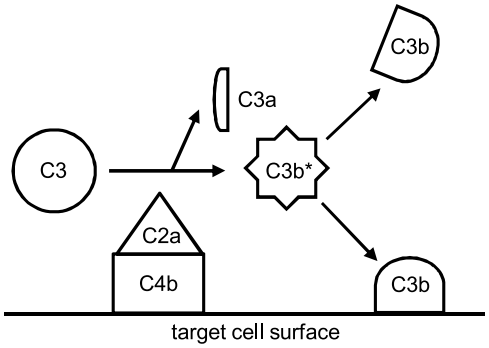

Fig. 1. The labile binding site of C3 (as understood in the early 1970s). The C3-convertase C4b2a was deposited on the target surface by (i) the binding of antibodies to surface antigen; (ii) the binding of C1 complex to the antigen-bound antibodies and the activation of C1 into the enzymatically active form; (iii) the activation of C4 by the activated C1 into C4a and C4b and the binding of C4b on the target surface; (iv) the binding of C2 to C4b and the activation of C2 by the activated C1 into C2a and C2b; (v) the formation of the C4b2a complex on the cell surface with C2a as the proteolytically active component with a serine protease module. (The scheme of the formation of the C4b2a complex, as described above, on the target surface is not shown).
C3 is then cleaved by the C4b2a complex into C3a and C3b. C3a is released into the fluid phase. About 5–10% of the C3b is bound to the target surface. The rest of the C3b is found in the fluid phase and loses its ability to bind to the target. The "activated" form of C3b, here shown as C3b*, was postulated to exist with the presence of the "labile binding site" by deduction. This description may be regarded as the summary of the work by Müller-Eberhard et al (1966). The $t_{1/2}$ of C3b* was later estimated to be in the order of 1 ms.

At the time, the C3b binding to target cell membranes was attributed to hydrophobic interactions. But it was a default description with no theoretical or experimental basis. Indeed, there were reports on C3b binding to Sepharose beads (Goldstein et al., 1976), and zymosan, the yeast cell wall polysaccharide (Nicholson et al., 1974). Neither of them had membranes. Using whole human serum on EAs, Bhakdi and co-workers had analysed the resultant membrane proteins and showed that some were of complement origin (Bhakdi et al., 1974a)[4], and they concluded that the complement proteins were likely to interact with erythrocyte membrane proteins by non-covalent forces which could survive the incubation with SDS (see our pursuit along this line later). In a subsequent paper, this conclusion was strengthened by the result that C3 and C4 peptides could be desorbed from the cell membranes by extensive incubation in high salt at 37 °C (Bhakdi et al., 1974b)[5] . Using oil droplets coated with lipopolysaccharides from *E. Coli* and human serum albumin, Stossel et al (1975) found that a bound C3 fragment (most likely iC3b, the next breakdown product of C3b by factor I and its cofactors, which was not well characterised at the time) could not be easily eluted under harsh conditions including boiling in 2 M NaCl.

We did not ignore the high molecular weight smear, but chased to

[2] In the complement nomenclature in the 1970s, when a component was cleaved into two fragments, the smaller fragment was called "a" and the larger one "b". For example, when C4 was cleaved into two fragments, the smaller fragment was C4a, and the larger fragment was C4b.

[3] EAC14 cells were prepared by the sequential addition to sheep erythrocytes (E) of hemolysin, a rabbit antibody (A) against a surface antigen on E, followed by functionally pure C1, and then C4. Similarly, EAC1423 cells were prepared by the further addition of C2 and C3 to EAC14.

[4] In their publication in 1974 (Bhakdi et al., 1974a), the authors did suggest that future work should make use of "radiolabelled complement components and/or with immune-fixation techniques". By the time it was published (August 1974), we had already started our work, and the preliminary result showing the high molecular weight smear was the key to my qualifying examination defense in September 1974. Incidentally, Bhakdi et al. did not pursue the work along the line that they had suggested.

[5] Following Bhakdi et al's second publication (Bhakdi et al., 1974b), I did similar experiments to show that C3b indeed can be desorbed from EAC1423 cells which was temperature dependent. The work was followed up by Yeldur Vankatesh, a postdoctoral fellow with Paul in Washington University. The results were published in 1984 (Venkatesh et al., 1984). We concluded that the release was due to the breaking of the ester bond. It can now be understood that it is the histidine attacking the ester bond between C3b and the acceptor molecule (Law and Dodds, 1997).

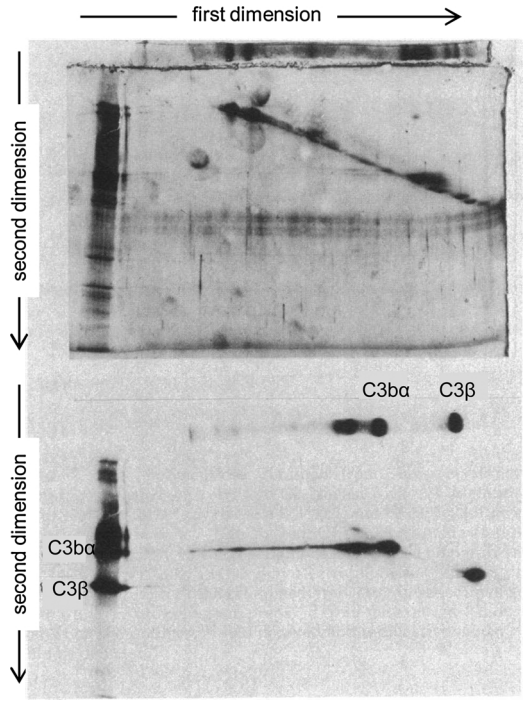

Fig. 2. Two-dimensional gel electrophoresis of EAC1423 (with ^{125}I-C3) membrane proteins. An EAC1423 membrane sample solubilized in SDS and β-mercaptoethanol was run on a SDS-polyacrylamide gel. A strip was cut out and treated with 1 M hydroxylamine, pH 9, for 1 h at room temperature. It was loaded onto a 6–12% polyacrylamide gel containing SDS for the second dimension electrophoresis. A solubilized EAC1423 membrane sample was run simultaneously with the hydroxylamine-treated strip in the second dimension electrophoresis. An identical, but untreated strip from the first dimension electrophoresis is shown horizontally on the top of the gel. Upper panel: the Coomassie blue stained gel; lower panel: the autoradiograph of the gel. The Figure was from Law and Levine (1977), but inverted horizontally (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

find out what it was. I remember the frustrations I had when I presented the results in lab meetings, to our lab members and those from other labs who were interested. The questions from the audience were all based on their reluctance to accept the idea of C3b binding via a covalent bond to cell surface molecules. They questioned repeatedly on the supposition that the interaction between C3b and molecules on target membrane was by some very stubborn non-covalent interactions able to survive the conditions designed to break them up.

"Did you have urea as well as SDS in the sample buffer when solubilizing the proteins? You should …" So I did. "Did you heat up the samples? You really should heat the sample at 100 °C for 15 min, not 5 min …" So I did. "Why did you use β-mercaptoethanol, and only at 1%? You should use a higher concentration …" So I did. "But β-mercaptoethanol is not as effective a reducing agent as dithiothreitol…" So I did. And the list went on and on, but the smear stayed. This series of queries made me realize that it was quite impossible to satisfy the critics, who would continue to find conditions that I should have considered. We needed another approach. I thought that if I could show what kind of bond it was, the question of whether the bond was covalent or not would disappear.

4. Breaking the ester bond

One possible candidate was an ester bond. I found the conditions that would break ester bonds. It would involve the incubation of the SDS solubilized sample with 1 M hydroxylamine, > pH 9.0 for 1 h at RT (or 30 min at 37 °C). When I did, the smear disappeared and only the two bands corresponding to the α and β chains of C3b remained.

If there was a covalent bond between C3b and another protein, it would require a chemical reaction to form the bond. And there should only be one bond. Thus, the active site on C3b can only be on the α chain or the β chain, but not both. If one examined the autoradiograph without hydroxylamine treatment, the smear was above the α chain, and there was nothing between the α and β chains. This gave a strong bias in the argument that the active site was in the α chain. But I thought of an experiment to settle this cleanly. This involved running a two-dimensional gel. The sample was run in one gel as usual. The gel strip was treated hydroxylamine to break the ester bonds. The gel strip would be loaded on the second gel. Proteins not affected by the treatment would appear on the diagonal. The crosslinked proteins would be dissociated into their components of lower molecular weight and would run as spots below the diagonal, and one of the spots would be radioactive and would run at the position of the α or β chain of C3b.

One evening I set out to do the experiment. I ran the sample in two lanes. Then I cut out one lane and treated it with hydroxylamine. After exchanging the buffer back into the sample buffer, the gel slice became so swollen to the point that I had to stuff it into the gap between the glass plates holding the second gel – the gel strip had broken up into pieces but they were "in place". I did anticipate that I would need a "control lane", and I made a slot in the second gel to run an untreated sample to provide some kind of a "reference". The gel was run and I left it staining before I "retired" very late in the evening.

After destaining I took the slab gel and placed it on a filter for drying. I had not forgotten the gel strip from the first gel that I had saved and put it in place. Then it was the usual autoradiography. Days later, I developed the film and the result was definitive (Fig. 2)[6] . The β chain stayed as one spot on the diagonal. But the smear had been reduced to a single line with the MW exactly as the C3bα chain, and there was no radioactivity on the diagonal above the C3bα chain. The smear was therefore the result of the C3bα chain binding to an assortment of proteins by ester bonds. In other words, we had located the "labile binding site" of C3 to the α chain, and not the β chain.

With these results, C3 had taken the centre stage of our research. Our original quest to understand how soluble complement proteins could become membrane-bound was completely derailed.

5. The Law and Levine publication

With these results, we prepared a manuscript and submitted it to the Journal of Biological Chemistry (JBC). After several weeks, when we did not receive any response from the journal, Paul placed a call to the editor. As Paul recalls, the editor told him that he did not believe our result. Soon after, the formal rejection letter came with a review. The review was quite long (as I recall, it was one-and-a-half page, single-spaced), and it made absolutely no sense. (It would make interesting reading now but Paul and I failed to track down a copy of the letter.) We had debated if we should publish the results without saying that the bond was covalent. It was late in the afternoon on the last day of 1976. After going through all my results I went to talk to Paul, who was sitting in his small office. I told him that the results all pointed to the bond being covalent, and most likely an ester. He agreed and we were to submit the manuscript to another journal. I also remember that I had friends visiting for the new year holiday and I left the lab to join the party.

[6] There were two other interesting points of the 2-d gel, which had nothing to do with the science. One was that the experiment was done in the mid-1970s, when we did not wear gloves as a standard practice. And I did not. In the Coomassie blue stained gel, there were smudges. They were my fingerprints – the definitive proof that I did the experiment. After the work was published, I can claim to have achieved something that may be considered unique among fellow scientists – i.e. I have my fingerprints published in the PNAS.

Eventually, we convinced Alwin Pappenheimer and he communicated for us to the Proceedings of the National Academy of Sciences USA (PNAS). The article was published in 1977 (Law and Levine, 1977). Looking back, we were extremely grateful to Professor Pappenheimer. Without his help, the article may never have been published. Alternatively, like many others, it may end up a watered-down version published in some obscure journal.

6. The 7th Complement Workshop in St Petersburg, Florida, 1977

There were regular complement workshops and it was the first one that I participated. Unlike these days, graduate students, provided that the work was good, would be treated like "professionals". Our abstract was chosen for an oral presentation, and I was going to give the talk.

The talk went well. I was also pleased to find that I was able fend off questions from the audience. At the time of the meeting, many of those present were unknown to us. Paul remembers that someone told him that the covalent binding of C3 to target cells was already known, but when he asked where it was published he got no answer. Paul also recalls a chat in the back of the room with Frank Austen, who said that no one would believe the bond was covalent unless we could prove it. Well, we were on the right track, but we needed more experimental support.

Putting things in perspective, our conclusion was not universally accepted, but we became the centre of attention in the conference. Essentially, there were two camps. One considered our work was a breakthrough in the biochemistry of complement in that we had provided, for a first time, a plausible account on the physical and chemical nature of complement fixation. The other thought we (Paul and I) were crack-pots, and our results were some kind of artefacts. Looking back, it was what made my career. Paul was already a full professor at Harvard, and I was just a graduate student. But everyone wanted to talk to us, and Paul was happy to stay on the sideline and watch. The consequence was that everyone in the complement field knew that there was someone called Alex Law doing something "interesting".

7. My PhD thesis

I still needed some more work to pad up my PhD thesis. An ester bond can have two orientations. Was it a carboxylic group on C3 forming an ester bond with a hydroxyl group on the target, or a hydroxyl group on C3 attacking a carboxylic moiety on the target surface?

Zymosan (Z)[7] was known to activate human complement via the alternative pathway efficiently. Since C3 can bind covalently to targets, we did not even need to purify C3 for this experiment. We incubated Z with serum, and C3 fragments would bind covalently to Z. The bound C3 fragments would be reduced to C3d by extensive treatment with trypsin. We postulated that C3d bound to Z would either be in the form of C3d-CO-O-Z, or C3d-O-CO-Z. C3d can be released by hydroxylamine from Z, either as a C3d-CO-NHOH (hydroxamate) from C3d-CO-O-Z, or C3d-OH from C3d-O-CO-Z. After a very complex experiment,[8] we did show that there was 0.66 mol of hydroxyamate per mole of C3d, and we were able to conclude that the orientation of the ester bond was C3d-CO-O-Z. This meant that there was an active carboxylic group on C3 that somehow bound to hydroxyl groups on the target surface (Law et al., 1979b).

The big question was how to form an ester bond. Forming a bond is energetically not favourable. Perhaps there was already a bond in C3 and the bond formation would be by a transesterification mechanism, making the reaction energetically neutral. This was not unthinkable as it was known that one way to inactivate C3 was to treat it with hydroxylamine and simple ammonia compounds (Gordon et al., 1926). But there was a publication reporting that C3 inactivated by hydroxylamine was by releasing C3a, i.e. breaking a peptide bond just like the C3-convertases (Budzko and Müller-Eberhard, 1969). I tried hard to formulate a scheme that would break a peptide bond as well as an ester bond but I got nowhere. At the end, I repeated their experiment to find out. C3 was inactivated (i.e. loss of haemolytic activity) by hydroxylamine, but there was no release of C3a. The results were not published, and they were not included in my PhD thesis. This had added another lesson in my scientific training: a claim needs the support of reliable experimental results as well as sound theoretical backing. If they are incompatible, one of them must be incorrect.

With these results, I had enough to write my thesis. Two things happened that were "memorable". The first was that *Nature* sent me two manuscripts to review[9] . Paul just let me handled it. As I recall, the experiments in the manuscripts were done properly, but they did not provide any new insights beyond what we had published. I did not recommend publication – I was still a graduate student waiting to get his PhD.

The other was a letter from Brian Tack. Brian was a member of a team led by Jim Prahl in Utah. They had been working on C3 and C4. Brian had left the group and was trying to establish himself in the National Institutes of Health (NIH). He asked me to continue the work on C3 with him at the NIH, realizing that I was about to get my PhD and would need to find a post-doctoral position. He invited me to visit him at Bethesda and I went. Paul did not stop me from going. Brian put an offer on the table.

During my visit, Brian told me that they (Prahl's team) had found an interesting thiol group, which was not detected in C3, but made its appearance when C3 was activated to C3b or when C3 was inactivated by other means. Together with our results, it became very clear that the "labile binding site of C3″ started as an internal thioester, and that the covalent binding is by way of a transesterification mechanism from the thioester. Brian was fully aware of the possibility that I may return to Harvard and do the experiment myself. (The story on the discovery of the thioester will be covered in an article in this volume by Jamila Janatova, another member of the Prahl team.)

I went back to Harvard and told Paul what I have learned. We both agreed that we would not work on the thiol group until Prahl's group published (Tack et al., 1980)[10] . Paul, in a fatherly manner, asked me how I felt. I told him that there must be others thing that we could do. It

[7] Zymosan is a protein-carbohydrate complex prepared from yeast cell wall. It has the advantage over sheep erythrocytes as C3 binding target because of its particulate form and is insoluble. To ensure that only C3d was released from the Z under alkaline (with or without hydroxylamine) and in SDS, Z was pretreated at 100 °C in 0.15 M NaCl for 30 min, followed by 0.1 M NaOH with 1% SDS at 37 °C for 1 hr, then again at 100 °C in 0.15 M NaCl for 30 min. After these treatments, the pellet size of Z would be reduced significantly, but it retained the capacity to activate the alternative pathway of complement (Law et al., 1979b).

[8] C3d released by hydroxylamine would be in the form of C3d-CO-NHOH. When treated with iodine, a nitrate molecule would be generated, to react with sulfanilic acid to form a diazonium salt. The diazonium salt, when coupled with α-naphthylamine, would yield a pinkish compound with an absorbance at 520 nm. The background level of hydroxamate on C3d determined on C3d be

(*footnote continued*)
released from Z by incubation at pH 11.5, and the resultant C3d-COOH treated with hydroxylamine. The background was high, as was the standard error, but we managed to show that there was a 0.66 ± 0.12 mole of hydroxamate per mole of C3d within acceptable limits.

[9] It was interesting that *Nature* sent the manuscripts to me to review, not Paul. At that time, there was no declaration of the work contribution of the authors. My guess was that they just sent to me as the "first" author. Today, there is no doubt that they would send them to Paul as the "corresponding" or "senior" author.

[10] The Tack et al publication was not out until 1980. At the time, many groups caught on and publish similar findings on C3, C4, and α_2-macroglobulin (see Law, 2008). We held out ours until late in 1980 (Law et al., 1980b), and we were able to cite the Tack et al paper.

was therefore most satisfying that I solved the whole covalent binding reaction problem some 18 years later (Dodds et al., 1996).

Is C3 some kind of an enzyme? In the draft of my thesis, I made a suggestion that it may be a "half-enzyme". The examination committee did not like it, citing the very basic property of an enzyme was that it had to "turnover", i.e. it would not be used up and go on to catalyze the conversion of more substrates to products. I was asked to take it out of my thesis, and I did. Even with the thioester, the standard description, until my definitive work, was that activation of C3 would involve the exposure of the internal thioester, followed by a nucleophillic attack by hydroxyl groups that would complete the binding reaction. However, the mechanism of the binding reaction is more intricate (Dodds et al., 1996; Gadjeva et al., 1998). It starts with an intramolecular attack of the thioester by a histidine, resulting in an acyl-imidazole group and a thiolate anion, which then catalyzes the reaction of a hydroxyl group, of any compound including water, with the acyl-imidazole to yield the final ester-bonded complex. There is but one conclusion that C3 does act as an enzyme on itself. To put it succinctly, C3 is not only an enzyme, but it is also the substrate and product of the reaction, taking on all three roles roll into one. I am not sure there is a term for it. Perhaps it could be called an autozyme[11] ?

It was 1978, and it came to us (members of Paul's lab) as a big surprise that he and Ursula (Goodenough, Paul's wife) were offered positions at the Washington University in St. Louis – Ursula at the University, and Paul at the Department of Genetics in the Medical School. With such a move, it was understandable that Paul would want his "capable" team members to go with him to set up his new lab. I had to decide to join Brian at the NIH or stay with Paul. At the end, I decided to go to St. Louis with Paul.

8. Washington University School of Medicine, St. Louis

We continued to work on this project after we moved to St. Louis. There were two pieces of work that were significant in the covalent binding story. The first one was the demonstration of C4 also bound to target via a covalent bond, and the other was the setting up of a fluid phase binding assay for C3 and C4.

9. C4 and C5

In the complement system, C3, C4, and C5 are similar. They have high molecular weight up in the region of 200 kDa. Like C3, C5 has two chains, α and β, and C4 has an additional γ chain. Activation is by the proteolysis of the α chain and a peptide of about 75 amino acids from their respective N-terminus is released. Do C4b and C5b also bind covalently to their targets? The criteria of an ester bond had been established with C3b, it was obvious to conduct similar experiments on C4 and C5. EAC14 cells were prepared with ^{125}I-labeled C4, and EAC14235 cells with ^{125}I-labeled C5. The membrane proteins of the cells were subjected to SDS-PAGE followed by autoradiography. The result for C5 was clear enough: there was no high MW smear, and therefore no covalent binding. But there was a high MW smear for C4. A two-dimensional gel was run for the C4 sample, and after treatment with hydroxylamine, the α chain, like that of C3b, did appear as a band off the diagonal (Fig. 3). We concluded that C4b also binds by way of an ester bond (Law et al., 1980a).

If we deserved all the credits for not ignoring the smear in the original experiment with C3, we were definitely guilty of jumping to conclusions in our C4 work. We focused our attention on the horizontal band at the C4bα position and concluded that C4b must bind to the erythrocyte membrane proteins with the same ester bond as C3b. We completely overlooked the radioactive spots along the diagonal. At the time, it was already known that there were two types of C4 coded by two tandem gene loci, called C4A and C4B respectively, in the Major Histocompatibility Complex (O'Neill et al., 1978). We failed to make the mental quantum leap to suggest that the two forms of C4 bound to targets differently. One was C3b-like, binding to targets by an ester bond, and the other by a hydroxylamine-resistant bond, most likely an amide bond. It had to wait a few more years before I returned to the problem after I moved to Oxford (Law et al., 1984a).

In retrospect, it is the only satisfactory way to account for the versatility of C3 and C4 to bind to all kinds of biological molecules, be they glycoproteins and glycolipids on membrane surfaces, or viruses with only a protein envelope, or immune complexes made up of proteins and glycoproteins. They would invariably have hydroxyl and amino groups that can serve as acceptor moieties for the active carboxylic group on C3 and C4. This is a very simple but universal solution for the immune system to put a mark on its targets, to label them for subsequent actions such as the activation of the terminal membrane attack complex of complement, and the ingestion by phagocytes via their receptors for C3 and C4. But how are C3 and C4 prevented from binding to cells of our own body? The binding of C3 and C4 is limited to the surfaces on which they are activated. Although not known to have a thioester, this concept was established as the labile binding sites of C3 (and C4) by Müller-Eberhard et al. (1966). Now we know the presence of a thioester in C3 and C4. Thus, when the activated C3 and C4 diffuse away from the activating surfaces, the water in the intercellular fluids would act as a protective layer by hydrolysis of the activated thioester. Our own cells, which do not activate complement except in pathological conditions, are essentially freed from the deposition of C3 and C4. Should C3 and C4, in their respective derivatives in the form of C3b and C4b, be somehow deposited on our own cells, there is a plethora of molecules in the intercellular fluid and on the surfaces of these cells that would act on C3b and C4b to prevent them from activating the later components of the complement sequence.

10. The fluid phase binding assay

C3b was known to bind to target cell surfaces via a "labile binding site" (Müller-Eberhard et al., 1966). After the description of the thioester, it was understood that binding was due to the transient exposure of the thioester, which either reacted with the hydroxyl groups on the cell surface, or underwent hydrolysis in the fluid phase. However, simply exposing the thioester was insufficient to account for the very transient nature of the "reactive" form of C3b, the half-life of which was too short to be measured. An exposed thioester of C3b, based on simple molecules with a thioester, should have a half-life in the order of 10 s of minutes. We needed to have a system to study the kinetics of the binding reaction.

Up to that point, most binding experiments were done on the standard sheep erythrocytes, or other particulate targets such as zymosan. Some estimates were made based on molecular diffusion in the vicinity of the cell surface and a half-life of 60 μs was reported for the reactive form of C3b (Sim et al., 1981). My argument against this approach was that the cell surface was like a jungle with all kinds of molecules and free diffusion was impossible to define. I therefore designed an experimental system such that the binding reaction can be measured in the fluid phase. C3 would be cleaved into C3a and C3b with trypsin, and C3b would not be further cleaved if we kept the time short, such as less than 5 min, at which point, an excess of soybean trypsin inhibitor would be added. The system would only have purified C3, trypsin, and a radioactive small molecule with a free hydroxyl group, such as glycerol. (Later on glycine would be used as a small molecule with an amino group.) After the reaction, the sample would be run in an SDS gel. The radioactivity associated with the gel slices containing the α and β chains of C3b would be determined. Specific binding would be the radioactivity associated with the α-chain whereas background would be that associated with the β-chain. The standard

[11] The word "autozyme" has been already used for some healthcare product to relieve gastrointestinal problems.

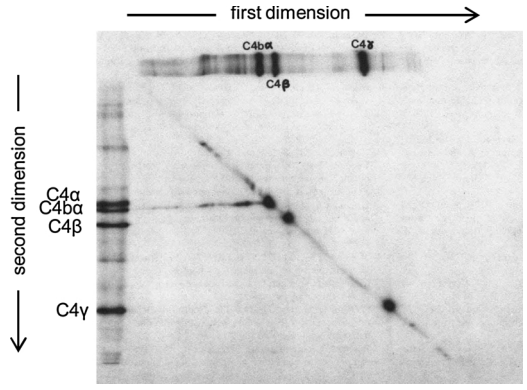

Fig. 3. Two-dimensional gel electrophoresis of EAC14 (with ^{125}-I-C4) membrane proteins. The result was obtained as with C3 in Fig. 2 except ^{125}I-labeled C4 was used on EAC1 cells. Only the autoradiograph (reproduced from Law et al., 1980a) is shown. The markers in the second dimension were a mixture of C4 (with C4α, C4β, and C4γ chains) and C4b (with C4bα, C4β, and C4γ chains).

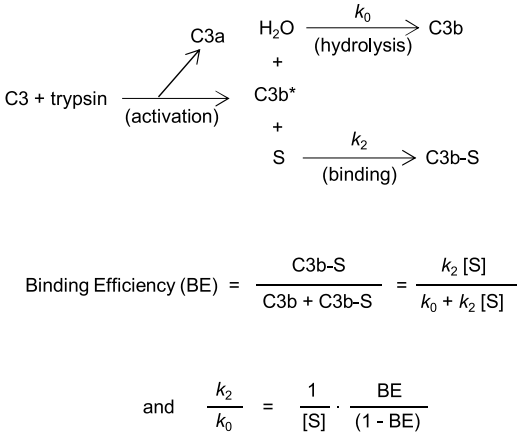

Fig. 4. The binding reaction of C3 with kinetics parameters. "S" is the (radioactive) small molecule (glycerol or glycine) in the reaction. The activated C3b, C3b*, either reacts with water, or bind to the S. The binding efficiency (BE) can be determined for a range of concentration of S, [S]. k_0 is the hydrolysis rate of the thioester in C3b*, and k_2 the reaction rate of C3b* with S. k_2/k_0 can be expressed as a function of BE and [S].

buffer[12] for the binding reaction was 10 mM phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.5.

The binding reaction would be written with the "activated C3b" binding to the radioactive small molecule with a second-order rate of k_2 (k' in earlier publications), and its hydrolysis with a first-order rate of k_0. With the binding efficiency only determinable at the end-point, what we can obtain was the ratio of the two rates. k_2/k_0 was first described in publication in *Biochemistry* (Law et al., 1981) (Fig. 4).

Using this assay system we were able to determine the binding profiles of C3 and C4 (still as a mixture of C4A and C4B) to hydroxyl group (glycerol) and amino group (glycine) were very different (Law et al., 1984b). It was also essential for the subsequent work in determining the very slow k_0 for C4A, and k_0 for C4B remained too fast to be determined. This led to the conceptual breakthrough that the binding reaction and hydrolysis of the thioester were catalyzed for C4B whereas that of C4A was passive (Sepp et al., 1993). The significance of this work had been under-appreciated with a total citation of only 23, but it was the crucial step leading to final determination of the chemical mechanism of the binding reactions of C4A and C4B (Dodds et al., 1996), and C3 (Gadjeva et al., 1998).

After three years in St Louis, I left for England to join Professor Rodney Porter's Immunochemistry Unit in the Department of Biochemistry at Oxford. The details can be found in part (II) of the story (Law, 2008).

[12] I have shown that Tris, with its three hydroxyl groups, would compete with the glycerol in the binding reaction (result not published). In the case when we need to determine the binding reaction as a function of pH, a composite buffer with acetate/phosphate/borate would be used (Law, 1983; Law et al., 1984b).

Declaration of Competing Interest

The authors have no competing interests to declare.

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