Autolytic fragmentation of complement components C3 and C4 under denaturing conditions, a property shared with α_2 -macroglobulin

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The α polypeptide chain of the complement protein C3 splits into two fragments of 74 000 and 46 000 apparent mol.wt. under certain conditions used to prepare the protein for SDS (sodium dodecyl sulphate)/polyacrylamide-gel electrophoresis. The cleavage reaction occurs over a wide range of temperatures and from pH 4.6 to 10.6 in the presence of denaturants such as urea, SDS and guanidine hydrochloride. It is also induced by heat-denaturation of C3 in the absence of chemical denaturants. The reaction occurs only with haemolytically active C3, and is not observed with hydroxylamine-inactivated C3 or with C3b. A similar cleavage of the α -chain of complement component C4 occurs under the same conditions, forming fragments of 53 000 and 41 000 apparent mol.wt. This reaction is again specific for haemolytically active C4, and does not occur with C4b or hydroxylamine-inactivated C4. The complement component C5, although structurally similar to C3 and C4, does not undergo a reaction of this type. The characteristics of the denaturation-induced cleavage of C3 and C4 match those described for the 'heat-induced' cleavage of α_2 -macroglobulin [Harpel, Hayes & Hugli (1979) *J. Biol. Chem.* **254**, 8669–8678]. Cleavage of α_2 -macroglobulin is also specific for the active form of the protein, and does not occur with chemically inactivated or proteinase-cleaved forms. The unusual conditions and specificity of the peptide-bond cleavage in all three proteins suggest that it is an autolytic process rather than being the result of trace proteinase contamination. The active forms of C3, C4 and α_2 -macroglobulin have the transient ability to form covalent bonds after activation. The autolytic cleavage reaction is likely to be related to the covalent-bond-forming reactions of these proteins.

The third (C3) and fourth (C4) components of serum complement and the serum proteinase inhibitor α_2 -macroglobulin have a number of properties in common. The physiological roles of the two complement proteins and of α_2 -macroglobulin do not appear superficially to be closely related, but all three proteins have important roles in clearance of exogenous material from the circulation and in inflammation. The complement system, of which C3 and C4 are components, is the major effector system in the humoral-immune-defence system, and is responsible for neutralization and clearance of invading micro-organisms and other foreign material from the blood and extracellular fluid. Contact of the complement system with foreign materials results in activation of complement-system proteinases, which, in turn, cleave and activate C3 and C4 (Lachmann,

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G. The nomenclature of complement components is that recommended by the World Health Organisation (1968).

1979; Porter & Reid, 1979). Once activated, C3 and C4 bind to the surface of the material that initiated complement activation, e.g. cell membranes and polysaccharides (Müller-Eberhard *et al.*, 1966; Mardiney *et al.*, 1968; Law & Levine, 1977; Law *et al.*, 1979, 1980) or immune complexes (Müller-Eberhard & Biro, 1963; Takahashi *et al.*, 1977; Goers & Porter, 1978; Campbell *et al.*, 1980). Once fixed to a surface, C3 and C4 function as non-catalytic subunits of the complex proteinases of the complement system (Porter & Reid, 1979). Bound C3 and C4 also participate in immune adherence and opsonization reactions by binding to specific receptors on certain cell types and so promote phagocytosis of foreign materials (Lachmann, 1979). It seems likely that the initial reaction of binding of C3 and C4 to surfaces involves very similar mechanisms for both proteins, and that the binding is by means of a covalent bond (ester or amide) of which the carbonyl group is donated by C3 and C4 (Law & Levine, 1977; Law *et al.*, 1979,

1980; Campbell *et al.*, 1980; Sim *et al.*, 1981; Twose *et al.*, 1980).

α_2 -macroglobulin is best known as a serum proteinase inhibitor of wide specificity, and its principal physiological role is likely to be the trapping of cellular and exogenous endopeptidases and removal of these proteins from the circulation (Starkey, 1979). A number of additional actions of α_2 -macroglobulin, and its possible role in modulation of the immune system, have been discussed by James (1980). Proteolytic enzymes were initially thought to interact with α_2 -macroglobulin by a non-covalent entrapment process that occurred after cleavage of α_2 -macroglobulin by the proteinase (Barrett & Starkey, 1973). It has become clear, however, that a proportion of the proteinase molecules that interact with α_2 -macroglobulin become covalently bound to the α_2 -macroglobulin molecule (Harpel & Hayes, 1979; Salvesen & Barrett, 1980).

Thus C3, C4 and α_2 -macroglobulin all take part in covalent binding reactions. All three proteins are also known to exhibit unusual sensitivity to inactivation by amines, e.g. methylamine, ammonium salts (Gordon *et al.*, 1926; Ratnoff *et al.*, 1954; Müller-Eberhard & Biro, 1963; von Zabern *et al.*, 1980) and strong nucleophiles, e.g. hydrazine and hydroxylamine. Recent studies have demonstrated covalent incorporation of methylamine into α_2 -macroglobulin (Swenson & Howard, 1979), putrescine into C4 (Campbell *et al.*, 1980) and phenylhydrazine and methylamine into C3 (Sim *et al.*, 1981; Twose *et al.*, 1980). Incorporation of small ligands is likely to occur by the same mechanism as the physiological covalent binding reactions discussed above.

Active α_2 -macroglobulin, but not amine-inactivated or proteinase-cleaved α_2 -macroglobulin, has been reported to undergo cleavage of a single peptide bond under denaturing conditions (Barrett & Salvesen, 1979; Barrett *et al.*, 1979; Harpel & Hayes, 1979; Harpel *et al.*, 1979). This reaction appears to be autolytic, and suggests the presence of an unusually reactive functional group within the molecule that may be associated with the covalent-binding mechanism. We report here that a similar autolytic reaction occurs with the active forms of C3 and C4.

Materials and methods

Chemicals and reagents

Materials for SDS/polyacrylamide-gel electrophoresis. Acrylamide was supplied by Fisons Ltd, Loughborough, Leics, U.K., and *N,N'*-methylenebisacrylamide, *NNN,N'*-tetramethylethylenediamine and Coomassie Brilliant Blue R250 were from

Sigma, Poole, Dorset, U.K. SDS was from BDH Ltd, Poole, Dorset, U.K.

Other materials. Hydroxylamine hydrochloride was supplied by Cambrian Chemicals, Croydon, Surrey, U.K. Guanidine hydrochloride (Ultrapure) was purchased from Bethesda Research Laboratories, Rockville, MD 20850, U.S.A. Sources of other reagents were as described previously (Sim *et al.*, 1981). Detergents were from Sigma.

Buffers

Where pH values are quoted for Tris buffers in the text, these values represent the pH of the buffer at 20°C, unless specified otherwise.

Proteins

C3 was isolated from outdated human plasma as described by Sim *et al.* (1980) or by the method of Tack & Prahil (1976).

Factors B and D, isolated as described by Kerr (1979) and by C. Parkes & M. A. Kerr (personal communication) were generously provided by Miss C. Parkes and Dr. D. L. Christie.

β_2 H was isolated by DEAE-Sephadex and CM-Sephadex chromatography of a 5–12% (w/v) poly(ethylene glycol) precipitate from human plasma (R. B. Sim, unpublished work).

C3b inactivator was prepared as described by Crossley & Porter (1980) and was provided by Miss E. M. Press.

C4 and C4b were purified as previously described (Gigli *et al.*, 1977; Campbell *et al.*, 1980) and were provided by Dr. R. D. Campbell and Dr. D. Chakravarti.

C5 was prepared by modifications of the method of Tack *et al.* (1979) and was provided by Mr. A. W. Dodds and Dr. R. G. DiScipio.

C3b was prepared from C3 by limited proteolysis with trypsin (Bokisch *et al.*, 1969) or by incubation of C3 at 37°C in 2.5 mM-sodium barbitone/HCl/72.5 mM-NaCl/2.5% (w/v) glucose/0.15 mM-CaCl₂/0.3 mM-MgCl₂, pH 7.5, with Factor B and Factor D in the molar proportions C3:B:D of 1:0.14:0.07. Molar quantities of these proteins were determined from the following values of $A_{280,1\text{cm}}^{1\%}$: Factor B, 12.7 (Curman *et al.*, 1977), Factor D, 10, and C3, 9.7 (Tack & Prahil, 1976). C3b was separated from uncleaved C3 by chromatography on sulphated Sepharose (C. Parkes, R. G. DiScipio & M. A. Kerr, personal communication).

Polyacrylamide-gel electrophoresis in buffers containing SDS

The SDS/polyacrylamide-gel method of Laemmli (1970) was used. Slab gels containing 7.5 or 10% (w/v) acrylamide were run in a Bio-Rad apparatus. Gels were stained for protein with Coomassie Blue and destained as described previously (Sim & Sim,

1979). Stained gels were scanned at 540nm, a Vitatron densitometer being used to quantify the intensity of staining. We are indebted to Dr. M. G. Ord for use of this instrument.

Preparation of samples for SDS/polyacrylamide-gel electrophoresis

Reduction and alkylation of protein samples was carried out routinely by addition of 1 vol. of protein sample to 1 vol. of 200mM-Tris/HCl (pH 7.5)/8M-urea/2% (w/v) SDS/40mM-dithiothreitol. Samples were incubated for 30min at 37°C and were then made 42mM with respect to iodoacetamide by addition of an appropriate volume of 500mM-iodoacetamide in 1M-Tris/HCl, pH 7.5. Incubation was continued for 20min at 20°C. Gel electrophoresis was carried out immediately or samples were stored overnight at -20°C.

Samples to be examined without reduction of the protein were mixed with 1 vol. of 200mM-Tris/HCl (pH 7.5) / 8M - urea / 2% (w/v) SDS / 42mM-iodoacetamide and incubated for 30 min at 37°C.

Other conditions for preparation of protein samples are stated, where appropriate, below.

Calculation of molecular weights from SDS/polyacrylamide-gel electrophoresis

Molecular weights of polypeptide chains were calculated from mobilities on SDS/polyacrylamide gels as described by Weber & Osborn (1969). Reduced and alkylated bovine serum albumin, catalase, ovalbumin, soya-bean trypsin inhibitor, trypsin (Sigma), IgG and plasminogen (prepared in this laboratory) were used as standards.

Haemolytic assays

The haemolytic activities of C3 and C4 were assessed as described by Tack & Prahl (1976) and by Gigli *et al.* (1977) respectively. C5 haemolytic activity was determined as described by Tack & Prahl (1976). C5 assays were performed by Mr. A. W. Dodds and Dr. R. G. DiScipio.

Treatment of C3, C4 and C5 with hydroxylamine

Purified C3, C4 and C5 in 150mM-NaCl were made 100mM-with hydroxylamine hydrochloride, pH 9.0, by addition of a 1M-hydroxylamine solution. Samples were incubated for 2h at 37°C, then dialysed against 5mM-sodium barbitone/HCl (pH 7.5)/145mM-NaCl to remove hydroxylamine. This treatment led to loss of greater than 95% of haemolytic activity of C3 and C4. The effect on C5 haemolytic activity was not tested.

Treatment of C3 with KBr

Purified C3 in 25mM-potassium phosphate/5mM-EDTA, pH 7.5, was mixed with an equal volume of saturated KBr solution and incubated for 18h at

4°C (Müller-Eberhard *et al.*, 1966). The sample was then dialysed against 5mM-sodium barbitone/HCl (pH 7.5)/145mM-NaCl to remove KBr. This procedure resulted in total destruction of C3 haemolytic activity.

Results

Investigation of the autolytic splitting of C3 was prompted by the observation that, in routine examination of reduced C3 samples by SDS/polyacrylamide-gel electrophoresis, two extra protein bands, in addition to the expected C3 α -chain band (116000 mol.wt.) and C3 β -chain band (70000 mol.wt.) (Nilsson & Mapes, 1973), were seen. The intensity of these additional bands on Coomassie Blue-stained gels was variable and appeared greater if samples were prepared for electrophoresis by incubation at 100°C for 5 min in 100mM-Tris/HCl

Fig. 1. SDS/polyacrylamide-gel electrophoresis of active and inactive C3 and of C3b

Identical samples of purified C3 were taken and converted into C3b by incubation with Factor B and Factor D, or inactivated by hydroxylamine, as described in the Materials and methods section, or left untreated. The samples were dialysed, in separate vessels, against 5mM-sodium barbitone/HCl (pH 7.5)/145mM-NaCl and adjusted to a concentration of 300 μ g/ml. The untreated (active) C3 sample was reduced for electrophoresis either at pH 7.5 (30 min, 37°C) as described in the Materials and methods section (track 1) or by incubation for 10 min at 100°C with an equal volume of 0.2M-Tris/HCl (pH 10.0)/8M-urea/2% (w/v) SDS/40mM-dithiothreitol (track 2). Hydroxylamine-treated C3 (track 3) and C3b (track 4) were reduced as described for the sample in track 2. Alkylation of the samples with iodoacetamide was done in each case as described in the Materials and methods section.

Fig. 2. Time course and extent of cleavage of C3 in SDS/urea: influence of dithiothreitol

C3 (7.5 µg), in 25 µl of 10 mM-sodium phosphate (pH 7.4)/145 mM-NaCl, was placed in 25 µl of 200 mM-sodium phosphate (pH 8.0)/2% (w/v) SDS/8 M-urea pre-equilibrated to 100°C (a) or 37°C (c). Incubation was carried out at 100°C (a) or 37°C (c) for various times. Samples were chilled rapidly to 0°C, made 20 mM with respect to dithiothreitol and incubated at 37°C for 30 min. An additional series of samples was treated identically to those incubated at 100°C except that 20 mM-dithiothreitol was present throughout the 100°C incubation. Samples were then alkylated with iodoacetamide as described in the Materials and methods section. Protein

(pH 8.0) / 4 M-urea / 1% (w/v) SDS / 20 mM-dithiothreitol, rather than incubation for 60 min at 37°C in the same buffer. Decreasing the time, temperature and pH of incubation in the Tris/SDS/urea buffer was found to prevent appearance of these additional bands. If the same C3 samples were inactivated with hydroxylamine, or converted into C3b before examination by SDS/polyacrylamide-gel electrophoresis, only the expected α -, α' - and β -chain bands were observed. An example of the appearance of additional bands is shown in Fig. 1. Haemolytically active C3, prepared for electrophoresis under the mild conditions specified in the Materials and methods section, shows only the α - and β -chain bands (track 1). Under more severe conditions of incubation, however, the intensity of the α -chain band is diminished and two new products, of apparent mol.wts. 74 000 and 46 000, are observed (track 2). If the same C3 sample is inactivated with hydroxylamine (track 3), or converted into C3b (track 4) before preparation for electrophoresis, no split products are seen in conditions in which they are created in active C3.

Time course and extent of C3 cleavage under denaturing conditions

C3 was incubated for various times at pH 8.0 and at 37°C or 100°C in the presence of SDS and urea to determine the rate of appearance of the cleavage products of 74 000 and 46 000 mol.wt. Time courses are shown in Fig. 2. Incubation at 100°C (Fig. 2a) results in a rapid decrease in the quantity of intact C3 α -chain observed on Coomassie Blue-stained polyacrylamide gels, and a concomitant increase in the quantity of the 74 000- and 46 000-mol.wt. fragments. The staining intensity of the C3 β -chain is unaffected.

samples were subjected to SDS/polyacrylamide-gel electrophoresis. Gels were stained with Coomassie Blue and scanned. The areas under peaks corresponding to the α -chain (▲), β -chain (●) and the 74 000-mol.wt. (○) and 46 000-mol.wt. (△) split products were measured and each expressed as a percentage of the total stain. In (b) results shown in (a) (●) and corresponding results for samples incubated at 100°C in the presence of 20 mM-dithiothreitol (○) were used to calculate the percentage of C3 cleaved by using the relationship:

$$\text{Percentage of C3 cleaved} = 100 \times \left[1 - \frac{\text{area under } \alpha\text{-chain peak}}{\text{area under } \alpha\text{-} + 74\,000\text{-mol.wt.} + 46\,000\text{-mol.wt. chain peaks}} \right]$$

In (d), results shown in (c) were used to calculate the percentage of C3 cleaved, as described above.

These findings demonstrate that the two fragments are derived from the C3 α -chain. The sum of the apparent molecular weights of the two split products is very close to the apparent molecular weight of the α -chain. It is therefore probable that the fragments are derived from the α -chain as a result of cleavage of a single peptide bond, without loss of additional peptides from the α -chain. Samples run on SDS/polyacrylamide gels without reduction of disulphide bonds demonstrate that the 74000- and 46000-mol.wt. products remain disulphide-linked within the C3 molecule.

At 37°C (Fig. 2c), a similar but much slower disappearance of the α -chain and corresponding increase in the quantity of split products is observed. Results from Figs. 2(a) and 2(c) were used to calculate the percentage of C3 cleaved at a given time (Figs. 2b and 2d). At both 37 and 100°C, cleavage of C3 appears to reach a maximum of about 70%. Purified C3 preparations undergo inactivation during purification and storage, as discussed by von Zabern *et al.* (1980) and Sim *et al.* (1981). The haemolytically inactive C3 thus formed is indistinguishable from active C3 on SDS/polyacrylamide gels, but is not cleaved by a fluid-phase alternative-pathway C3 convertase (C. M. Parkes, R. G. DiScipio & M. A. Kerr, personal communication). It is likely to be similar in properties to hydroxylamine-inactivated C3 (von Zabern *et al.*, 1980; C. Parkes, & E. Sim, unpublished work). It was therefore considered likely that the 30% of the C3 remaining uncleaved after prolonged incubation in SDS and urea (Figs. 2b and 2d) corresponds to inactive C3.

This point was investigated further by comparing the proportion of C3 in various preparations cleaved in denaturing conditions with the proportion that is susceptible to proteolysis by a C3bBb convertase. A number of C3 preparations that had been stored at 4 or -70°C (Sim *et al.*, 1981) for periods ranging from 2 weeks to 15 months were tested for the presence of haemolytically inactive C3 by incubation with Factors B and D, as shown in Fig. 3(a). Digestion of samples with convertase for 1 h at 37°C led to complete loss of C3 haemolytic activity. Extending the incubation time further did not lead to conversion of residual inactive C3 into C3b. The same C3 preparations were also treated by prolonged incubation in SDS and urea. The degree of cleavability of C3 by convertase and the percentage of the C3 split by incubation in SDS and urea show a very close correlation (Fig. 3b). This strongly suggests that the haemolytically inactive C3, which does not undergo cleavage by C3 convertase, likewise does not split on incubation under denaturing conditions. The results also indicate that complete cleavage of haemolytically active C3 in SDS and urea is possible.

Fig. 3. Correlation between cleavage of C3 samples by

C3 convertase and C3 fragmentation in SDS/urea. Samples of different C3 preparations, stored at 4°C or -70°C for 0.5–15 months, were dialysed against 5 mM-sodium barbitone / HCl (pH 7.5) / 145 mM-NaCl/0.15 mM-CaCl₂/0.3 mM-MgCl₂. Portions of each preparation were incubated with Factor B and Factor D, as described in the Materials and methods section, in a total volume of 60 μ l of the same buffer. Incubation was for 60 min, and prepared for SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. Gel scans of the pattern of cleavage of C3 to C3b and of Factor B to Ba and Bb during incubation are shown (a). The percentage of C3 cleaved to C3b was calculated by measuring areas under the α - and α' -chain peaks on the gel scans. The same C3 samples (25 μ l of each) were incubated for 150 min at 100°C in 25 μ l of 200 mM-Tris/HCl (pH 9.0)/2% (w/v) SDS/8 M-urea, then made 20 mM with respect to dithiothreitol by addition of 100 mM-dithiothreitol in 1 M-Tris/HCl, pH 7.5. Incubation was continued for 30 min at 37°C. The samples were finally made 42 mM with respect to iodoacetamide and incubated as described in the Materials and methods section. Samples were examined by SDS/polyacrylamide-gel electrophoresis. The proportion of the C3 α -chain cleaved into 74000- and 46000-mol.wt. fragments was estimated as described in Fig. 2. The correlation between convertase and denaturation cleavage is shown in (b).

Effect of dithiothreitol on rate of C3 cleavage

Comparisons of the rates of cleavage of the α -chain of C3 on incubation in SDS and urea at 100°C (Fig. 2*b*) shows that the presence of 20mM-dithiothreitol slows the rate of cleavage approx. 10-fold, but does not appear to decrease the final extent of cleavage. In Fig. 2(*d*), it is shown that incubation in SDS and urea, without dithiothreitol, at 37°C for 30 min results in about 40% cleavage of the α -chain. The standard incubation described in the Materials and methods section (SDS and urea with 20mM-dithiothreitol, pH 7.5, for 30 min at 37°C) does not, however, cause significant cleavage of the α -chain. The difference in rate between these two sets of conditions is due to both a difference in pH (Fig. 4) and the inhibitory effect of dithiothreitol.

Effect of pH and temperature on cleavage of C3 in SDS and urea

General observation, as discussed above, indicated that high pH and high temperature promoted the cleavage of the α -chain of C3 into 74 000- and 46 000-mol.wt. fragments. The effect of varying pH is shown in Fig. 4. The cleavage occurs over the entire pH range studied, but shows a general increase in extent as pH increases.

The effect of temperature on the cleavage reaction is shown by an Arrhenius plot (Fig. 5) of the initial

Fig. 4. Effect of pH on cleavage of C3 in SDS/urea. Samples of C3 (7.5 μ g in 25 μ l of 150mM-NaCl) were incubated for 16 h at 37°C with 25 μ l of 40mM-Tris/40mM-sodium acetate/40mM-diethanolamine/2% (w/v) SDS/8M-urea adjusted (at 37°C) to pH values in the range 4.6–10.6. The samples were then adjusted to pH 7.5 and made 20mM with respect to dithiothreitol by addition of 100mM-dithiothreitol in 1M-Tris/HCl, pH 7.5. Incubation was continued for 30 min at 37°C and samples were then alkylated as described in the Materials and methods section. The percentage of C3 α -chain cleaved into 74 000- and 46 000-mol.wt. fragments was estimated as described in Fig. 2.

rates of cleavage at various temperatures. The activation energy for the bond cleavage, determined from Fig. 5, is 72.8 kJ (17.4 kcal)/mol. Initial rate of cleavage at 100°C is 115 times faster than the rate of cleavage at 37°C.

Other conditions producing cleavage of C3 α -chain into 74 000- and 46 000-mol.wt. fragments

The cleavage of C3, and the similar cleavage of α_2 -macroglobulin (Barrett *et al.*, 1979; Harpel *et al.*, 1979), were initially observed on incubation in SDS and urea. Incubation of C3 in other denaturants and detergents, and the effect of heat denaturation alone was examined. Samples of C3 were incubated under various conditions to produce bond cleavage and were subsequently reduced and alkylated under the mild conditions specified in the Materials and methods section. As shown in Fig. 1 (track 1), the final reduction and alkylation step does

Fig. 5. Effect of temperature on cleavage of C3 in SDS/urea.

Samples of C3 (7.5 μ g in 25 μ l of 150mM-NaCl) were incubated with 25 μ l of 200mM-sodium phosphate (pH 8.0)/2% (w/v) SDS/8M-urea at 25, 37, 56, 75 and 100°C. Individual samples were removed at various times, chilled to 0°C, then reduced and alkylated as described in Fig. 2. Samples were examined on SDS/polyacrylamide gels and the proportion of the C3 α -chain cleaved into 74 000- and 46 000-mol.wt. fragments was estimated as described in Fig. 2. The initial rate of C3 cleavage was determined from graphs of the form shown in Figs. 2(*b*) and 2(*d*). A plot of log(initial rate of cleavage) versus inverse temperature (K⁻¹) is shown.

not itself cause significant bond cleavage. A comparison of the effects of denaturants and detergents is shown in Table 1. Incubation of C3 in guanidine hydrochloride, or in urea/SDS, causes a similar degree of cleavage of the α -chain of C3. Incubation in SDS alone, or in urea alone causes the fragmentation to occur, but to a lesser extent than when urea and SDS are both present. The anionic detergent, sodium deoxycholate, at high concentration, is as effective as SDS and urea, but no cleavage was found on incubation of C3 with a low concentration of sodium deoxycholate. No effect was found with non-ionic detergents at a concentration of 1% (w/v), nor with buffer alone.

Incubation of C3 at elevated temperature in the absence of any denaturant or detergent also causes formation of the 74 000- and 46 000-mol.wt. fragments of the α -chain. A time course of cleavage of the α -chain of C3 on incubation in sodium phosphate buffer at 100°C is shown in Fig. 6. Comparison of Fig. 6 (incubation without denaturant) with Fig. 2(b) (incubation with denaturant) shows that the rate of C3 cleavage at 100°C is approx. 2-fold greater if SDS and urea are present.

Cleavage of C3 on heating in the absence of denaturants was readily observed at other temperatures above 64°C. At 56°C, however, only 6–8% of C3 was detectable after 8 h incubation, and at 37°C cleavage could not be detected even after incubation of C3 for 24 h. An Arrhenius plot of C3 cleavage in the absence of denaturants is shown in Fig. 7. This plot shows a discontinuity at 64°C. The activation energy for the overall reaction above 64°C is 76.6 kJ (18.3 kcal)/mol, a value close to that obtained in the

presence of denaturants (Fig. 5). Below 64°C, however, the minimum activation energy is about 377 kJ (90 kcal)/mol.

In separate experiments, it was shown that incubation of C3 for 15 min at 64°C or above led to protein precipitation and loss of haemolytic activity (R. G. DiScipio & E. Sim, unpublished work), whereas 15 min incubation below 60°C did not cause changes in C3 activity or solubility. The discontinuity in the Arrhenius plot (Fig. 7) therefore corresponds to the temperature at which C3 becomes susceptible to heat denaturation. This observation, together with the data presented in Table 1, shows that the bond-cleavage reaction is denaturation-dependent. In experiments of the type shown in Figs. 6 and 7, it was established that, at temperatures of 64°C and above, C3 precipitation precedes bond cleavage. When denaturants such as

Fig. 6. Time course of C3 cleavage at 100°C without denaturant

Samples of C3 (15 μ g) in 50 μ l of 10 mM-sodium phosphate (pH 8.0)/145 mM-NaCl were incubated for various times at 100°C. Samples were chilled to 0°C, then reduced and alkylated as described in the Materials and methods section. Samples were run on 7.5% polyacrylamide gels in buffers containing SDS. The percentage of C3 cleaved was calculated as described in Fig. 2.

Fig. 7. Arrhenius plot of C3 cleavage in the absence of denaturing agents

The time course of C3 cleavage at 100, 92, 75, 64, 56 and 37°C was studied as described in Fig. 6. Initial rates of C3 cleavage were calculated from graphs of the type shown in Fig. 6. C3 cleavage was readily detectable at temperatures above 64°C and this portion of the Arrhenius plot is shown as a solid line. Of temperatures studied below 64°C, cleavage was detected only at 56°C. The portion of the Arrhenius plot below 64°C is therefore shown as a broken line, formed by extrapolation from 64°C, through the single experimental point at 56°C.

Fig. 8. Comparison of the 74 000- and 46 000 mol.wt. C3 chain fragments with the C3 cleavage products formed by β_1H and C3b inactivator

(a) Comparison of the cleavage products of C3 and C3b. Haemolytically active C3 (30 μ g) or C3b (30 μ g) were incubated (30 min, 37°C) either alone, or with β_1H (5 μ g) or with β_1H (5 μ g) + C3b inactivator (8 μ g) in a total volume of 150 μ l of 5 mM-sodium barbitone / HCl (pH 7.5) / 145 mM-NaCl/0.15 mM- $CaCl_2$ /0.3 mM- $MgCl_2$. Samples were then reduced and alkylated as described in the Materials and methods section, and examined on an SDS/polyacrylamide gel. Samples shown are: 1, C3 + β_1H + C3b inactivator; 2, C3b alone; 3, C3b + β_1H ; 4, C3b + β_1H + C3b inactivator. Alternatively, C3 (30 μ g) or C3b (30 μ g) in 150 μ l of sodium barbitone buffer (as above) were diluted with 150 μ l of 200 mM-Tris/HCl (pH 9.0)/2% (w/v) SDS/8 M-urea and incubated for 10 min at 100°C. Samples were cooled and made 20 mM with respect to

SDS/urea or guanidine are present, however (Table 1), C3 does not precipitate.

The 74 000- and 46 000-mol.wt. products are distinct from those produced by C3b inactivator and β_1H

A comparison of the products of active C3,

Table 1. Production of 74 000- and 46 000-mol.wt. fragments of the C3 α -chain under various conditions

Samples of C3 (60 μ g) were adjusted to final concentrations of denaturants and detergents as shown below, in a total volume of 500 μ l of 10 mM-Tris/acetate (pH 7.0)/10 mM-NaCl/2.5 mM-EDTA. The samples were incubated for 5 h at 37°C. Mixtures containing guanidine hydrochloride were then dialysed against 5 mM-sodium barbitone/HCl (pH 7.5)/145 mM-NaCl. All samples were then adjusted to final concentrations of 1% (w/v) SDS/4 M-urea/20 mM-dithiothreitol, maintaining the same volumes in all samples. The samples were incubated (30 min, 37°C), alkylated as described in the Materials and methods section, and analysed by SDS/polyacrylamide-gel electrophoresis. Gels were stained with Coomassie Blue and scanned, and the percentage of C3 cleaved was calculated as in Fig. 2. 0% = not detectable. The C3 preparation used was the same as shown in Fig. 2.

Denaturant/detergent	Cleavage of C3 α -chain (%)
4 M-Urea + 1% (w/v) SDS	53
4 M-Urea	31
1% (w/v) SDS	44
4 M-Guanidine hydrochloride	55
1% (w/v) Triton X-100	0
1% (w/v) Tween 80	0
1% (w/v) Lubrol	0
0.02% (w/v) Sodium deoxycholate	0
2% (w/v) Sodium deoxycholate	54
Buffer alone	0

dithiothreitol by addition of 100 mM-dithiothreitol in 1 M-Tris/HCl, pH 7.5. Samples were incubated (30 min, 37°C), then alkylated as described in the Materials and methods section. Samples shown are: 5, C3; 6, C3b. (b) Comparison of active C3 and hydroxylamine-inactivated C3. Active C3 (11.5 μ g) or inactivated C3 (11.5 μ g) were incubated (30 min, 37°C) either alone, or with β_1H (2 μ g) + C3b inactivator (1 μ g) in a total volume of 65 μ l of sodium barbitone buffer, as above. Alternatively, active C3 or inactivated C3 in 65 μ l sodium barbitone buffer were diluted with 65 μ l of 200 mM-Tris / HCl (pH 9.0) / 2% (w/v) SDS / 8 M-urea and incubated for 15 min at 100°C. Samples were prepared for electrophoresis as in (a) above. Samples shown are: 1, active C3 incubated in sodium barbitone buffer; 2, active C3 incubated at 100°C in SDS/urea; 3, active C3 + β_1H + C3b inactivator; 4, inactivated C3 incubated in sodium barbitone buffer; 5, inactivated C3 incubated at 100°C in SDS/urea; 6, inactivated C3 + β_1H + C3b inactivator.

Fig. 9. Effect of incubation of C4 and C4b under denaturing conditions

C4 (10 μ g), hydroxylamine-treated C4 (10 μ g) or C4b (10 μ g) in 25 μ l of 5 mM-sodium barbitone/HCl (pH 7.5)/145 mM-NaCl were diluted with 25 μ l of 200 mM-Tris/HCl (pH 9.0)/2% (w/v) SDS/8 M-urea and incubated for 10 min at 100°C. Samples were cooled, made 20 mM with respect to dithiothreitol by addition of 100 mM-dithiothreitol in 1 M-Tris/HCl, pH 7.5, and incubated 30 min at 37°C. Samples were then alkylated as described in the Materials and methods section, and run on 10% polyacrylamide gels in buffers containing SDS. Scans of the Coomassie Blue-stained gels are shown: (a) haemolytically active C4; (b) hydroxylamine-treated C4; (c) C4b.

hydroxylamine-treated C3 and C3b, formed after incubation with SDS/urea or with β_1 H and C3b inactivator are shown in Fig. 8. Incubation in SDS/urea causes cleavage of haemolytically active C3 (Fig. 8a, track 5; Fig. 8b, track 2) but not of C3b (Fig. 8a, track 6) or of inactivated C3 (Fig. 8b, track 5). In contrast, C3b inactivator and β_1 H do not cleave active C3 (Fig. 8a, track 1; Fig. 8b, track 3), but instead, in agreement with Crossley & Porter (1980), cleave only haemolytically inactive C3 (Fig. 8b, track 6) and C3b (Fig. 8a, track 4). The products of cleavage of the α -chain of active C3 incubated in SDS/urea have apparent mol.wts. of 74 000 and 46 000, whereas products of cleavage of the α -chain of inactive C3, or the α' -chain of C3b, by C3b inactivator and β_1 H, have apparent mol.wts. of 76 000 + 43 000, and 68 000 + 43 000 respectively. The two cleavage reactions, although producing superficially similar fragments, are therefore entirely distinct.

Effect of incubation of C4 and C5 with denaturants

C3, C4 and C5 are widely regarded as being structurally homologous proteins (for discussion, see Porter & Reid, 1979). The effect of incubation of C4, C5, C4b and hydroxylamine-treated C4 and C5 under conditions in which the α -chain of C3 was cleaved was therefore investigated. C4 also undergoes cleavage of the α -chain on incubation in SDS/urea, producing fragments of apparent mol.wts. 53 000 and 41 000 (Fig. 9a). As is the case for C3, the combined molecular weights of these fragments is equivalent to the molecular weight of the intact α -chain and so the cleavage is likely to be at a single site with production of only two fragments. Hydroxylamine-treated C4 and C4b do not show any cleavage of the α - or α' -chain under the same conditions (Figs. 9b and 9c). This cleavage pattern of haemolytically active C4 has also been observed on incubation in guanidine (Gigli *et al.*, 1977).

C5, whether native or hydroxylamine-treated does not undergo any cleavage under conditions that cause cleavage of active C3.

Discussion

The results presented demonstrate that haemolytically active C3 and C4 undergo a peptide-bond cleavage in the α -chain when incubated under denaturing conditions. The calculated molecular weights of the cleavage products suggest that, in each case, only a single peptide bond is split. The characteristics of the cleavage reaction have been fully investigated for C3, and these conditions correspond closely to the conditions for 'heat-induced' (Harpel *et al.*, 1979) or 'alkali-sensitive' (Barrett *et al.* 1979) cleavage of α_2 -macroglobulin.

Fig. 10. Suggested fragmentation patterns of C3, C4 and α_2 -macroglobulin

Details are discussed in the text. The polypeptide-chain segments corresponding to the α - and β -chains of C3 and C5 and of the α -, β - and γ -chains of C4 are indicated. The site of activation of these proteins by limited proteolysis is shown by a light arrow (i) and the suggested site of denaturation-induced cleavage, by a heavy arrow (V). The limits of the regions of the polypeptide chains involved in covalent bonding reactions are shaded. N, N-terminus; C, C-terminus.

Under denaturing conditions, the 185 000-mol.wt. monomer polypeptide chain of α_2 -macroglobulin undergoes cleavage to form two fragments of 62 000–68 000 and 125 000–139 000 apparent mol.wt. This fragmentation is seen only with active α_2 -macroglobulin (S- α_2 -macroglobulin), whereas proteinase-cleaved α_2 -macroglobulin or the apparently intact, but inactive F- α_2 -macroglobulin, which is formed during purification and storage, or by methylamine, ammonia or hydrazine treatment (Hall & Roberts, 1978; Barrett *et al.*, 1979; Harpel *et al.*, 1979) do not split. The same pattern is seen with C3 and C4. C3 and C4 both have proteolytically activated forms (C3b and C4b) and both have inactive forms that arise during purification and storage or by chemical treatment as described for F- α_2 -macroglobulin. Only active C3 and C4, like α_2 -macroglobulin, are cleaved under denaturing conditions, whereas C3b, C4b and the inactive forms do not undergo cleavage.

The cleavage conditions for C3 described here and those determined for α_2 -macroglobulin by Harpel *et al.* (1979) are comparable in all respects. The cleavage of the α -chain of C3 to form 74 000- and 46 000-mol.wt. fragments occurs only on denaturation of C3, whether by heat or by chemical denaturants. This is emphasized by the similar values of activation energy for the reaction with denaturants (Fig. 5) or without denaturants above 64°C (Fig. 7). In the absence of denaturants at

temperatures below 64°C, where denaturation is energetically unfavourable, the activation energy is much higher. Extrapolation of the Arrhenius plot as shown in Fig. 7 suggests that cleavage of C3 at 37°C in the absence of denaturant may occur, but at a maximum rate of 0.05%/day. The bond-cleavage reaction of α_2 -macroglobulin has been demonstrated only under conditions where the protein would be chemically or heat-denatured (Barrett *et al.*, 1979; Harpel *et al.*, 1979) and thus the bond-cleavage reaction of α_2 -macroglobulin, like that of C3 is a 'denaturation-induced' rather than 'heat-' or 'alkali-' sensitive. Cleavage of C3 in 2% sodium deoxycholate but not in 0.2% sodium deoxycholate or in other detergents (Table 1) may also represent a denaturant activity of this anionic detergent. The C3-cleavage reaction occurs under a wide range of denaturing conditions. Temperatures of 25°C or above are sufficient to cause fragmentation in the presence of SDS/urea, as is the case for α_2 -macroglobulin (Harpel *et al.*, 1979). α_2 -Macroglobulin is also known, like C3, to undergo fragmentation on incubation with SDS alone, with guanidine or at high temperature in the absence of denaturants (Barrett *et al.*, 1979; Harpel *et al.*, 1979). The C3 fragmentation occurs at all pH values between 4.6 and 10.6 (Fig. 4), but is accelerated at higher pH. The rate of fragmentation of α_2 -macroglobulin also increases in the pH range 7.5–10.5 (Harpel *et al.*, 1979). Neither cleavage reaction

appears to be dependent on any particular buffer component, since C3 cleavage has been shown to occur in both Tris (Fig. 1) and phosphate (Fig. 2) buffers, and Harpel *et al.* (1979) found no difference in α_2 -macroglobulin cleavage in Tris, carbonate or glycine buffers.

The presence of dithiothreitol or other reducing reagent during C3 denaturation is not necessary for cleavage of the α -chain of C3 to occur, although subsequent reduction is, of course, necessary to observe the cleavage products on SDS/polyacrylamide gels. The presence of dithiothreitol during denaturation slows the rate of the cleavage reaction (Fig. 2b). Incubation of C3 with low concentrations (5 mM) of dithiothreitol in the absence of denaturant destroys C3 haemolytic activity (R. B. Sim, unpublished work) and is likely to render C3 resistant to the denaturation-induced cleavage. Reduction of C4 in the absence of denaturants before exposure of the protein to guanidine hydrochloride has been shown to prevent denaturation-induced cleavage of C4 (Gigli *et al.*, 1977). The overall effect of dithiothreitol on the cleavage reaction may therefore depend both on an inhibitory effect on the rate of cleavage and on a separate reaction of inactivation of C3 that prevents cleavage. The rates of these two effects may differ in pH- and temperature-dependence and so the result shown in Fig. 2(b) cannot be extrapolated to other incubation conditions. Studies of α_2 -macroglobulin cleavage have generally been done with denaturant and reductant present simultaneously (Barrett *et al.*, 1979; Harpel *et al.*, 1979), but cleavage occurs in guanidine hydrochloride without reducing agent (Harpel *et al.*, 1979). The rate of C3 cleavage at 100°C in the presence of SDS, urea and dithiothreitol (Fig. 2b) is similar to that seen with α_2 -macroglobulin (Harpel *et al.*, 1979). Both C3 (Fig. 3b) and α_2 -macroglobulin (Harpel *et al.*, 1979) fragmentation can be driven close to 100% yield. Since this is the case and, as discussed above, the bond-cleavage reaction occurs only with the active forms of C3, C4 and α_2 -macroglobulin, the denaturation-induced cleavage reaction provides a method for determining the amount of inactive forms of these proteins present.

The phenomenon being studied involves cleavage of a peptide bond, and so it is difficult to eliminate the possibility that the reaction is due to trace contamination with an unusual proteolytic enzyme. Harpel *et al.* (1979) have argued against proteinase contamination in relation to cleavage of α_2 -macroglobulin, and the comments of these authors apply also to the C3-cleavage reaction. If it is envisaged that the cleavage of C3 is due to a contaminating proteinase, such a proteinase would require: (a) absolute specificity for one site present in denatured C3, but not in native C3, or in denatured or

undenatured inactive C3 or C3b; (b) unusual temperature-stability and resistance to the action of 4 M-guanidine hydrochloride, 4 M-urea and 1% (w/v) SDS for up to 30 min at 100°C (Fig. 2b) or 20 h at 37°C (Fig. 2d); (c) resistance to 20 mM-dithiothreitol at 100°C (Fig. 2b); (d) unusual pH optima (Fig. 4). These requirements do not rule out participation of a proteinase, but make it extremely unlikely. The cleavage reaction occurs predictably and controllably in different samples of three distinct proteins that are isolated by different methods. We therefore consider the likelihood of a constant degree of contamination of C3, C4 and α_2 -macroglobulin with proteinases of similar properties to be negligible, and conclude, in agreement with Harpel *et al.*, (1979) that the cleavage reactions are autolytic.

Contamination of C3 preparations with more 'normal' proteinases appears to be insignificant, since during the long incubation reported here, cleavage products other than the 74 000- and 46 000-mol.wt. fragments were not observed. C3 haemolytic activity is also stable on prolonged (6 h) incubation in physiological buffers at 37°C. (R. B. Sim, unpublished work).

The denaturation-induced fragmentation of the active forms of C3, C4 and α_2 -macroglobulin suggest the presence of chemical groupings of unusual reactivity in these proteins, which are absent or destroyed in the chemically inactivated or proteinase-cleaved forms. Similarly, the active forms of these proteins possess the ability to form covalent bonds after activation by limited proteolysis. This activity is transient, and decays rapidly in the proteinase-cleaved forms and is absent in the chemically inactivated forms. C5, although structurally and functionally similar to C3 (Porter & Reid, 1979) does not undergo denaturation-induced cleavage and apparently also lacks the ability to form covalent bonds (Law *et al.*, 1980). It appears likely, therefore, that the reactive groupings involved in the denaturation-cleavage reaction may be the same as those involved in covalent binding reactions. The covalent binding reaction of C3 has been suggested to occur by transfer of an electrophilic carbonyl group from a thioester bond in C3 to form an ester or amide bond with a suitable binding surface (Law *et al.*, 1979; Tack *et al.*, 1980a; Sim *et al.*, 1981; Twose *et al.*, 1980). The postulated thiol-ester group in C3 is likely to be in a hydrophobic environment (Law *et al.*, 1979; Sim *et al.*, 1980). α_2 -Macroglobulin is also known to bind methylamine through the side-chain carbonyl group of a glutamic acid residue (Swenson & Howard, 1979), and the C4 covalent binding reaction also appears very similar to that of C3 (Campbell *et al.*, 1980; Tack *et al.*, 1980b). It is possible that, in all three proteins, full denaturation of a hydrophobic region containing the reactive carbonyl group occurs

relatively slowly (i.e. at a rate similar to the denaturation-cleavage reaction) and causes the carbonyl group to attack a nitrogen atom involved in the peptide bond. This would result in formation of an imide, which may then hydrolyse in two ways: (a) to cleave the peptide bond, leaving a new, cyclized *N*-terminal group, or (b) to release the original reactive carbonyl group. The nature of the products formed by imide hydrolysis would depend on the exact identity of the amino acid residues involved. Further structural studies will be required to define the mechanism of this reaction more precisely.

All of the proteins discussed undergo activation by limited proteolysis and all but C5 form covalent bonds on activation and cleave on denaturation. The α_2 -macroglobulin monomer is a single polypeptide chain of 180 000–190 000 mol.wt. (Barrett *et al.*, 1979), whereas C3, C4, and C5 are known to be synthesized as single-polypeptide-chain pro-proteins of 180 000–200 000 mol.wt. (reviewed by Porter & Reid, 1979). Although there is relatively little structural and sequence data on any of these proteins or the fragments discussed here, a pattern in the gross structure of the four proteins can be predicted by assuming alignment of the proteinase-sensitive sites, denaturation-sensitive cleavage sites and the known regions involved in covalent binding. A suggested pattern for the alignment of the large fragments of the four proteins is shown in Fig. 10. The data shown for α_2 -macroglobulin are from the results of Salvesen & Barrett (1980). This alignment predicts an order for the known chains of C3, C4 and C5 in the pro- forms of these proteins and in support of this model, Abraham *et al.* (1980) have recently shown that the *N*-terminus of the β -chain of C4 does correspond to the *N*-terminus of pro-C4.

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