The human complement system: assembly of the classical pathway C3 convertase

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The assembly of the classical pathway C3 convertase in the fluid phase has been studied. The enzyme is assembled from C2 and C4 on cleavage of these proteins by C1s. Once assembled, the enzyme activity decays rapidly. Kinetic evidence has been obtained that this decay is even more rapid than previously suggested (k_{decay} is 2.0 min^{-1} at 37°C). As a result, optimal C3 convertase activity is only observed with high C1s levels, which result in rapid rates of cleavage of C2 and increased rates of formation of the C3 convertase. Using high concentrations of C1s at lower temperatures (22°C) in the presence of excess substrate we have demonstrated kinetically that the enzyme comprises an equimolar complex of C4b and cleaved C2. We have obtained direct evidence from gel-filtration experiments for the role of C2a as the catalytic subunit of the enzyme. C2b appears to mediate the interaction between C4 (or C4b) and C2 at pH 8.5 and at low ionic strength where the interactions can easily be detected. It may therefore be important in the assembly of the enzyme, though it is not involved in the catalytic activity. The decay of the C3 convertase reflects the release of C2a from the C4b·(C2b)·C2a complex, and the stabilizing effect of iodine on the C3 convertase is therefore apparently one of stabilizing the C4b·C2a interaction, which is otherwise weak. C1s is not a part of the C3 convertase enzyme.

Activation of the third component of complement (C3) is the central event of the complement system. It is effected *in vivo* by either of two complex proteolytic enzymes, the C3 convertases, which are generated by two separate enzyme cascade systems, the classical and alternative pathways. Both C3 convertases, in the presence of the product of the reaction they catalyse, namely C3b, are subsequently able to cleave the fifth component of complement, C5. (For reviews see Porter & Reid, 1979; Müller-Eberhard, 1978.) The classical pathway C3 convertase is generated from the second and fourth components of complement in an Mg^{2+} -dependent reaction catalysed by the serine protease C1s of the C1 complex. The enzyme, once assembled, is highly unstable, decaying with a half-life of 10–20 min. Although the individual components have been determined, the mechanism of assembly and mechanism of action of this complex enzyme remain unclear, and the molecular basis of the biologically important decay process is also uncertain.

Abbreviations used: EAC14 cells are sheep erythrocytes sensitized with rabbit anti-(sheep erythrocyte) antibody coated with C1 (guinea-pig) and C4 (human) (Nelson *et al.*, 1966); Pipes, 1,4-piperazinediethanesulphonic acid.

Müller-Eberhard *et al.* (1967) first showed that the normally membrane-bound C3 convertase could be assembled in cell-free solution from a mixture of C2, C4 and C1s. C1s was shown to cleave C2 into C2a (mol.wt. 80000) and a proposed, though undetected, fragment C2b (Polley & Müller-Eberhard, 1968a) and to cleave C4 into C4a (mol.wt. 8000) and C4b (mol.wt. 198000). Polley & Müller-Eberhard (1967) also demonstrated that the C3 convertase could be stabilized by prior treatment of the C2 with low concentrations of iodine. Although the molecular basis of this stabilizing reaction is not fully understood, it is believed to involve the oxidation of thiol groups to a disulphide bond (Polley & Müller-Eberhard, 1968b). Using this chemically modified enzyme Polley & Müller-Eberhard (1968a) were able to isolate the enzyme and to propose from the apparent molecular weight of the C3 convertase that it was an equimolar complex of C4b and C2a. Further progress has been hampered by the unavailability of sufficient quantities of highly purified C2, but the development of purification schemes that yield milligram amounts of C2 (Nagasawa & Stroud, 1977; Kerr & Porter, 1978) have allowed the detection of both fragments of C2, thus making possible a more detailed study of the function of C2 in the C3 convertase.

Nagasawa & Stroud (1977) suggested that the C2b fragment may play a rôle in the assembly of the C3 convertase in that under certain conditions a molecular complex of C4b and C2b could be detected. I have since isolated and characterized both fragments of C2 with regard to molecular weight, amino-acid composition and *N*-terminal sequence analysis, identifying the two fragments as representing separate domains within the C2 molecule, C2b being the *N*-terminal region (Kerr, 1979).

In the light of these results I have re-investigated several of the properties of the fluid-phase C3 convertase. I have studied the kinetics of assembly and decay of the enzyme and have obtained from gel filtration studies direct evidence for the separate function of the two fragments of C2 in the enzyme.

Materials and methods

Materials

The sources of materials used in the purification and characterization of the proteins have been described previously (Kerr & Porter, 1978; Kerr, 1979).

Methods

C2 was purified from outdated human plasma by the method of Kerr & Porter (1978) as modified by Kerr (1979). In some cases, C2, C3 and C4 were purified from the same preparation with the following modifications.

(a) Euglobulin precipitations at pH 7.4 and 5.5 were carried out by the method of Gigli *et al.* (1977).

(b) The pseudoglobulin fraction was subjected to chromatography on CM-Sephadex C50 and to $(\text{NH}_4)_2\text{SO}_4$ fractionation as described in Kerr & Porter (1978); the pellet from the 50%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation step was then used for the purification of C3 and C4 and the pellet from the 75%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation was used for the purification of C2 (and factor B).

(c) For the purification of C3 and C4, the pellet from the 50%-satd. $(\text{NH}_4)_2\text{SO}_4$ step was resuspended in 0.4 M-sodium phosphate, pH 6.0, and equilibrated with 0.05 M-sodium phosphate pH 8.0, by passage through Sephadex G-25. The sample was then applied to a column (20 cm $\times$ 5.0 cm) of DEAE-Sephadex A-50 equilibrated in the same buffer. The column was developed with a linear gradient (2 $\times$ 500 ml) from 0.05 M-sodium phosphate, pH 8.0, to 0.05 M-sodium phosphate/0.15 M-NaCl, pH 8.0. The C3 was eluted by a further 500 ml wash with this limit buffer and then C4 was eluted by 0.4 M-sodium phosphate, pH 6.0. [All buffers contained 0.5% (w/v) benzamidinium hydrochloride.]

(d) C3 was further purified by chromatography on hydroxyapatite as described by Tack & Prahll (1976). C4 was purified by gel filtration on C1-

Sephadex 6B. Both proteins were homogeneous by the criterion of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

C1s was purified by the method of Gigli *et al.* (1976).

C4b was prepared by incubation of C4 with C1s (molar ratio 100:1) at 37°C for 15 min. The C1s was inactivated by two treatments with di-isopropyl fluorophosphate (10 mM, 37°C for 30 min) and then the inhibitor was removed by gel filtration on Sephadex G-200, which also removed aggregates of C4b (Reboul *et al.*, 1979). The product, C4b with C4a non-covalently bound (Budzko & Müller-Eberhard, 1970; Patrick *et al.*, 1970), is for simplicity referred to throughout this paper as C4b. There is no evidence that C4a plays any role in convertase activity (Patrick *et al.*, 1970).

Iodine-treated C2 was prepared by incubation of C2 in 0.1 M-sodium phosphate, pH 6.0, with iodine (10 μM in 24 μM -KI at 4°C for 15 min). The product was subjected to gel filtration on Sephadex G-25 equilibrated in 5 mM-veronal buffer (pH 7.5)/0.3 mM- CaCl_2 /1.0 mM- MgCl_2 /0.15 M-NaCl and was used immediately.

In the absence of other data all protein concentrations were determined by using an assumed $A_{280}^{1\%}$ of 10.0.

Fractions from gel filtration columns were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The gels were run according to Laemmli (1970), stained using Coomassie Brilliant Blue and scanned using a Gilford scanning densitometer.

Haemolytic assays for C2 and C4 were carried out as described previously (Kerr & Porter, 1978) except that all volumes were decreased to one-fifth. C3 haemolytic assays were a modification of the method of Tack & Prahll (1976). C3 solutions, 0.1 ml of dilutions in gelatin/veronal/ Ca^{2+} / Mg^{2+} buffer (Nelson *et al.*, 1966) were incubated for 1 h at 37°C with 0.2 ml of 1:40 diluted KBr-treated human serum (Tack & Prahll, 1976), 0.1 ml of EAC14 cells (10^6 cells/ml) and 0.1 ml of functionally pure guinea-pig C2 (10000 haemolytic units/ml) in the same buffer. After 1 h, 1.0 ml of cold (0–2°C 0.15 M-NaCl) was added and, after centrifugation, the degree of lysis was measured from the A_{410} of the supernatant. The addition of guinea-pig C2 increased the sensitivity of the assay and overcame anomalous results due to excess human C2 in those convertase assays where it was present.

Results

Cleavage of C2 and C4 by C1s

Incubation of highly purified C2 with catalytic amounts of C1s leads to the loss of haemolytic activity. This loss of activity is the result of cleavage

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified components*

Sodium dodecyl sulphate/polyacrylamide gels were run as described by Laemmli (1970). Samples of (a) C2, 2.5 μ g; (b) C2, 2.5 μ g incubated with C1s, 5 ng, at 37°C for 1 h; (c) C4, 5.0 μ g; (d) C4, 5.0 μ g, incubated with C1s, 5 ng, at 37°C for 1 h; (e) C4, 5.0 μ g, and C2, 2.5 μ g, incubated with C1s, 5 ng, at 37°C for 1 h were treated with either 20 mM-iodoacetamide/8 M-urea/2% (w/v) sodium dodecyl sulphate/0.2 M-Tris/HCl, pH 8.0 (Non-reduced) or 10 mM-dithiothreitol in the same buffer (Reduced) before electrophoresis.

of the single polypeptide chain into two fragments C2a and C2b (see Fig. 1). The rate of inactivation of C2 by C1s in the presence of C4 (molar proportions C4:C2:C1s, 100:100:1) is the same as in the absence of C4 and the products of C2 cleavage, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, are also the same. The rate of destruction of C4 haemolytic activity in such mixtures, which is some 10–20 times faster than that of C2, is similarly unaffected by the presence or absence of C2. This loss of haemolytic activity reflects the cleavage of a peptide from the largest of the three polypeptides of C4 (Schreiber & Müller-Eberhard, 1974) (see Fig. 1). The rates of cleavage of C2 and C4 independently, or in a mixture of the two, are the same in buffers containing Ca^{2+} and Mg^{2+} or in buffers containing 10 mM-EDTA.

Generation of C3-cleaving activity in solution from C4, C2 and C1s

In preliminary experiments, combinations of C4, C2 and C1s in the presence of Ca^{2+} and Mg^{2+} were incubated for 5 min at 37°C and then samples were removed and added to a solution of C3 in 10 mM-EDTA. After incubation at 37°C for 15 min, the remaining C3 haemolytic activity was assayed, or

the amount of conversion of C3 α -chain to C3b α' -chain was determined by scanning of sodium dodecyl sulphate/polyacrylamide gels. This assay for C3 convertase, which was chosen for its similarity to the haemolytic assays used for the classical pathway components, confirmed the basic properties of the enzyme characterized in previous studies, namely (a) the fluid-phase C3 convertase could be assembled from purified C4, C2 and C1s in the presence of Ca^{2+} and Mg^{2+} , but not in the presence of 10 mM-EDTA; (b) in the absence of any one of the components no convertase was formed and if the C1s was inactivated by prior incubation with di-isopropyl fluorophosphate (10 mM for 1 h at 37°C) no C2 or C4 cleavage occurred and no convertase was formed; (c) prior incubation of C2 or C4 with di-isopropyl fluorophosphate had no apparent effect; (d) incubation of C2 with C1s followed by the addition of C4 resulted in the formation of no convertase, whereas incubation of C4 with C1s followed by the addition of C2 had no deleterious effect on the formation of convertase.

Time course for the generation of fluid-phase C3 convertase

The preliminary experiments were extended to

study the time course for the assembly and decay of the C3 convertase and to investigate the molecular state of the C2 and C4 during these reactions. C2, C4 and C1s (molar proportions 500:500:1) were incubated for various times at 37°C in buffers containing Ca^{2+} and Mg^{2+} , then samples were removed and added to C3 and C3 destruction was determined as described previously. The results are shown in Fig. 2. C3 convertase was generated rapidly and then decayed with a half-life of around 20 min, behaving similarly to the cell-bound enzyme (Kabat & Mayer, 1963). Maximal C3 convertase activity was observed when most of the C4 had been cleaved but at this time only a small percentage of the C2 had been cleaved. The decay of convertase activity was complete when all the C2 have been cleaved, but, upon the addition of a second sample of C2 to a fully decayed C3 convertase preparation, a second activation-decay curve was seen of equal magnitude to the first. Addition of a second sample of C4 or C4b to decayed convertase produced no such activity. C3 convertase activity could also be assembled from C4b and C2 on incubation with C1s. Under these conditions considerable convertase was generated within the mixing time of the proteins, although a true zero-time value obtained by addition of each component directly to C3 in EDTA gave no C3 destruction.

Increased C1s and therefore increased C2 and C4 cleavage rates resulted in the formation of more C3 convertase activity at early time points, but the activity decayed more rapidly. In those samples containing less C1s, where activity remained after 30 min, uncleaved C2 was detected by sodium dodecyl sulphate/polyacrylamide gels or haemolytic assay. Incubation of C4 or C4b with C2 in the presence of C1s inactivated by prior incubation with di-isopropyl fluorophosphate or *p*-nitrophenylguanidinobenzoate gave no C3 convertase activity.

Kinetics of assembly of the C3 convertase

The observation of maximal C3 convertase in samples where only a small amount of C2 cleavage had occurred led us to investigate in more detail the early events of the activation-decay cycle to exclude the possibility of a rôle for uncleaved C2 (or C1s) in the convertase activity. The C3 convertase assay was also modified such that rates of C3 destruction could be measured. Because of difficulties in measuring initial rates of C3 destruction (due to limitations of the haemolytic assay) and because the relatively low substrate concentration (50–200 times that of the enzyme) probably precludes such measurements, we have expressed C3 destruction in terms of first-order kinetics. Subsequent studies using C3 convertase made stable over several days by prior treatment of the C2 with iodine showed the validity of such an approach. This stable enzyme showed

Fig. 2. Formation and decay of C3 convertase activity. C4 (80 µg), C2 (40 µg) and C1s (80 ng) in 850 µl of 5 mM-veronal (pH 8.5)/0.5 mM- CaCl_2 /2.0 mM- MgCl_2 /40 mM- NaCl were incubated at 37°C. (a) Samples (20 µl) were removed at different times and added to 20 µl of 10 mM-dithiothreitol/8 M-urea/2% (w/v) sodium dodecyl sulphate/0.2 M-Tris/HCl, pH 8.0. The degree of conversion of C2 to C2a (O) or C4 α-chain to C4bα'-chain (●) was quantified by scanning of sodium dodecyl sulphate/polyacrylamide gels. (b) Samples (20 µl) were simultaneously assayed for C3 convertase activity by addition to 100 µl of C3 (150 µg/ml) in 5 mM-veronal/10 mM-EDTA, pH 7.5. After incubation at 37°C for 15 min, the degree of C3 destruction was determined by haemolytic assay (●) or by scanning of sodium dodecyl sulphate/polyacrylamide gels of samples treated with the same dithiothreitol/sodium dodecyl sulphate/urea/Tris buffer (O).

first-order kinetics with regard to substrate (C3) cleavage over at least five half-lives (M. Kerr, unpublished work).

The effect of increased C1s and therefore increased C2 cleavage on the assembly of C3 convertase from equimolar C2 and C4b in the presence of excess substrate is shown in Fig. 3(a), and the amounts of C2 remaining in the incubation mixtures are shown in Fig. 3(b). Clearly the

increasing $\text{C}\bar{\text{I}}\text{s}$ concentration increases the amount of C3 convertase formed. At lower $\text{C}\bar{\text{I}}\text{s}$ concentrations there is a marked downward deviation from first-order kinetics which is due to continual formation of the C3 convertase by cleavage of the C2; at higher concentrations of $\text{C}\bar{\text{I}}\text{s}$ the cleavage of C2 is rapid and the lag phase is removed. Under such conditions it is evident that for short periods after the cleavage of all the C2 there is no apparent decay of C3-cleaving activity, and the properties of this stable C3 convertase can therefore be studied. The rate of cleavage of C3 after the cleavage of all the C2 is independent of $\text{C}\bar{\text{I}}\text{s}$ concentration, suggesting no role for the $\text{C}\bar{\text{I}}\text{s}$ in the C3 convertase other than that of cleaving C2 and C4.

Similar experiments carried out with constant equimolar C4b and $\text{C}\bar{\text{I}}\text{s}$, varying C2, or constant C2 and $\text{C}\bar{\text{I}}\text{s}$, varying C4b, showed no deviation from first-order kinetics for C3 destruction. Both results show increasing C3 convertase activity with increasing C2 or C4b up to an approximate ratio of 1:1 (Fig. 4). The lack of further increase in C3

convertase activity on increasing C2 to above the level of C4b is further evidence for the lack of decay of the convertase under these conditions, since if cleaved C2 in decayed convertase was replaced by uncleaved C2 then it would be expected that excess C2 would increase C3 convertase activity. The results are therefore compatible with a hypothesis that C3 convertase comprises an equimolar complex of C4b with cleaved C2. $\text{C}\bar{\text{I}}\text{s}$ is essential for the cleavage of C4 and C2, but plays no catalytic rôle in the subsequent C3 convertase.

The apparent stability of the C3 convertase for these short periods raises the possibility that C3 might stabilize the enzyme. However, the possibility that C2, $\text{C}\bar{\text{I}}\text{s}$ and excess C3, pre-incubated to form C2a and C2b and then added to C4b, might form convertase was tested and found not to be the case. A small, though reproducible, C3 conversion was found on incubation of C2, $\text{C}\bar{\text{I}}\text{s}$ and C3. The rate was less than 1% of that with C4b present and probably reflects contamination of the C3 with a trace of C4b.

Fig. 3. Relationship between C3 convertase activity and C2 cleavage

C2 ($0.5\mu\text{g}$), C4b ($1.0\mu\text{g}$) and C3 ($150\mu\text{g}$) in $100\mu\text{l}$ of 5mM -veronal ($\text{pH } 7.5$)/ 0.3mM - CaCl_2 / 1.0mM - MgCl_2 / 0.15M - NaCl were incubated with various amounts of $\text{C}\bar{\text{I}}\text{s}$ at 22°C : $\blacktriangle$, zero; $\blacksquare$, $0.1\mu\text{g}$; $\bullet$, $0.5\mu\text{g}$; $\circ$, $1.0\mu\text{g}$; $\square$, $5.0\mu\text{g}$. At different times samples ($1\mu\text{l}$) were removed and added to 1ml of ice-cold gelatin/veronal/ Ca^{2+} / Mg^{2+} (Nelson *et al.*, 1966). The C3 (Fig. 3a) and C2 (Fig. 3b) haemolytic activity was assayed as described in the Materials and methods section.

Fig. 4. Effect of various concentrations of C2 and C4b on the convertase activity

(a) C4b (1.0 μg), C1s (0.5 μg) and C3 (150 μg) in 100 μl of 5 mM-veronal (pH 7.5)/0.3 mM-CaCl₂/1 mM-MgCl₂/0.15 M-NaCl were incubated with various amounts of C2 at 22°C. At different times samples (1 μl) were removed, added to 1 ml of ice-cold gelatin/veronal buffer (Nelson *et al.*, 1966) and then assayed for C3 haemolytic activity. The results of two experiments are shown. (b) C2 (0.5 μg), C1s (0.5 μg) and C3 (150 μg) were incubated with various amounts of C4b in a similar manner.

Effect of di-isopropyl fluorophosphate on C3 convertase

The effect of the serine protease inhibitor di-isopropyl fluorophosphate on the activity of C3 convertase measured in the presence of excess substrate is shown in Fig. 5. In the presence of C4b, C2 and C1s in molar proportions 1:1:1, di-isopropyl fluorophosphate had no effect on C3 convertase activity, despite gradual inhibition of C1s as indicated by the loss of C4-cleaving activity or by the loss of ability to cleave C2 in the incubation mixture. In a similar experiment, in which a molar proportion of C4b:C2:C1s of 1:1:0.4 was used, the C3 convertase activity was apparently inhibited

by di-isopropyl fluorophosphate. This inhibition was correlated with a slowing of the rate of C2 cleavage and is therefore a reflection of the amount of convertase formed and not of the activity of the convertase. The result may explain the different reports on the inhibition of C3 convertase by di-isopropyl fluorophosphate. It adds further weight to the idea that C1s plays no direct role in C3 convertase activity *per se*, but is essential for the cleavage of C2 to form the convertase.

Interaction of C2 and fragments C2a and C2b with C4 and C4b

It has been shown in earlier studies (Polley & Müller-Eberhard, 1968a) that, under certain conditions, C2 and C4b can form a complex demonstrable in sucrose-density gradients. We have obtained similar results by gel filtration of mixtures of C2 and C4 or C4b on Sephadex G-200 equilibrated in 5 mM-veronal (pH 8.5)/0.6 mM-CaCl₂/2.0 mM-MgCl₂/40 mM-NaCl. Under these conditions, C2 was co-eluted with C4 or C4b. However, when the C4b and C2 were run separately, or when a mixture of the two was run in an identical column equilibrated in 5 mM-veronal (pH 8.5)/10 mM-EDTA, C2 was eluted after C4b, as would be expected from their molecular weights.

Gel filtration of a mixture of C1s-digested C2 and C4 (decayed C3 convertase) in the Mg²⁺-containing buffer resolved two protein (A_{280}) peaks; the first was shown on analysis of the fractions by sodium dodecyl sulphate/polyacrylamide gels to contain C4b and C2b, whereas the second peak contained C2a with C2b in the trailing edge (Fig. 6). In the EDTA-containing buffer the three protein fragments were eluted separately. These results are therefore direct evidence for the existence of the C4b-C2b complex shown by Nagasawa & Stroud (1977) from their studies using polyacrylamide gels and affinity techniques, and they suggest that the interaction between C4b and C2 may be mediated by the C2b part of the molecule. The interaction between C4b and C2b is, however, not only inhibited by EDTA, it is also, like the C4b-C2 interaction, strongly dependent on the pH and ionic strength of the buffer. The C4b-C2b complex could not be detected on gel filtration of decayed convertase on Sephadex G-200 equilibrated in 5 mM-veronal/ (pH 7.5)/0.3 mM-CaCl₂/1.0 mM-MgCl₂/0.15 M-NaCl. When a mixture of C4b and C2 was subjected to gel filtration under these conditions, the two proteins also were eluted separately, although the shape of the C2 peak suggested weak association with C4b. Therefore, because of the similarity in the behaviour of the C4b-C2 and C4b-C2b complexes on gel filtration and because no evidence for any interaction between C4b and C2a has been observed, it appears likely that it is the C2b part of the C2

Fig. 5. Effect of di-isopropyl fluorophosphate on C3 convertase activity

C2 (0.5 µg), C4b (1.0 µg) and C3 (150 µg) in 100 µl of 5 mM-veronal/100 mM-Pipes/HCl (pH 7.5)/0.3 mM-CaCl₂/1 mM-MgCl₂/0.15 M-NaCl were incubated with C1s [0.5 µg (triangles) or 0.2 µg (circles)] at 22°C in the presence (closed symbols) or absence (open symbols) of 10 mM-di-isopropyl fluorophosphate. At different times samples (1 µl) were removed and added to 1 ml of ice-cold gelatin/veronal/Ca²⁺/Mg²⁺ buffer (Nelson *et al.*, 1966). C3 and C2 haemolytic assays were carried out as described in the Materials and Methods section.

molecule that provides most of the interaction between C2 and C4b.

The observation that treatment of C2 with small amounts of iodine resulted in the formation of a stable C3 convertase allowed Polley & Müller-Eberhard (1968a) to demonstrate the enzyme activity in sucrose-density gradients and gel filtration columns. From the sedimentation coefficient and diffusion constant they estimated a molecular weight for the enzyme of 305 000, which suggested an equimolar complex of C4b and C2a. By the optimization of the conditions for the treatment of C2 with iodine we have now been able to show directly the existence of this complex. Stable C3 convertase was prepared by prior treatment of the C2 with 10 µM-iodine, and was then subjected to gel filtration on Sephadex G-200 in 5 mM-veronal (pH 8.5)/10 mM-EDTA (Fig. 7). The yield of activity was greater than 80% and C2a was clearly identified as a component of the convertase. Under these conditions C2b was not associated with the C3 convertase activity. However, if gel filtration was carried out under conditions that favour the interaction of C4b and C2b, that is, in the higher pH/lower ionic strength veronal buffer, then the C4b-C2b-C2a complex can be demonstrated. It therefore appears that the effect of iodine on C2, which stabilizes the C3 convertase, is one of greatly enhancing the affinity of the C2a portion of C2 for

C4b. No effect on the strength of interaction between C2b and C4b or C2a and C2b has been observed.

Preliminary results with guinea-pig C2, which is known to form a more stable C3 convertase, have shown that the enzyme can be isolated by gel filtration in a similar manner to that for iodine-treated human C2. The difference in stability between the C4b-C2a interaction for the two species may help to identify the structure in C2 modified by the iodine treatment.

Discussion

The availability of milligram quantities of highly purified C2 has made possible characterization of the role of C2 and the fragments C2a and C2b in the classical pathway C3 convertase. By gel filtration it has been possible to isolate sufficient quantities of the iodine-stabilized C3 convertase to allow identification of the subunit structure of the enzyme by sodium dodecyl sulphate/polyacrylamide gels. The enzyme comprises a complex of C4b, (C4a), C2a, and under certain conditions, C2b. The reversible interaction between C4b and C2 at pH 8.5 at low ionic strength is apparently mediated by the N-terminal C2b part of the molecule, and therefore C2b may be important in the assembly of the complex enzyme. Since the interactions between

Fig. 6. Gel filtration of decayed C3 convertase C4b (1.6 mg), C2 (800 μ g) and C1s (8 μ g) in 5 mM-veronal (pH 8.5)/0.5 mM-CaCl₂/2.09 mM-MgCl₂/40 mM-NaCl were incubated at 37°C for 30 min. The sample was then chromatographed on a column (1.6 cm $\times$ 100 cm) of Sephadex G-200 equilibrated in (a) 5 mM-veronal (pH 8.5)/0.5 mM-CaCl₂/2.0 mM-MgCl₂/40 mM-NaCl or (b) 5 mM-veronal (pH 8.5)/10 mM-EDTA. Fractions were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and C4b α' chain (O), C2a, ($\blacktriangle$) and C2b ($\triangle$) were quantified by gel scanning.

C4b and C2, and C4b and C2b, under more physiological conditions are weak, the complexes could not be detected by gel filtration and therefore more extensive physical studies are needed to assess the importance of the interactions in the function of the C3 convertase. However, removal of the C2b does not have any effect on the activity of the stabilized C3 convertase and it is therefore likely that C2b plays no part in the C4b-C2a interaction. It is also apparent that the effect of iodine treatment of the C2 is of stabilizing the C4b-C2a interaction and not of stabilizing the C2a-C2b interaction.

C1s is not a part of the C3 convertase, although it is suggested from the lack of interaction between C2a and C4b that it is the cleavage of the C2 in a C4b-C2 complex by C1s that results in effective

Fig. 7. Gel filtration of iodine-stabilized C3 convertase C4b (1.6 mg), iodine-treated C2 (800 μ g) and C1s (800 μ g) in 5 mM-veronal (pH 7.5)/0.5 mM-CaCl₂/2.0 mM-MgCl₂/0.15 M-NaCl were incubated at 37°C for 30 min. The sample was then chromatographed on a column (1.6 cm $\times$ 100 cm) of Sephadex G-200 equilibrated in the same buffer. Fractions were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis for C2a ($\blacktriangle$) and C2b ($\triangle$) and C3 convertase activity ($\bullet$) as described in the legend to Fig. 2. C4b was eluted at 60–80 ml.

convertase. In a study of the rates of cleavage of C4 and C2 by C1s we were unable to detect any effect of C4 on the rate of cleavage of C2. This is in agreement with the results of Gigli & Austen (1969) who also found no effect of C4 on the rate of cleavage of C2 by C1s, although the rate of cleavage of C2 by the C1 complex or by EAC1 cells was increased by C4.

We have attempted to analyse the kinetics of assembly and decay of the C3 convertase in terms of two first-order processes:

In such a model the concentration of convertase (Y) varies according to the equation:

$$Y = \frac{[\text{C2}] k_1}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t}) \quad (1)$$

and the time at which maximal C3 convertase activity is formed ($t_{\max}$) is given by:

$$t_{\max} = \frac{1}{k_1 - k_2} \ln \frac{k_1}{k_2} \quad (2)$$

See also Kabat & Mayer (1963).

Using k_1 as the rate constant for the apparent first

Table 1. *Kinetics of formation and decay of fluid-phase C3 convertase activity*

C4 (80 µg), C2 (40 µg) and C1s (80 ng–12 µg) in 850 µl of 5 mM-veronal (pH 8.5)/0.5 mM-CaCl₂/2.0 mM-MgCl₂/40 mM-NaCl were incubated at 37°C. Samples were removed at different times and assayed for C2 haemolytic activity and for C3 convertase activity as described in the legend to Fig. 2. The k_1 values are the apparent first-order rate constants for the destruction of C2 haemolytic activity. The k_2 value was that decay rate constant that gave best correlation between the observed time at which maximum C3 convertase activity ($t_{\max.}$) was obtained and that time predicted from eqn. (2). The $Y_{\max.}$ value is that percentage of the C2 existing as part of the C3 convertase at the time of maximum activity. N.D., not determined.

Molar proportion C4b:C2:C1s	Measured k_1 (min ⁻¹)	Estimated k_2 (min ⁻¹)	Theoretical values		Observed values	
			$t_{\max.}$ (min)	$Y_{\max.}$ (% of total C2)	$t_{\max.}$ (min)	C3 destroyed at $t_{\max.}$ (%)
100:100:0.2*	0.01	2.0	2.7	0.5	N.D.	25
100:100:0.6	0.02	2.0	2.3	1.0	2–3	40
100:100:3.0	0.1	2.0	1.6	4.3	1–2	80
100:100:30	0.5	2.0	0.9	15.7	<1	100

* Data taken from Fig. 2.

order rate of cleavage of C2 by C1s, we have obtained experimental values for $t_{\max.}$ consistent with predicted values over a 50-fold range of C1s concentration (Table 1) allowing the estimation of the decay rate constant k_2 of 2.0 min⁻¹ at 37°C. This decay rate, which is greater than previously suggested, is independent of C1s concentration. The measured decay rate for C3 convertase activity (see Fig. 2) reflects both the rate of formation of the enzyme and the rate of decay; it is therefore dependent on the C1s concentration. As a result, for catalytic amounts of C1s, the time for assembly of maximal C3 convertase activity ($t_{\max.}$) is short and the amount of convertase ($Y_{\max.}$) assembled at this time is low, occurring when only a small amount of the C2 has been cleaved (see Table 1 and Fig. 2).

Optimal C3 convertase is expressed only with high concentrations of C1s. Under such conditions, in the presence of excess substrate and at lower temperatures, the C3 convertase activity was apparently stable for short periods. By study of the kinetics of this enzyme we have been able to obtain evidence of an approx. 1:1 complex of cleaved C4 and C2. The apparent lack of decay of the enzyme and the shape of the curves relating the effect of varying either C2 or C4b at a constant concentration of the other with the C3 convertase activity formed both suggest a greater affinity of C4b for C2 than observed in the gel filtration experiments. This increased affinity may be the result of stabilisation of the complex by C3 or C3b. An investigation of this effect and its importance in the subsequent assembly of the C5 convertase is needed.

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