Studies on C3 Convertases

INHIBITION OF C5 CONVERTASE FORMATION BY PEPTIDES CONTAINING AROMATIC AMINO ACIDS

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The influence of various peptides containing the aromatic amino acids phenylalanine and tyrosine on the formation of the enzyme EAC1423 of the complement system from component C3 and enzyme EAC142 was investigated. Kinetic analysis of enzyme EAC1423 formation and studies on the binding of the C3b fragment of ¹²⁵I-labelled component C3 to enzyme EAC142 both showed that binding of the C3b fragment of component C3 was decreased by the peptides. Kinetic studies on component-C3 turnover in the fluid phase of enzyme EAC142 failed to reveal effects of the peptides. However, an initial lag in component-C3 turnover occurred that at constant component-C3 concentration was inversely proportional to enzyme EAC142 concentration. This lag in enzyme EAC142 activity is considered as an indication that the interaction of enzyme EAC142 with component C3 possibly does not follow simple Michaelis–Menten kinetics, as was previously assumed. It is shown that the stages after enzyme EAC1423 formation are not influenced by the peptides, suggesting a high degree of specificity of the peptides for the inhibition of enzyme EAC1423 formation.

Complement is a complex system of serum proteins that, together with antibody, constitutes a major humoral defence mechanism of the organism. Activation of complement leads to the sequential interaction of its components that, at several stages, is of an enzymic nature. As a result of the subsequent cleavage of peptide bonds, the complement components undergo transition from being soluble to being membrane-bound proteins. This finally leads to the build-up of macromolecular assemblies on biological membranes. Thus complement may cause irreversible damage to the membrane and cell death; or, by the generation of peptides during its activation, it may initiate specialized cell functions. During evolution, two pathways of complement activation have evolved, which reflect its important role. The classical pathway comprises at least nine components or, more precisely, 11 proteins, since the first component (C1) is a macromolecule composed of three non-covalently linked subunits. Its activation is initiated by complexes formed by antigen and antibody of the IgG‡

‡ Abbreviations: IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A; iPr, (S) isopropyl. For complement-component nomenclature, see the text.

or IgM type. The alternative, or properdin, pathway might be termed the 'first humoral defence line' of the organism. For, unlike the classical pathway, it does not require the participation of antibody for its activation. Rather, it is activated by certain polysaccharides and lipopolysaccharides found in bacterial cell walls, and also by some polyanions, e.g. dextran sulphate (Burger *et al.*, 1975). It may, however, also be activated by IgA aggregates. So far, five serum proteins have been found to function in the alternative pathway, one of which, component C3, also participates in the classical pathway.

Since the function of complement may also adversely affect the hosts cells (as is manifested, for instance, in patients suffering from certain complement dysfunctions), Nature has provided a means of controlling complement action; hence, some of the complement factors, when activated, or the complexes formed from activated factors, have only a short life. In addition, there exist three proteins regulating complement activity, namely an inhibitor and two inactivating enzymes.

Further background information can be obtained from a review by Müller-Eberhard (1975); from this the relationship between the classical and alternative

pathways will become apparent. The review also indicates that component C3 fulfils multiple functions in both pathways, and in fact represents the stage at which they merge. Cleavage of component C3 by the enzymes of the classical and alternative pathway leads to events of considerable biological significance. Hence there is justification for investigating in some detail the properties of the enzymes participating in component-C3 cleavage, or in the formation of component-C3-cleaving enzymes. In the past years, there have been several reports on compounds acting on C3 convertase. For instance, Basch (1965) demonstrated the inhibition, by several aromatic compounds, of the lysis of EAC142 cells (see under 'Nomenclature' below) by the late-acting components of guinea-pig complement. Owing to incomplete knowledge of the complement sequence at that time, however, he was unable to identify the reaction step at which inhibition occurred. Later, Shin & Mayer (1968) suggested that, in the presence of *N*-acetyltyrosine ethyl ester, the fixation of the C3b fragment of component C3 by enzyme EAC142 was inhibited, whereas the cleavage of component C3 was not affected.

In the present studies on component-C3-cleaving enzymes we have been following two lines of approach: a search for synthetic low-molecular-weight substrates on the one hand, and the design of inhibitors of these enzymes on the other. Compounds of this kind should prove useful tools in the gaining of a deeper insight into the molecular mechanism governing the action of component-C3 cleavage. The present paper is concerned with a study of enzyme EAC142 (C3 convertase), the component-C3-cleaving enzyme of the classical pathway of complement activation. It reports the effects of peptides containing aromatic amino acids, and of minor fragments thereof, on the formation of enzyme EAC1423. These findings were presented at the Third European Complement Workshop held at Paris in November 1976.

Nomenclature

The nomenclature used follows that recommended in *Immunochemistry* (1970), hence only some brief comments on terminology will be made. 'EA' refers to sheep erythrocyte sensitized with rabbit antibody (IgG or IgM type) against boiled sheep erythrocyte stromata. 'EAC142' refers to an enzyme formed on sensitized sheep erythrocytes that is composed of activated components C1, C4 and C2 respectively; it is termed 'C3 convertase', since it cleaves component C3 of plasma into a major (C3b) and a minor (C3a) fragment. On binding of the former to enzyme EAC142, the intermediate enzyme EAC1423 is formed, which acts enzymically on component C5, the next one in the complement-activation sequence.

Materials and Methods

Materials

Buffer. Unless otherwise indicated, iso-osmotic veronal/NaCl buffer (I 0.15, pH 7.3) containing 0.1% gelatin, 0.15 mM-Ca²⁺ and 1 mM-Mg²⁺ (Mayer, 1961a) was used throughout.

Reagents. The reagents used for buffers etc. were of analytical grade and were products of Merck, Darmstadt, Germany.

Cobra venom. Freeze-dried cobra (*Naja naja*) venom was used. It was purchased from Miami Serpenterium Laboratories (Miami, FL, U.S.A.).

Peptides. The preparation of some of the peptides used was as described by Schnabel & Oberdorf (1968a,b). These peptides are marked with an asterisk in Table 1. The preparation of the other peptides was by established methods of peptide synthesis using protective reagents. The purity of the peptides was checked by elemental analysis. Since they usually were obtained as trifluoroacetates or hydrochlorides, the pH of their buffer solutions generally ranged between 3 and 4, owing to hydrolysis. A few drops of 1 M- and 0.1 M-NaOH was used to adjust the pH to 7.3. Within the range of the peptide concentrations used, no significant influence on the conductivity of the buffer was detectable. The peptides investigated are listed in Table 1.

Methods

Assay system. The principles underlying the assay of complement activity were delineated by Mayer (1961a) and since by Rapp & Borsos (1970). Despite the extreme sensitivity of the haemolytic assay, the procedure suffers from drawbacks, owing to the biological nature of the substrate (erythrocytes) used.

Site-forming units. This term was introduced by Hoffmann & Meir (1967). It may be used to indicate the haemolytic effectiveness of complement components, and is based on the 'one-hit' hypothesis of immune haemolysis proposed by Mayer (1961b).

Haemolytic assay. Unless otherwise indicated, all experiments were performed by using a microtitration system supplied by Eppendorf, Hamburg, Germany. Haemoglobin was determined at 412 nm with a Gilford model 240 spectrophotometer.

Reagent for the preparation of enzyme EAC142. Cobra venom contains about 0.5% (w/w) of a factor (VF) (Bitter-Suermann *et al.*, 1972) that in the presence of factor D of the alternative pathway and Mg²⁺ forms an enzymically active complex with factor B of the alternative pathway. This complex cleaves components C3 and C5. The early acting complement components are not affected. More details can be obtained from a study by Cooper (1973). In our laboratory we used this property of

factor VF to obtain a reagent for the preparation of enzyme EAC142. It was prepared by incubating guinea-pig serum with freeze-dried cobra venom (1 mg/ml of guinea-pig serum) for 1 h at 37°C. The VFB enzyme thus formed inactivated components C3 and C5. The reaction mixture was left for a further 1 h at room temperature (20–22°C). Thereafter, portions were frozen and kept at –70°C until use.

Preparation of enzyme EAC142. A 1:40 dilution of the reagent described above was incubated with an equal volume of optimally sensitized EA erythrocytes (5×10^8 cells/ml) for 10 min at 37°C. Ice-cold buffer was added to the reaction mixture, and the cells were washed twice and adjusted to the desired concentration. On incubating component C3 with enzyme EAC142, the former is cleaved. Thereby the C3b fragment of component C3 is incorporated into the complex, forming enzyme EAC1423.

For determining component C3 turnover in the fluid phase of enzyme EAC142, cells for titrating component C3 in the secondary assay were prepared at 1/0.075. This procedure yields considerably more C142 sites/cell and thus allows the use of smaller volumes of the dilutions of the assay mixture for titrating residual haemolytically active component C3.

Converting reagent. Lysis of EAC1423 cells was achieved with a reagent supplying an excess of components C5–C9. It is prepared from guinea-pig serum treated with hydrazine as described by Klein & Wellensiek (1965). Thereby components C3 and C4 are destroyed, whereas the other complement components are left functionally active.

Kinetics of the formation of enzyme EAC1423 from enzyme EAC142 and component C3. To 1000 μ l of an ice-cold enzyme EAC142 suspension (2.2×10^8 cells/ml), 500 μ l of the ice-cold solution of the respective peptide was added in an appropriate concentration so as to yield the desired concentration in the final reaction volume. By immersion in a 37°C water bath and with vigorous shaking, the temperature of the reaction mixture was quickly brought to 25°C. The reaction vessel was transferred to a 25°C water bath, the shaking continued for 1 min, and the reaction initiated by adding 500 μ l of component C3 (containing 4.4×10^8 site-forming units/ml), prewarmed to 25°C. At the time intervals indicated in the Figures, 100 μ l samples were withdrawn, immediately pipetted into 1000 μ l of ice-cold buffer and centrifuged at 9950g for 30 s in a high-speed Eppendorf bench centrifuge. At the end of the centrifugation, 1000 μ l of the supernatant was withdrawn and 1000 μ l of ice-cold buffer added. The cells were washed and centrifuged. Again, 1000 μ l of the supernatant was withdrawn and replaced by 1000 μ l of a 1:1500 dilution of the converting reagent. This procedure ob-

viates the necessity to remove the total supernatant of the cells, and hence prevents the eventual loss of cells. Thereby a 1:1331-fold dilution of the component C3 originally present in the reaction mixture was achieved, which had no detectable effect with respect to enzyme EAC1423 formation in the final incubation at 37°C, as ascertained by appropriate control experiments. As a control, the procedure outlined above was followed, replacing the inhibitor solution by 500 μ l of buffer. The cells were suspended in the converting reagent and incubated for 60 min at 37°C, with repeated shaking. At the end of this period the cells were centrifuged, and the haemoglobin released was determined by measuring the A_{412} of the supernatant. Cell blank controls were set up that contained enzyme EAC142 treated in an identical manner together with converting reagent; component C3 was replaced by 100 μ l of buffer. Lysis due to traces of component C3 contained in the converting reagent was usually less than 3%. Complete lysis controls were prepared in water.

Consumption of component C3 from the fluid phase of enzyme EAC142. The supernatant of the cells that was obtained after the first centrifugation step of the procedure outlined above was kept (at 0°C) for component-C3 titration, which was performed in the following manner. Depending on the sensitivity of the EAC142 cells used for titration, 100–200 μ l of the supernatant was added to 1000 μ l of an ice-cold 1:1500 dilution of the converting reagent in a buffer at 1/0.075. To this mixture 200 μ l or 100 μ l of enzyme EAC142 (containing 0.65×10^8 or 1.3×10^8 cells/ml respectively) suspended in the same buffer was added. The reaction vessels were transferred to a 37°C water bath and incubated for 60 min, with occasional agitation. Subsequently the reaction vessels were centrifuged for 30 s in an Eppendorf table centrifuge, and the haemoglobin released by the cells was determined. To establish identical conditions for the titration, the control contained the identical peptide concentration, resulting from the 1:11 or 1:5.5 (respectively) dilution of the assay mixture containing the peptide.

Kinetic analysis of the effects of the peptides on the stages of the haemolytic sequence after enzyme EAC1423 formation. Enzyme EAC1423 was prepared by incubating equal volumes of enzyme EAC142 (5×10^8 cells/ml) and component C3 (5×10^{10} site-forming units/ml, prewarmed to 30°C) for 5 min at 30°C. At the end of this period, ice-cold buffer was added to the reaction mixture, and the cells were centrifuged, washed twice and adjusted to a concentration of 0.65×10^8 cells/ml. To a series of Eppendorf vessels containing 1000 μ l of an ice-cold 1:3000 dilution of the converting reagent and 100 μ l of peptide solution (13 mM), 200 μ l of cells (0.65×10^8 cells/ml) was added. In the control, the peptide solution was replaced by

buffer. The vessels were transferred to a 37°C water bath and incubated for 60 min, with occasional shaking. At the time intervals referred to in the inset of Fig. 2, samples were removed from the water bath, centrifuged, and the haemoglobin in the cell supernatant was determined. Cell blanks were included in which the converting reagent was replaced by buffer. The haemoglobin released by the cell blanks was less than 3% of the complete lysis controls.

Binding of the C3b fragment of 125 I-labelled component C3 to enzyme EAC142. This was investigated by incubating 500 μ l of enzyme EAC142 (8.4×10^8 cells/ml) with 100 μ l of solutions containing 125 I-labelled component C3 (50.4, 25.2, 12.6, 6.3 and 3.15 μ g/ml) at 0°C in the presence of 100 μ l of Phe-Tyr-Gly (35, 17.5 and 7 mM). In the control experiments, the peptide solution was replaced by 100 μ l of buffer. Component C3 dilutions were performed in buffer containing 2% (w/v) bovine serum albumin. The tubes containing the reaction mixture were transferred to a water bath at 37°C and incubated for 60 min with occasional shaking. At the end of this period, ice-cold buffer was added, the cells were centrifuged for 5 min at 1200g, and the supernatant was carefully removed by suction. The cells were washed three more times with buffer. Finally, 500 μ l of buffer was added to the cell pellet. The cells were suspended, 500 μ l was withdrawn, pipetted into a glass tube and the radioactivity bound was determined in a Berthold well-type scintillation counter. Unspecific binding, which was maximally 10% of specific binding, was determined by following the procedure outlined above with EAC14 cells obtained from enzyme EAC142 by prolonged incubation at 37°C. After counting for radioactivity, the cells were lysed in water to determine the cell number.

Purification of component C3. Component C3 was purified as described by Bitter-Suermann *et al.* (1970). All experiments were repeated with different component-C3 batches.

125 I-labelling of component C3. This was done by the chloramine-T method (McConahey & Dixon, 1965). The haemolytic activity of component C3 was not affected by the procedure.

Additional control experiments. Additional control experiments (results not shown) were performed which showed that the peptides neither had any influence on component-C3 activity nor impaired the stability of the respective cellular intermediates.

Results and Discussion

The curves shown in Fig. 1(a) and the inhibition values listed in Table 1 suggest that the degree of inhibition of enzyme EAC1423 formation critically depends on the presence of the Phe-Tyr sequence within the amino acid backbone of the peptides; the

Fig. 1. (a) Kinetics of enzyme EAC1423 formation in the presence of various peptides and (b) quantification of the decrease of haemolytically active component C3 in the fluid phase of various concentrations of enzyme EAC142

(a) $\circ$, Control; $\bullet$, Asn-Lys-Phe-Tyr-Gly-Leu-iPr-Cys-NH₂ (iPr = *S*-isopropyl); ∇ , Phe-Tyr; $\square$, Tyr-Gly; Δ , Leu-iPr-Cys-NH₂. Lysis values obtained on 60 min incubation of enzyme EAC1423 with converting reagent are given as 'Z' values (Mayer, 1961b) [$Z = -\ln(1 - y)$, where y = degree of haemolysis]. The inset shows the dose-response curve for Phe-Tyr, for the inhibition of enzyme EAC1423 formation obtained at 16 min. The conditions are described under 'Methods'. (b) Component-C3 content is given on the ordinate and is expressed as percentage lysis obtained in the secondary assay. $\circ$, 1.1×10^8 cells/ml; Δ , 2.2×10^8 cells/ml; ∇ , 2.75×10^8 cells/ml; $\square$, 5×10^8 cells/ml. The concentration of component C3 that was added to the reaction mixture was constant, and was chosen so as to contain about 1.1×10^8 site-forming units when incubated for 16 min with the lowest concentration of enzyme EAC142, in the absence of Phe-Tyr. Closed symbols ($\bullet$, Δ , ∇ , $\blacksquare$) show the curves obtained for the respective enzyme concentrations in the presence of 1 mM-Phe-Tyr. The assays were done in duplicate and the symbols represent means $\pm$ s.e. Being almost identical, the experimental points obtained for the respective [enzyme EAC142] in the presence and absence of the peptide are connected by single curves. The conditions are described under 'Methods'.

Table 1. Inhibition of enzyme EAC1423 formation at 16 min

The inhibition of enzyme EAC1423 formation was followed kinetically (see under 'Methods'). The inhibition values obtained at 16 min, which did not change significantly on prolonged incubation, are given for the peptides listed below. Abbreviations: Et, (*S*)-ethyl; OMe, methyl ester; Pr, (*S*)-propyl; Eth, ethionine.

Peptide (1 mM in the reaction mixture)	Inhibition (%)
Asn-Lys-Phe-Tyr-Gly-Leu-Et-Cys-NH ₂ *	47
Asn-Lys-Phe-Tyr-Gly-Leu-Pr-Cys-NH ₂ *	46
Asn-Lys-Phe-Tyr-Gly-Leu-Met-OMe*	39
Asn-Lys-Phe-Tyr-Gly-Leu-D-Eth-NH ₂	29
Asn-Lys-Phe-Tyr-Gly-Leu-Thr-NH ₂ *	47
Pro-Glu-Lys-Phe-Tyr-Gly-Leu-NH ₂	31
Phe-Tyr-Gly-Leu-Et-Cys-NH ₂	46
Asn-Lys-Phe-Tyr-Gly	47
Lys-Phe-Tyr-Gly	45
Tyr-Gly-Leu-Et-Cys-NH ₂	23
Phe-Tyr-Gly-OMe	45
Phe-Tyr-Gly	46

peptide fragments in which phenylalanine is missing are less effective; Leu-iPr-Cys-NH₂, which lacks both aromatic amino acids, is completely ineffective. The decrease in inhibitory potency seen with some of the long-chain peptides containing the Phe-Tyr sequence shows, however, that certain structural requirements also have to be met by the other amino acids contained within the molecule; presumably in these instances the conformation of the peptide molecule, a function of its primary sequence, prevents proper orientation of the Phe-Tyr sequence relative to the site at which they exert their effect. Fragments of the active peptides lacking tyrosine were not available. Therefore an estimation of the individual contributions of the aromatic amino acids to the overall inhibition is not possible. It might be, however, that tyrosine is the more effective; besides binding by hydrophobic interaction, it also offers the possibility of hydrogen-bond formation via the phenolic oxygen. Tyrosine or phenylalanine alone did not show any effect, indicating that peptide-bonding of an aromatic amino acid to at least an additional amino acid residue is required for inhibition to occur (see, e.g., Tyr-Gly; Fig. 1*a*). As concluded from the inhibition data, the contribution of the non-aromatic amino acids in the 'active' long-chain peptides is of minor importance. The inset of Fig. 1*a* shows the dose-response curve for Phe-Tyr, for the inhibition of enzyme EAC1423 formation at 16 min, where a 'plateau' value for enzyme EAC1423 formation was reached. Although increasing with increasing concentrations of the peptide, the degree of inhibition appears to approach a limiting value.

The results of binding of the C3b fragment of ¹²⁵I-labelled component C3 to enzyme EAC142 (Fig. 2)

show that uptake of the C3b fragment is inhibited by the peptides in a concentration-dependent manner, as suggested by the haemolysis experiments. The possibility has to be considered, however, that these experiments reveal binding of the C3b fragment to different categories of sites, i.e. enzyme EAC142 sites (thus forming enzyme EAC1423) and distinct membrane sites. The curves shown in the inset of Fig. 2 show that the peptides do not influence the stages of the complement sequence after enzyme EAC1423 formation.

In this connexion the inhibition, by *N*-acetyltyrosine ethyl ester, of component-C5 turnover by enzyme EAC1423 (Shin & Mayer, 1968) is noteworthy. This difference might be explained by the fact that this compound contains the ester function and is additionally acetylated at the nitrogen of the α -amino group. The high degree of specificity of inhibition of EAC1423 formation by the Phe-Tyr sequence renders this peptide, and possibly also structurally similar compounds, potentially useful in the design of specific inhibitors for this stage of the complement sequence.

In Fig. 1(*b*) the results of an experiment are shown in which the decrease of haemolytically active component C3 in the fluid phase of enzyme EAC142 was quantified in the presence of various concentrations of enzyme EAC142. At low enzyme concentrations there is an initial lag in the decrease of haemolytically active component C3. With increasing enzyme EAC142 concentrations the lag phase becomes shorter until, at high enzyme concentrations, it is no longer detectable. The Figures show that the presence of Phe-Tyr sequence apparently does not influence component-C3 turnover. Determinations of enzyme EAC1423 formation performed in parallel at the lowest enzyme EAC142 concentration (results not shown) showed that this, unlike component-C3 turnover, proceeded without a lag phase.

Various possibilities can be thought of which may explain the occurrence of lags in enzyme activity. One explanation might be a conformational change induced by the binding forces of the substrate, as in the induced-fit and strain mechanisms, occurring at a rate lower than the turnover of the substrate. But there also exist more involved treatments of the case, as, for instance, put forward by Frieden (1970), Shill & Neet (1971) and Ainslie *et al.* (1972). At present it is not possible, on grounds of the limited amount of data, to choose between these alternatives. To investigate the matter more closely it will be necessary to perform experiments on the turnover of component C3 by enzyme EAC142 under conditions less restricted than those used in the present study; here the conditions had to be chosen so that formation of enzyme EAC1423 could be followed. It also appears

Fig. 2. Binding of the C3b fragment of ^{125}I -labelled component C3 to enzyme EAC142 in the presence and absence of various Phe-Tyr concentrations

○, Control; ●, 1 mM; □, 2.5 mM; ▲, 5 mM-Phe-Tyr. The experimental conditions are described under 'Methods'. Specifically bound radioactivity is plotted on the ordinate. The assays were done in duplicate and the symbols represent means $\pm$ S.E. The inset shows the results of a kinetic experiment on the effects of some peptides on the stages of the haemolytical sequence after enzyme EAC1423 formation. The concentrations of the inhibitors used were all 1 mM. ○, Control; ●, Phe-Tyr; △, Phe-Tyr-Gly; □, Asn-Lys-Phe-Tyr-Gly-Leu-Et-Cys-NH₂ (Et = S-ethyl). Lysis values are given as 'Z' values (Mayer, 1961b). Experimental conditions were as described under 'Methods'.

worth investigating why the formation of enzyme EAC1423, in contrast with component-C3 turnover by enzyme EAC142, proceeds without a detectable lag. Possibly it might turn out that the interaction of C3 convertase with component C3 is not governed by simple Michaelis-Menten kinetics, as was previously reported (Shin & Mayer, 1968). The 'lag' behaviour of enzyme EAC142 reported here may indicate that a more complex mechanism is involved.

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