Purification of the human complement control protein C3b inactivator

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(Received 18 February 1980/Accepted 28 May 1980)

An alternative method of isolation from human plasma is described for C3b inactivator, C3bINA, the proteinase that in conjunction with either β 1H or C4b-binding protein will hydrolyse respectively C3b or C4b, the activation products of the third, C3, and fourth, C4, components of complement. The purification is by chromatography of plasma on columns of QAE-Sephadex, wheat-germ agglutinin–Sephadex, hydroxyapatite and Sephacryl S-200. The yield of C3bINA (6 mg from 500 ml of plasma) is severalfold higher than in previously described methods. The sensitivity of the assay for C3bINA has been increased by including optimal amounts of β 1H, and it was observed that β 1H was essential for hydrolysis by C3bINA of C3b, whether the C3b was in solution or bound to a cell surface. Native C3 is not hydrolysed by C3bINA + β 1H, but the haemolytically inactive form that appears on prolonged storage at 4°C or on freezing and thawing is hydrolysed and gives fragments of the α -chain of 75000 and 43000 apparent mol.wt. As the α' -chain of C3b, which has lost an *N*-terminal peptide C3a, gives fragments of 67000 and 43000 apparent mol.wt. when incubated with C3bINA + β 1H, this suggests that the larger fragment is *N*-terminal and the smaller one *C*-terminal. The pH optimum of C3bINA with soluble substrates is 6.0, but no clear classification of the type of proteinase to which this enzyme belongs has been obtained.

When complement is activated, by either the classical or the alternative pathway, the components C3b and C4b are formed by proteolytic cleavage of C3 and C4. The products C3b and probably C4b are important, not only in continuing the sequence of events leading to cytolysis, but also in acting as opsonins with other major biological roles. Regulation of these functions of C3b and C4b is achieved via the C3b inactivator (C3bINA), which apparently acts as a proteinase to cleave the α' -chains of C3b and C4b, in conjunction with two cofactors, β 1H and C4b-binding protein respectively. The functions associated with C3b and C4b are lost as a result of the action of C3bINA (for review see Porter & Reid, 1979).

In the case of C3b, C3bINA cleaves a single peptide bond in the α' -chain to produce two

fragments of mol.wts. 67000 and 43000, both of which are disulphide-linked to the β -chain (Pangburn *et al.*, 1977).

C3bINA has been purified to homogeneity and shown to have a structure of two covalently linked chains of mol.wts. 50000 and 38000, both of which contain carbohydrate, as detected by the periodic acid/Schiff stain (Pangburn *et al.*, 1977). Multiple bands between pH 5.7 and 6.1 are observed on isoelectric focusing (Fearon, 1977). C3bINA has been reported to resist denaturation at pH 2.2 or by 2M-guanidine (Pangburn *et al.*, 1977). The low concentration of C3bINA in serum has resulted in only small amounts of highly purified C3bINA being isolated.

The present work reports a method employing mild isolation techniques with precautions against proteolysis for the purification of C3bINA, with a substantially higher yield than has previously been reported. Further information on the properties of C3bINA and the cleavage products formed from C3 derivatives has been obtained.

Materials and methods

Materials

Outdated human plasma was obtained from the

Abbreviations used: the nomenclature of complement components and control proteins is that recommended by the World Health Organisation (1968); activated components are indicated by an overbar, e.g. $\overline{\text{C1}}$, or by the activation product, e.g. C3b or C4b; C3b inactivator is C3bINA; IgG, immunoglobulin G; IgM, immunoglobulin M; iPr₂P-F, di-isopropyl phosphorofluoridate; DGV buffer, dextrose/saline/veronal/gelatin buffer; GV buffer, saline/veronal/gelatin buffer; QAE-Sephadex, quaternary aminoethyl-Sephadex.

Churchill Hospital, Oxford, U.K. Chemicals were obtained as follows: QAE-Sephadex A-50, Sepharose 4B, Sephacryl S-200, CM (carboxymethyl)-Sephadex C-50 and Sephadex G-75, Pharmacia Fine Chemicals, Uppsala, Sweden; hydroxyapatite (DNA-grade Bio-Gel HTP), Bio-Rad Laboratories, Bromley, Kent, U.K.; wheat-germ, local grocers and *N*-acetyl-D-glucosamine, Sigma Chemical Co., Poole, Dorset, U.K.; anti-(human C3bINA) (goat) serum and anti-(human transferrin) serum (goat), Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.; anti-[human (IgG + IgM)] serum (goat), Behring Diagnostics, Marburg, Germany. The sources of other reagents have been described previously (Gigli *et al.*, 1976, 1977).

Complement components

Component C2 and factor B were purified by the method of Kerr & Porter (1978) as modified by Kerr (1979). C3 was isolated by the procedure of Tack & Prahl (1976). β 1H was obtained as a by-product of the C3 preparation, being eluted between IgM and C3/C5 during gel filtration on Sepharose 6B, and was purified to homogeneity by chromatography on hydroxyapatite. Factor $\bar{D}$ was purified by Sephadex G-75 chromatography of a fraction eluted with 0.4 M-sodium phosphate buffer, pH 6.0, from CM-Sephadex C-50 during the procedure for the purification of C2 (Kerr & Porter, 1978; Johnson *et al.*, 1980).

C3b was prepared by incubation of C3 (5.8 mg) with 150 μ g of factor B and 20 μ g of factor $\bar{D}$ in 3.2 ml of 0.15 M-NaCl/25 mM-imidazole buffer, pH 7.0, containing 5 mM-MgCl₂ and 2 mM-CaCl₂, at 37°C for 30 min. Polyacrylamide-gel electrophoresis of samples of the incubation mixture taken at 5 min intervals indicated that conversion of C3 into C3b was complete after 10 min, and less than 1% of the C3 haemolytic activity (Tack & Prahl, 1976) remained at the end of the 30-min incubation period. The incubation mixture was dialysed against 0.1 M-sodium phosphate buffer, pH 6.0, and applied to a column (1.5 cm $\times$ 5 cm) of CM-Sephadex equilibrated with the same buffer. C3b was not adsorbed on the gel, and was separated from inactive C3, factor B and $\bar{D}$, which were adsorbed and eluted on the addition of 0.5 M-NaCl to the buffer. The C3b preparation was homogeneous on polyacrylamide-gel electrophoresis.

Preparation of L-lysine-Sepharose 4B

L-Lysine-Sepharose 4B was prepared by the method of Deutsch & Mertz (1970). The incorporation of lysine was determined by amino acid analysis of a washed and dried sample to be 5 μ mol/ml of packed Sepharose.

Plasminogen adsorbed on L-lysine-Sepharose

from plasma (see below) was eluted with 0.2 M- ϵ -aminohexanoic acid in 0.3 M-potassium phosphate buffer, pH 7.0, and the gel was washed extensively and stored in water containing 0.02% NaN₃.

Preparation of *N*-acetyl-D-glucosamine-Sepharose 4B

Oxirane-Sepharose 4B was prepared by the method of Sundberg & Porath (1974) and immediately coupled with *N*-acetyl-D-glucosamine as described by Vretblad (1976). The gel was washed successively with water, 0.1 M-sodium borate buffer, pH 8.0, 0.1 M-sodium acetate buffer, pH 4.0, and water and finally equilibrated in a column with 50 mM-sodium cacodylate buffer, pH 7.2.

Preparation of wheat-germ agglutinin

Wheat-germ agglutinin was purified by modification of the procedure of Shaper *et al.* (1973). Wheat-germ (780 g) was washed on a Buchner funnel with Whatman no. 4 paper with 4 litres of *n*-hexane and dried overnight. The defatted wheat-germ was ground in a Waring blender in several lots for 3 $\times$ 15 s each. The dry ground wheat-germ was suspended in 5 litres of water at 4°C, stirred for 2 h and centrifuged at 1000 g for 30 min at 4°C. The supernatant was made 55% saturated with (NH₄)₂SO₄ (33 g/100 ml) and stirred at 4°C for 2 h, then centrifuged at 8000 g for 30 min at 4°C. The precipitate was suspended in 800 ml of ice-cold water and centrifuged as before. The resulting supernatant was heated in a water bath at 63°C for 15 min and centrifuged as before to yield a clear golden supernatant.

The extract was applied to a column (2.2 cm $\times$ 15 cm) of *N*-acetyl-D-glucosamine-Sepharose 4B equilibrated with 50 mM-sodium cacodylate buffer, pH 7.2, at 35 ml/h. The column was washed with 600 ml of buffer until the A_{280} of the eluate had fallen to 0.06, after which the agglutinin remaining adsorbed on the gel was eluted with buffer containing 100 mg of *N*-acetyl-D-glucosamine/ml. The fractions containing the agglutinin were combined. The recovery was 27 mg of agglutinin/100 g of wheat-germ, on the basis of $A_{280}^{1\%} = 14.3$ (Nagata & Burger, 1974). Several preparations of wheat-germ agglutinin were combined, precipitated with (NH₄)₂SO₄ (40 g/100 ml) and redissolved in the minimal volume of 50 mM-sodium cacodylate buffer, pH 7.2.

After each use, the *N*-acetyl-D-glucosamine-Sepharose 4B was washed successively on a sintered-glass funnel with water, 6 M-urea containing 50 mM-EDTA, pH 7.0, water, 30% (w/v) ethanol and water and stored in water containing 0.02% NaN₃.

Preparation of wheat-germ agglutinin–Sephacrose 4B

Sephacrose 4B (100ml) was activated with 10g of CNBr by the procedure of Cuatrecasas *et al.* (1968), the pH being maintained at 11 with 4M-NaOH for 8min. The Sepharose was then washed on a sintered-glass funnel with 2 litres of cold 0.1M-NaHCO₃, and the moist Sepharose was transferred to a beaker containing 100ml of 0.15M-NaCl/0.1M-NaHCO₃, pH 8.3. A concentrated solution of wheat-germ agglutinin (500mg in 40ml), dialysed against 0.15M-NaCl/0.1M-NaHCO₃, pH 8.3, containing 0.1M-N-acetyl-D-glucosamine was added and the mixture was stirred at 20°C for 3h. The wheat-germ agglutinin–Sephacrose was washed extensively with 0.15M-NaCl/0.1M-NaCO₃, pH 8.3, and the same buffer containing 100mg of N-acetyl-D-glucosamine/ml, and stored at 4°C in 0.2M-NaCl/50mM-sodium phosphate buffer, pH 7.0, containing 0.02% NaN₃.

Buffers used in haemolytic assay of C3bINA

Buffers of the following compositions were used: GV buffer, iso-osmotic veronal buffer, pH 7.5, containing 0.1% gelatin (Nelson *et al.*, 1966); EDTA/DGV buffer, pH 6.5, 5% (w/v) glucose in water/GV buffer/0.1M-EDTA, pH 7.5 (15:5:2, by vol.) adjusted to pH 6.5 with 1M-HCl; DGV buffer, equal volumes of GV buffer and 5% (w/v) glucose; DGV²⁺ buffer, DGV buffer containing 0.15mM-CaCl₂ and 0.5mM-MgCl₂; EDTA/GV buffer, GV buffer/0.1M-EDTA, pH 7.5 (1:9, v/v).

Preparation of EAC43 cells

EAC43 cells were prepared by the method of C. Parkes, R. G. DiScipio & M. A. Kerr (personal communication) as follows: Sheep erythrocytes sensitized by antibody and components C1 and C4 (EAC14) (Borsos & Rapp, 1967) were incubated in DGV²⁺ buffer (1 × 10⁸ cells/ml) with human component C2 (0.4 µg/ml) and component C3 (5 µg/ml) for 5 min at 30°C, then chilled on ice for 10 min. The cells were then centrifuged at 1000g for 10 min at 4°C and resuspended in EDTA/GV buffer. This latter step was repeated, and the mixture was incubated for 15 min at 37°C, centrifuged, resuspended in EDTA/GV buffer and incubated for a further 15 min at 37°C. The resulting EAC43 cells were washed twice with DGV²⁺ buffer and stored at 4°C.

Assay of C3bINA

C3bINA activity was assayed by modification of the method of Whaley *et al.* (1976). EAC43 cells in DGV²⁺ buffer (1 × 10⁸/ml) were pelleted by centrifugation at 1000g for 10 min, washed twice by resuspension to the same concentration of cells in

EDTA/DGV buffer, pH 6.5, and finally resuspended in EDTA/DGV buffer, pH 6.5, containing 10 µg of β1H/ml immediately before use. Dilutions (0.1 ml) of C3bINA in EDTA/DGV buffer, pH 6.5, were incubated at 37°C for 30 min with 0.1 ml of the washed EAC43 cells (1 × 10⁸/ml). Ice-cold DGV buffer (0.5 ml) was added to stop the reaction, and the cells were washed twice with ice-cold DGV buffer and once with ice-cold DGV²⁺ buffer. The pelleted cells were resuspended in DGV²⁺ buffer (0.3 ml) containing factors B (7 µg/ml) and $\bar{D}$ (0.4 µg/ml) and incubated at 30°C for 30 min. The amounts of components B and $\bar{D}$ were predetermined to give maximum lysis of EAC43 cells in the absence of C3bINA in the first incubation mixture. After addition of 0.3 ml of guinea-pig serum diluted 1:30 with 0.04M-EDTA in GV buffer and further incubation at 37°C for 1 h, 1 ml of ice-cold 0.15M-NaCl was added and the mixture centrifuged at 1000g for 10 min. The degree of lysis was measured from the A_{410} of the supernatant and expressed as (Z') haemolytic units, 1 unit being the reciprocal of the dilution of C3bINA that gives $Z' = 1$, where $Z' = \ln(1 - \% \text{ inactivation})$ (Whaley *et al.*, 1976).

Preparation of C3bINA

All procedures were performed at 4°C and all buffers contained 0.02% NaN₃. Tris buffers were prepared by dilution of 1M-Tris adjusted to the stated pH with HCl at 20°C. Each of the columns was used repeatedly after washing as described without repacking of the column, with the exception of the QAE-Sephadex resin, which was regenerated by washing successively with 0.1M-HCl, water, 0.1M-NaOH and water.

Plasma (500 ml), to which 0.5 ml of 2.5M-iPr₂P-F in propan-2-ol was added, was clarified by centrifugation at 10000g for 30 min. The supernatant was applied to a column (5 cm × 15 cm) of lysine–Sephacrose equilibrated with 0.1M-potassium phosphate buffer, pH 7.0, containing 0.15M-NaCl and 15mM-EDTA at a flow rate of 200 ml/h. The column was washed with buffer, and the A_{280} of the eluate was monitored and the non-absorbed protein eluted after the void volume was collected until the A_{280} of the eluate was less than 1.0. The plasminogen-depleted protein pool was made 2.5mM with respect to iPr₂P-F and dialysed against three 3-litre changes of 60mM-NaCl/20mM-Tris/HCl buffer, pH 7.8. The non-diffusible material was passed through a column (7 cm × 36 cm) of QAE-Sephadex equilibrated with the dialysis buffer at a flow rate of 300 ml/h. Fractions (25 ml) were collected and the absorption at 280 nm of the eluate was recorded. After washing of the column with 1.5 column volumes of dialysis buffer, a linear gradient (2 × 2 litres) of the buffer containing 60mM-NaCl and of buffer containing 0.35M-NaCl was applied, followed

by a step to buffer containing 1.0M-NaCl. The fractions containing C3bINA activity (Fig. 2a below) were combined and made 60% saturated (36g/100ml) with $(\text{NH}_4)_2\text{SO}_4$ and stirred for 2 h at 4°C. After centrifugation of the mixture at 8000g for 20min, the precipitate was dissolved in 60ml of 50mM-sodium phosphate buffer, pH 7.0, containing 0.2M-NaCl, and then $\text{iPr}_2\text{P-F}$ was added to 2.5 mM. The concentrated solution was dialysed against three 1-litre changes of buffer and passed through a column (4cm $\times$ 7.5cm) of wheat-germ agglutinin-Sephacrose equilibrated with the same buffer at 15 ml/h. After an initial wash with 1 column volume of buffer, the flow rate was increased to 30 ml/h, and 25 ml fractions were collected until the A_{280} of the eluate was less than 0.15. Adsorbed protein was eluted with buffer containing 100mg of *N*-acetyl-D-glucosamine/ml (Fig. 2b below), or alternatively with a linear gradient of 150ml of buffer and 150ml of buffer containing 30mg of *N*-acetyl-D-glucosamine/ml followed by a step to buffer containing 100mg of the sugar/ml (Fig. 2c below). The active fractions from stepwise elution were pooled, made 2.5 mM with respect to $\text{iPr}_2\text{P-F}$ and dialysed against three 2-litre changes of 25 mM-potassium phosphate buffer, pH 7.5. The solution was applied to a column (3.2cm $\times$ 15cm) of hydroxyapatite equilibrated with the dialysis buffer at a flow rate of 35 ml/h, and 10ml fractions were collected. The column was washed with buffer until the A_{280} of the eluate was less than 0.05, when a linear gradient (2 $\times$ 300ml) of 25mM-0.2M-potassium phosphate buffer, pH 7.5, was applied (Fig. 2d below). The pooled fractions containing C3bINA activity were concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (40g/100ml) as before, and the precipitate was redissolved in 3–5 ml of 25mM-Tris/HCl buffer, pH 7.0. The concentrated solution was passed directly through a column of Sephacryl S-200 equilibrated with 25mM-Tris/HCl buffer, pH 7.0, containing 0.15M-NaCl at 12 ml/h, and 4ml fractions were collected (Fig. 2e below). The active fractions were combined, made 2.5 mM with respect to $\text{iPr}_2\text{P-F}$ and concentrated by

ultrafiltration with a Diaflo PM10 membrane to 2–3 mg/ml and stored at 4°C.

Electrophoresis in polyacrylamide gels

Gel electrophoresis in 7.5% (w/v) polyacrylamide slabs in the presence of sodium dodecyl sulphate was performed by the method of Laemmli (1970). Apparent molecular weights were assigned by comparison with proteins of known molecular weights (β 1H, mol.wt. 150000; transferrin, mol.wt. 90000; bovine serum albumin, mol.wt. 67000; ovalbumin, mol.wt. 45000; bovine β -lactoglobulin, mol.wt. 18400). Gel slabs were scanned with a Vitatron TLD 100 densitometer equipped with a peak-area integrator.

Protein concentration and amino acid analysis

The recovery of protein during the purification of C3bINA was determined by a modification of the Lowry method (Hartree, 1972), with bovine serum albumin as the reference standard. Other protein concentrations were calculated from the A_{280} , assuming that an absorption value of 1.0 equals 1 mg/ml or, for factor $\bar{D}$, 0.5 mg/ml. Amino acid analyses were carried out as described by Gigli *et al.* (1977).

Results

Haemolytic assay of C3bINA

The optimal conditions for the inactivation of C3b on EAC43 cells by C3bINA were investigated by using pure C3bINA and β 1H and veronal buffers with a range of ionic strength and pH compatible with cell viability. EDTA was included in the veronal buffers, as it is required to prevent C3b formation during assays of crude fractions. At physiological pH, decreasing the ionic strength from 0.15M-NaCl to 0.038M-NaCl resulted in a 3-fold increase in the specific activity of C3bINA. In the presence of 0.038M-NaCl, however, decreasing the pH from 7.5 to 6.5 resulted in a 30-fold increase in the specific activity of C3bINA (Table 1). Accordingly the

Table 1. *Effect of ionic strength and pH on C3bINA haemolytic activity*

The activity of pure C3bINA was determined by incubation of C3bINA with EAC43 cells and 1 μ g of β 1H in the buffers indicated at 37°C for 30 min, followed by the addition of factors B and $\bar{D}$ and guinea-pig serum as described in the Materials and methods section. The activity (units/mg) obtained for C3bINA in each buffer is expressed as a percentage of the activity obtained in the standard assay buffer.

Composition of buffer (by vol.)		C3bINA activity (%)
5% Dextrose/GV buffer/0.1 M-EDTA, pH 7.5	pH	
0:10:1	7.5	1
5:5:1	7.5	2
7.5:2.5:1	7.5	3
7.5:2.5:1	7.0	20
7.5:2.5:1	6.5	100

low-ionic-strength buffer at pH 6.5 was adopted as the standard assay buffer.

The effect of varying the concentration of β 1H on C3bINA activity is shown in Fig. 1. In the absence of β 1H there was no inhibition of lysis by C3bINA and similarly, in the absence of C3bINA, even high concentrations of β 1H ($4\mu\text{g}/\text{assay}$) did not result in inhibition of lysis. The decrease in the number of C3b haemolytic sites was directly proportional to the concentration of β 1H in the range 0.0625 – $1\mu\text{g}$ of β 1H/assay for a given concentration of C3bINA. The amount of β 1H added to the standard assay was therefore fixed at 0.1 ml of solution containing $10\mu\text{g}$ of β 1H/ml, i.e. $1\mu\text{g}/\text{assay}$. Relatively more β 1H was required to achieve near-maximal inactivation of cell bound C3b as the concentration of C3bINA was decreased. Thus, in the presence of $1\mu\text{g}$ of β 1H, near-maximal inactivation of cell bound C3b was observed only at the highest concentration of C3bINA, when the molar ratio β 1H/C3bINA was $20:1$. At the two lower concentrations of C3bINA, even though the molar ratios of β 1H to C3bINA were increased to $40:1$ and $80:1$, the inactivation was not complete.

Purification of C3bINA

The recovery of C3bINA activity was found to be optimal when plasma depleted of plasminogen rather than serum was used as the starting material for the purification scheme. Chromatography on the affinity adsorbent lysine-Sepharose gave quantitative recovery of C3bINA activity and prevented activa-

tion of the clotting factors. As an additional precaution against proteolysis, repeated addition of $i\text{Pr}_2\text{P-F}$ was made during the early stages of the purification. Attempts to obtain an initial fractionation of the C3bINA activity in plasma by using either poly(ethylene glycol) or $(\text{NH}_4)_2\text{SO}_4$ were unsatisfactory, since C3bINA activity was precipitated over a wide range of poly(ethylene glycol) concentrations at pH 7.4, affording little purification, and similarly at least 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ was required to precipitate 80% of the C3bINA activity.

Chromatography of the plasminogen-depleted plasma on QAE-Sephadex A-50 removed 95% of the protein with little loss of C3bINA activity (Table 2) and was therefore adopted as the initial purification step. Under the conditions specified, C3bINA, together with transferrin, which serves as a visual marker, is separated from the bulk of the protein bound to the resin by extended washing before application of the salt gradient (Fig. 2a). Analysis by immunodiffusion showed that the QAE-Sephadex A-50 pool of C3bINA activity did not contain β 1H, which is eluted at higher salt concentration. Removal of β 1H is advantageous before chromatography on wheat-germ agglutinin-Sepharose, as this protein is present in large amounts in plasma and was found to bind to the affinity resin as does C3bINA.

The QAE-Sephadex pool of C3bINA activity was concentrated by precipitation by 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ before chromatography on wheat-germ agglutinin-Sepharose. Most of the transferrin stays in solution during the $(\text{NH}_4)_2\text{SO}_4$ step, and the remainder is removed by chromatography on the affinity adsorbent. The protein bound to wheat-germ agglutinin-Sepharose was eluted by the inclusion of *N*-acetyl-D-glucosamine in the buffer either as a step to a sugar concentration of 100 mg/ml (Fig. 2b) or as a gradient to a sugar concentration of 30 mg/ml , which eluted the C3bINA activity as a diffuse peak, followed by a step to 100 mg/ml to remove residual adsorbed protein (Fig. 2c). The simple step-elution method gave a more concentrated protein solution with a better recovery of C3bINA activity than the gradient elution method, and was used routinely. In either case, two further steps were required to remove remaining contaminants: chromatography on hydroxyapatite (Fig. 2d) was followed by gel filtration on Sephacryl S-200 (Fig. 2e) as a final step. The solution of purified C3bINA was stable at 4°C over the pH range 6–8 for days and at pH 7 for a period of weeks, even in the absence of proteinase inhibitors.

The purification scheme is summarized in Table 2, and electrophoresis patterns in polyacrylamide gels containing sodium dodecyl sulphate are shown in Fig. 3. Electrophoresis of the C3bINA preparation obtained after fractionation on Sephacryl S-200

Fig. 1. Effect of increasing concentration of β 1H on C3bINA inhibition of haemolytic activity

EAC43 cells were incubated in EDTA/DGV buffer, pH 6.5, containing various concentrations of homogeneous preparations of C3bINA [$0\mu\text{g}$ (○); $0.007\mu\text{g}$ (●); $0.015\mu\text{g}$ (□); $0.029\mu\text{g}$ (■)] and β 1H, and the haemolytic activity was determined as described in the Materials and methods section.

Table 2. *Purification of C3bINA from plasma*
The purification was performed with 500 ml of plasma as described in the text.

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Activity yield (%)
Plasma	500	440 000	29 000	15	100
QAE-Sephadex pool	770	385 000	1600	240	88
Wheat-germ agglutinin-Sephadex pool	223	152 000	150	1000	35
Hydroxyapatite pool	98	118 000	20	5900	27
Sephacryl S-200 pool	23	88 000	6	15 000	20

Fig. 2. *Chromatography of C3bINA*

(a) The dialysed preparation of plasminogen-depleted plasma (750 ml) was loaded on a column (7 cm $\times$ 36 cm) of QAE-Sephadex and eluted with buffer containing 20 mM-Tris/HCl, pH 7.8, and an increasing concentration of NaCl applied as a gradient (first arrow) and 1.0 M-NaCl step (second arrow) as described in the Materials and methods section. Active fractions were pooled as shown. —, A_{280} ; O, C3bINA activity (percentage inhibi-

tion of lysis); ----, conductivity (mS). (b) The pool from (a) was concentrated and dialysed (80 ml), then applied to a column (4 cm $\times$ 7.5 cm) of wheat-germ agglutinin-Sephadex and eluted with 0.2 M-NaCl/50 mM-sodium phosphate buffer, pH 7.0, as described in the Materials and methods section. The arrow indicates the addition of 100 mg of *N*-acetyl-D-glucosamine/ml to the eluting buffer. Symbols are as for (a). (c) A pool obtained in a separate preparation, but by the same procedure as that in (a), was loaded on wheat-germ agglutinin-Sephadex as in (b). Elution of adsorbed protein was effected by increasing concentration of *N*-acetyl-D-glucosamine in buffer applied as a gradient (first arrow) followed by a step to 100 mg/ml (second arrow). Symbols are as for (a). (d) After dialysis, the pool from (b) was loaded on a hydroxyapatite column (3.2 cm $\times$ 15 cm) in 25 mM-potassium phosphate buffer, pH 7.5, and eluted by a gradient of increasing phosphate concentration as described in the Materials and methods section. Symbols are as for (a). (e) The concentrated pool from (d) was chromatographed on a Sephacryl S-200 column (2.5 cm $\times$ 100 cm) in 0.15 M-NaCl/25 mM-Tris/HCl buffer, pH 7.0. Symbols are as for (a).

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Fig. 3. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of samples taken at various stages of the C3bINA purification

For experimental details see the text. Reduced samples: (1) plasma; (2) QAE-Sephadex pool; (3) wheat-germ agglutinin pool; (4) hydroxyapatite pool; (5) Sephacryl S-200 pool (4 μ g). Non-reduced samples: (6) Sephacryl S-200 pool (2 μ g); (7) Sephacryl S-200 pool (10 μ g).

Cleavage of fluid-phase C3b and inactive C3 by C3bINA and β 1H

As shown in Fig. 4, the α' -chain of C3b in solution was cleaved by C3bINA and β 1H to produce two fragments (which required reduction for dissociation from the β -chain) of apparent mol.wts. 67000 and 43000. The α' -chain of C3b was unaffected by either C3bINA or β 1H alone. No other degradative products have been observed even in the absence of iPr_2P-F treatment of the purified components. With equimolar concentrations of C3b, C3bINA and β 1H at pH 7, 50% cleavage of C3b α' -chain occurred after about 1 min at 37°C (Fig. 5). When the molar concentration of either C3bINA or β 1H was decreased 10-fold, the time required for 50% cleavage was increased approx. 10-fold and more than 30-fold respectively.

Native C3, unlike C3b, is not cleaved by C3bINA and β 1H. However, preparations of C3 lose haemolytic activity on standing at 4°C or on slow-freezing at -20°C (von Zabern *et al.*, 1980; M. A. Kerr, unpublished work). The loss of haemolytic activity correlates with the loss of hydrolysis of the α -chain by C3 convertases of both activation pathways (C. Parkes, R. G. DiScipio & M. A. Kerr, unpublished work). This denaturation of native C3 is accompanied by exposure of a reactive thiol group, as occurs when C3b is formed (Janatova *et al.*, 1979). Such inactive C3 was also found to be cleaved by

Fig. 4. Cleavage of fluid-phase C3b by C3bINA and β 1H

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis of reduced samples was performed as described in the text: (1) C3b (1.3 μ g); (2) C3bINA (4 μ g); (3) β 1H (1.5 μ g). Other samples (20 μ l) in 0.15 M-NaCl/0.1 M-sodium phosphate buffer, pH 7, containing equimolar concentrations of C3b (1.3 μ g), C3bINA (0.6 μ g) or β 1H (0.9 μ g) added as indicated, were incubated at 37°C for various times, then reduced and electrophoresed: (4) C3b + C3bINA, 30 min; (5) C3b + β 1H, 30 min; (6)–(12) C3b, C3bINA + β 1H [(6) 2 min; (7) 4 min; (8) 6 min; (9) 10 min; (10) 15 min; (11) 20 min; (12) 30 min]. Arrows indicate the C3bINA cleavage products of apparent mol.wts. 67000 and 43000.

Fig. 5. Rate of cleavage of C3b as a function of C3bINA and β 1H concentration

Samples in 0.15M-NaCl/0.1M-sodium phosphate buffer, pH7, containing C3b, C3bINA and β 1H in various molar proportions [1:1:1 (O); 1:0.1:1 (□); 1:1:0.1 (Δ)] were incubated for various times at 37°C, then electrophoresed. The percentage cleavage of C3b α' -chain was determined by densitometric scanning of the slab gels.

Fig. 6. Cleavage of fluid-phase inactive C3 by C3bINA and β 1H

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of reduced samples was performed as described for Fig. 4 except that inactive C3 instead of C3b was examined. Arrows indicate the C3bINA cleavage products of apparent mol.wts. 75 000 and 43 000.

C3bINA and β 1H to yield the pattern shown in Fig. 6. As was observed with C3b, the α -chain of inactive C3 was unaffected by either C3bINA or β 1H alone. Two fragments were produced by cleavage of the α -chain, and these had apparent mol.wts. of 75 000 and 43 000. Since the C3a region of C3 is at the N-terminus of the α -chain (Hugli, 1975; Tack *et al.*, 1979), and since cleavage of C3b yields fragments of the α' -chain of mol.wts. 67 000 and 43 000, this result indicates that the 43 000-mol.wt. fragment is the C-terminal fragment of the α - and α' -chains. Comparison of the rates of cleavage of the α' -chain of C3b and of the α -chain of inactive C3, at two different concentrations of C3bINA, indicated that inactive C3 was cleaved at about one-quarter of the rate of C3b (Fig. 7).

pH optimum for C3bINA activity

The pH optimum for the cleavage of C3b by C3bINA and β 1H was found to be 6 when examined in various buffers in the pH range of 5–9 (Fig. 8).

Fig. 7. Comparison of the rate of cleavage of inactive C3 and C3b

Samples in 0.15M-NaCl/0.1M-sodium phosphate buffer, pH7, containing inactive C3, C3bINA and β 1H [in molar proportions 1:1:1 (O) and 1:0.1:1 (□)] or C3b, C3bINA and β 1H [in molar proportions 1:1:1 (●) and 1:0.1:1 (■)] were incubated for various times at 37°C, then electrophoresed. The percentage cleavage of inactive C3 α -chain or C3b α' -chain was determined by densitometric scanning of the gel slab.

Fig. 8. Effect of pH on the cleavage of fluid-phase C3b by C3bINA and β 1H

Samples containing C3b, C3bINA and β 1H (in molar proportions 1:0.02:1) were incubated for 7.5 min at 37°C in buffers [A, 0.1 M-Mes (4-morpholine-ethanesulphonic acid); B, 0.1 M-Pipes (1,4-piperazinediethanesulphonic acid); C, 0.1 M-Tris] adjusted to the indicated pH and conductivity (----) with NaOH or HCl. After electrophoresis, the percentage cleavage of the C3b α' -chain was determined by densitometric scanning of the gel slabs.

This agrees with the optimum pH of 6.5 (in the limited range 6.5–7.5) that gave the maximal inactivation of C3b on EAC43 cells (Table 1).

Effect of proteinase inhibitors on C3bINA

Preliminary attempts to identify the class of proteinase to which C3bINA belongs were unsuccessful. As judged by splitting of the C3b α' -chain, C3bINA was not inhibited by i Pr₂P-F (10 mM), 7-amino-1-chloro-3-tosylamidoheptan-2-one (5 mM), phenylmethanesulphonyl fluoride (0.1 mM) or benzamidine (5 mM), and hence may not be a serine esterase-type proteinase. However, C2 and factor B, which have been reported to be serine proteinases, are also resistant to some of these reagents (Kerr, 1979). Chelating reagents such as 1,10-phenanthroline (1 mM), EDTA (50 mM) and EGTA (50 mM) had no effect, and further evidence against a functional role for metals was observed in the acidic pH optimum of 6 for the reaction. There was no evidence for an essential thiol group, as there was no inhibition by HgCl₂ (1 mM), iodoacetic acid (1 mM) or 4-chloromercuribenzoate (0.1 mM), although *N*-ethylmaleimide showed some inhibition at a concentration of 1 mM.

Strong inhibition of C3bINA was observed with 1 mM-dithiothreitol and 10 mM-2-mercaptoethanol, presumably as a result of the reduction of interchain

disulphide bonds. Surprisingly, ZnCl₂, but not MgCl₂, MnCl₂, CaCl₂, CoCl₂ or NiCl₂, all at 1 mM, strongly inhibited C3bINA. The mechanism of this effect of ZnCl₂ has not been investigated.

The C3bINA proteolytic activity is unusual in that it appears to be dependent on prior interaction of either β 1H or C4-binding protein with the substrates C3b or C4b and in the specificity of the hydrolysis of a single peptide bond in C3b α' -chain and of two bonds in the C4b α' -chain.

Discussion

Inactivation of C3b

The assay of C3bINA activity measures the inactivation of C3b bound to EAC43 cells. On addition of factors B and $\bar{D}$, in the presence of Mg²⁺ and Ca²⁺, the alternative-pathway C3 convertase is formed, and the subsequent addition of C3–C9 as guinea-pig serum diluted in EDTA results in activation of the terminal components causing cell lysis. In the presence of limiting amounts of C3b on the EAC43 cells and an excess of factors B, $\bar{D}$ and guinea-pig serum, cell lysis is inhibited to a degree determined by the extent of inactivation of C3b by C3bINA. When pure C3bINA was assayed with pure β 1H, the inactivation of cell bound C3b was enhanced 100-fold in a low-ionic-strength pH 6.5 buffer as compared with an iso-osmotic pH 7.5 buffer. This result is in agreement with similar studies with serum as a source of C3bINA and β 1H (Whaley *et al.*, 1976).

The inactivation of C3b in both solid and fluid phases does not proceed in the absence of β 1H. An absolute requirement for β 1H by C3bINA for cleavage of C3b in solution was first reported by Pangburn *et al.* (1977). In other studies, however, both C3bINA alone and/or β 1H alone have been reported to produce concentration-dependent inhibition of solid-phase C3b, with β 1H serving to accelerate the effect of C3bINA (Whaley & Ruddy, 1976; Fearon & Austen, 1977; Fujita & Nussenzweig, 1979).

Evidence suggesting that C3bINA binds preferentially or exclusively to a bimolecular complex of C3b and β 1H, as compared with C3b alone, was obtained in experiments with both solid-phase and fluid-phase C3b. Thus relatively more β 1H was required to achieve maximum inactivation of cell bound C3b as the concentration of C3bINA was decreased. Similarly, the cleavage of C3b in solution was slowed more by a decrease in β 1H concentration than by a corresponding decrease in C3bINA concentration. The conclusion that the rate of inactivation of C3b by C3bINA is a function of the concentration of the C3b– β 1H complex is supported by the results reported for binding studies with the solid-phase EC3b. At 0°C, C3bINA

binding was proportional to the amount of β 1H bound and approached equimolar amounts at saturation of the sites binding C3bINA, with up to 30-fold enhancement of C3bINA binding in the presence of β 1H (Pangburn & Müller-Eberhard, 1978).

Purification of C3bINA

Loss of C3bINA activity during the isolation was little more than handling losses, except in the binding and desorption from the wheat-germ agglutinin-Sephadex column, where it was about 50% even with stepwise elution (Table 2). This may have been due to increased susceptibility of C3bINA to proteolytic digestion when bound even though inhibitors were added, but perhaps is more probably due to irreversible adsorption. Attempts to improve this yield are continuing, but the overall 20% recovery, described here, is higher than in previously described methods (Pangburn *et al.*, 1977; Fearon, 1977).

Hydrolysis of inactive C3 by C3bINA and β 1H

The hydrolysis of the α' -chain of C3b by C3bINA and β 1H and of C4b by C3bINA and C4-binding protein has been described previously (Pangburn *et al.*, 1977; Nagasawa & Stroud, 1977; Fujita *et al.*, 1978), and has been confirmed here. Native C3 and C4 are not hydrolysed by these enzymes, but both proteins lose haemolytic activity on standing in the cold, on freezing and thawing or on treatment with amines. In the last-mentioned case, the inactive protein is antigenically similar to C3b or C4b, though no peptide bond is split (von Zabern *et al.*, 1980). We have found that inactive C3 is hydrolysed by C3bINA and β 1H, though more slowly than C3b, to give chain products of 75000 and 43000 mol.wt., rather than 67000 and 43000 mol.wt. as from the α' -chain of C3b (Fig. 7). As discussed above, this suggests that the 43000-mol.wt. fragment is C-terminal and that the larger fragment is N-terminal.

Similarly, inactive C4 but not native C4 is hydrolysed by C3bINA and C4-binding protein (E. M. Press, unpublished work), and this suggests that the denaturation of C3 and C4 results in a change of conformation similar to that of C3b and C4b. This is in agreement with the evidence obtained by von Zabern *et al.* (1980) and with the observation by Janatova *et al.* (1979) that a thiol group unreactive in native C3 or C4 becomes reactive in both C3b or C4b and in inactive C3 and C4.

The gifts of anti-(human β 1H) serum (sheep) by Dr. A. R. Bradwell of the Immunodiagnostic Research Laboratory, Department of Immunology, University of Birmingham, Birmingham, U.K., and of complement compon-

ents by Dr. D. L. Christie, Dr. R. G. Di Scipio, Dr. M. A. Kerr and Dr. R. B. Sim of this laboratory are gratefully acknowledged.

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