The Purification and Characterization of Subcomponent C1s of the First Component of Bovine Complement

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Bovine C1s, a subcomponent of the first component of complement, was purified in good yield by a combination of euglobulin precipitation and ion-exchange and molecular-sieve chromatography. Approx. 10 mg can be obtained from 3 litres of serum, representing a yield of 11%. The C1s is obtained in zymogen form, with a mol.wt. of 85000–88000, determined by gel filtration and SDS/polyacrylamide-gel electrophoresis. It is haemolytically active when tested with human C1q and C1r. Activation can be achieved by incubation with human C1r, resulting in cleavage of the C1s chain into two chains of 65000 and 27000 mol.wt. and the generation of an isoleucine *N*-terminal residue on the smaller chain. Active C1s binds an equimolar amount of di-isopropyl phosphorofluoridate to the smaller chain, which is the *C*-terminal part in the zymogen. The chains can be separated by ion-exchange in 8M-urea. All of these characteristics show that bovine C1s is very similar to its human counterpart.

Complement is a complex mixture of plasma proteins that play an important part in defence against infection. Activation of the system can take place by two main routes, the classical and alternative pathways, both leading to activation of the membrane-attack system, which can result in cytolysis (reviewed by Fothergill & Anderson, 1978; Porter & Reid, 1978). The classical pathway is activated mainly by antigen–antibody aggregates, which interact with the first component of complement, C1, a Ca^{2+} -dependent complex of three proteins, C1q, C1r and C1s (Lepow *et al.*, 1963). The binding of the first component of complement to the Fc portion of immunoglobulin takes place through the C1q subcomponent (Yasmeen *et al.*, 1976). The binding of subcomponent C1q causes activation of subcomponent C1r in the C1 complex, by the splitting of probably a single peptide bond (Gigli *et al.*, 1976;

Sim *et al.*, 1977), which in turn allows activation of subcomponent C1s proenzyme (Ziccardi & Cooper, 1976; Sim *et al.*, 1977) to yield the fully active C1 complex.

Human subcomponent C1s occurs as a single-chain zymogen of about 85000 mol.wt. (Gigli *et al.*, 1976) that is cleaved on activation to give two disulphide-linked chains of mol.wts. 55000 and 27000 (Arlaud *et al.*, 1977; Sim *et al.*, 1977). C1s is inhibited by $\text{iPr}_2\text{P-F}$ (Becker, 1956) and inactivation is accompanied by equimolar binding of the reagent to the smaller chain (Barkas *et al.*, 1973; Sim & Porter, 1976), which also binds the C1 inhibitor (Harpel & Cooper, 1975). The small chain shows a number of homologies of amino acid sequence with serine proteinases, both in the active-site region (Barkas *et al.*, 1973) and at the *N*-terminus (Sim *et al.*, 1977). The *N*-terminal sequence of the large chain shows no obvious homologies with other proteins (Sim *et al.*, 1977).

Detailed structural studies of human complement components have been hampered by the poor availability of blood. Similar disadvantages apply to small animals such as the rabbit and the guinea pig. Although very little characterization has been carried out on the complement system in larger animals, it seemed worthwhile to investigate their potential as a source of complement components. The ready availability of bovine blood made this the system of choice, as it has also been for detailed study of many coagulation factors. Comparison of the structures of human and bovine components may help to elucidate

Abbreviations used: the symbols for complement components conform to the recommendations of the World Health Organisation (1968). Activated components are indicated by a bar, e.g. $\bar{\text{C1s}}$. SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; $\text{iPr}_2\text{P-F}$, di-isopropyl phosphorofluoridate; $\text{iPr}_2\text{P-}$, di-isopropylphosphoryl-; *N*-Boc-Tyr-ONp, *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester.

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those features which are important for biological activity.

The present paper describes the purification of bovine subcomponent C1s in high yield by rapid and simple chromatographic procedures. Characterization has also been carried out, and comparison of subcomponent C1s with human subcomponent C1s suggests that the bovine and human subcomponents are very similar. Together with the bovine C1q (Campbell *et al.*, 1979) and C1r subcomponents, which are also analogous to their human counterparts, bovine C1s provides adequate material for a detailed study of the C1 complex, and of its interaction with bovine C4 [Booth *et al.* (1979a) and the preceding paper (Booth *et al.*, 1979b)].

Materials and Methods

Materials

The sources of reagents were as follows: sheep erythrocytes in Alsever's solution, Tissue Culture Services, Slough, Bucks., U.K.; guinea-pig serum, Wellcome Reagents, Beckenham, Kent, U.K.; benzamidine hydrochloride hydrate, Aldrich Chemical Co., Gillingham, Dorset, U.K.; DEAE-Sephadex A-50, DEAE-Sephadex CL-6B, Sephadex G-200, Pharmacia (G.B.) Ltd., London W.5, U.K.; Ultrogel AcA 34, LKB Instruments Ltd., South Croydon, U.K.; agarose, SDS, polyacrylamide, *NN'*-methylenebisacrylamide, BDH, Poole, Dorset, U.K.; *N*-Boc-Tyr-ONp, Coomassie Brilliant Blue R (C.I. No. 42660), Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.; [1,3-³H]diisopropyl phosphorofluoridate (3.4 Ci/mmol), The Radiochemical Centre, Amersham, U.K.; Soluene 350, Packard Instrument Co., Downers Grove, IL, U.S.A.

Functionally pure human C1q was prepared as described by Reid (1974), purified human C1r was prepared by the method of Ziccardi & Cooper (1976), and purified bovine C4 was prepared by the method of Booth *et al.* (1979a).

Methods

Isolation of subcomponent C1s. The methods and conditions used for the collection of blood and for the preparation of the euglobulin were as previously described (Campbell *et al.*, 1979).

The euglobulin fraction from 3 litres of serum in 300 ml of 100 mM-NaCl/10 mM-EDTA/10 mM-benzamidine/2.5 mM-iodoacetamide/0.2 mM-phenanthroline/25 mM-potassium phosphate buffer, pH 7.4, conductivity 16 mmho/cm, was applied to a column (5 cm × 30 cm) of DEAE-Sephadex A-50, equilibrated with the same buffer, at a flow rate of 75 ml/h. The column was washed with starting buffer until the first protein peak had eluted. This peak contained all the subcomponent C1q haemolytic activity and was

used for the subsequent purification of this subcomponent (Campbell *et al.*, 1979). The protein bound to the column was eluted with a linear gradient composed of 2.5 litres of 100 mM-NaCl/10 mM-EDTA/10 mM-benzamidine/2.5 mM-iodoacetamide/0.2 mM-phenanthroline/25 mM-potassium phosphate buffer, pH 7.4, and 2.5 litres of 300 mM-NaCl/10 mM-EDTA/10 mM-benzamidine/2.5 mM-iodoacetamide/0.2 mM-phenanthroline/25 mM-potassium phosphate buffer, pH 7.4. The fractions containing the subcomponent C1s haemolytic activity were pooled, concentrated by ultrafiltration to 40 ml and dialysed against 2 × 1 litre changes of 200 mM-NaCl/10 mM-EDTA/10 mM-benzamidine/50 mM-sodium acetate buffer, pH 5.5, conductivity 22 mmho/cm, for 18 h at 4°C. The pool was then made 10 mM in CaCl₂ by the careful addition of 0.4 ml of a 1 M stock solution. After dialysis against 3 × 2 litre changes of 200 mM-NaCl/10 mM-CaCl₂/10 mM-benzamidine/50 mM-sodium acetate buffer, pH 5.5, conductivity 22 mmho/cm, for 18 h at 4°C, the protein was applied to a column (5 cm × 139 cm) of Ultrogel AcA 34 equilibrated in the same buffer, and eluted by upward flow at a rate of 40 ml/h. The fractions containing subcomponent C1s haemolytic activity were pooled and made 10 mM with respect to EDTA by the careful addition of a portion of a 1 M stock solution. This pool was concentrated to 10 ml by ultrafiltration and dialysed against 3 × 1 litre changes of 200 mM-NaCl/10 mM-EDTA/10 mM-benzamidine/50 mM-sodium acetate buffer, pH 5.5, for 18 h at 4°C. The protein was then loaded on to a column (2.5 cm × 137 cm) of Ultrogel AcA 34 equilibrated with the same buffer and eluted by upward flow at a rate of 15 ml/h. The fractions containing subcomponent C1s haemolytic activity were pooled, concentrated and stored at -20°C in buffers containing 10 mM-benzamidine.

Activation of bovine C1s by human C1r. Bovine C1s, in 100 mM-NaCl/10 mM-EDTA/25 mM-potassium phosphate buffer, pH 7.4, was activated by incubation with human C1r as described by Booth *et al.* (1979a).

Haemolytic and enzymic activities of subcomponents C1s and C1s. Veronal-buffered saline, I 0.065, pH 7.3, containing 1 mM-MgCl₂ and 0.15 mM-CaCl₂, and veronal-buffered saline, I 0.065, pH 7.3, containing 10 mM-EDTA, were prepared by the method of Rapp & Borsos (1970). EAC4 cells were prepared from sheep erythrocytes, rabbit antiserum and guinea-pig serum as described by Mayer (1961). Functionally pure guinea-pig component C2 was prepared as described by Nelson *et al.* (1966). The late components of complement (C3-C9) were supplied in the form of whole guinea-pig serum diluted 1:25 with veronal-buffered saline/EDTA.

To assay for subcomponent C1s haemolytic activity, 0.1 ml serial dilutions of the solution to be tested were made in veronal-buffered saline; 0.1 ml of

a dilution of human subcomponent C1q and 0.1 ml of a dilution of human subcomponent C1r were added along with 0.1 ml of EAC4 cells (1.5×10^8 cells/ml). The dilutions of human subcomponents C1q and C1r added were such that these subcomponents were present in excess over subcomponent C1s. After incubation at 37°C for 15 min, 0.1 ml of component C2 in veronal-buffered saline was added and the cells incubated at 37°C for 10 min. Then 0.5 ml of whole guinea-pig serum diluted 1:25 with veronal-buffered saline/EDTA, was added and the mixture was incubated at 37°C for 30 min. The cells were then centrifuged at 1500g for 2 min and the $A_{412}^{1\text{cm}}$ values of the supernatants read. Results were expressed in CH_{50} units (the reciprocal of the dilution of test material causing 50% lysis of the EAC4 cells).

The enzymic activities of subcomponents C1s and C1s were determined by measuring the rates of hydrolysis of *N*-Boc-Tyr-ONp at 25°C and pH 8.1 by a modification of the method of Bing (1969), as described by Sim *et al.* (1977). A unit of esterase activity was defined as the amount of enzyme required to hydrolyse 1 nmol of substrate/min at 25°C. A molar absorption coefficient of $16600 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for *p*-nitrophenol was used (Chase & Shaw, 1967).

Treatment of bovine C4 with subcomponents C1s and C1s. Purified bovine C4 was incubated with subcomponents C1s or C1s as described by Booth *et al.* (1979a). The extent of the reaction was determined by the appearance of cleavage products of bovine C4 on SDS/polyacrylamide-gel electrophoresis and by the loss of C4 haemolytic activity (Booth *et al.*, 1979a,b).

Protein determination. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Quantitative amino acid and carbohydrate analysis. These were carried out as described by Campbell *et al.* (1979).

Polyacrylamide-gel-electrophoresis. Electrophoresis in 5.8% polyacrylamide gels at pH 8.3 was performed by the method of Davis (1964).

SDS/polyacrylamide-gel electrophoresis was performed by the method of Weber *et al.* (1972) as previously described (Campbell *et al.*, 1979). For molecular-weight calibration, standard proteins of known molecular weight were treated in an identical manner to the sample.

Molecular-weight determinations. The molecular weights of subcomponents C1s and C1s were determined by gel filtration on columns of Sephadex G-200 or Ultrogel AcA 34 as described by Andrews (1965). Molecular weights were estimated from a standard plot with the following proteins: glutamate dehydrogenase (mol.wt. 315000), pyruvate kinase (245000), lactate dehydrogenase (140000), bovine serum albumin (68000) and ovalbumin (44000). The molecular weights of subcomponents C1s and C1s,

and the heavy and light chain of subcomponent C1s, were also determined by SDS/polyacrylamide-gel electrophoresis as described above.

Preparation of antisera. Antisera to a crude preparation of subcomponent C1s and to purified C1s were raised in rabbits by the immunization regime described by Campbell *et al.* (1979).

Immunological methods. Immunodiffusion analysis was carried out by the method of Ouchterlony (1958), using 1% (w/v) agarose in 2.4 mM-sodium acetate/2.4 mM-sodium barbitone buffer, pH 8.6.

Immuno-electrophoresis was performed by a modification of the method of Scheidegger (1955), using 1% (w/v) agarose in 0.6 mM-sodium acetate/0.6 mM-sodium barbitone buffer, pH 8.6. Electrophoresis was performed for about 1 h on a water-cooled plate with a potential gradient of 6 V/cm.

'Rocket' immuno-electrophoresis was carried out by a modification of the method of Laurell (1966) as described by Weeke (1973). Agarose [1% (w/v)] in barbital buffer, pH 8.6, 1.025, containing 2 mM-EDTA and 0.6% (v/v) antiserum to subcomponent C1s was used. Electrophoresis was performed at 4–5 V/cm for 16 h, and the plates were standardized by using dilutions of a purified preparation of C1s, the concentration of which was determined by amino acid analysis.

Treatment of subcomponents C1s and C1s with [^3H]iPr₂P-F. Subcomponents C1s or C1s (0.4–2 mg/ml) in 100 mM-NaCl/10 mM-EDTA/25 mM-potassium phosphate buffer, pH 7.4, were incubated with a 2–5-fold molar excess of [^3H]iPr₂P-F (specific activity 3.4 Ci/nmol) for 2 h at 37°C. Excess [^3H]iPr₂P-F was removed by exhaustive dialysis at 4°C against large volumes of the NaCl/EDTA/phosphate buffer, pH 7.4. Aqueous samples (10–50 μl) to be counted were made up to 1 ml with distilled water, 10 ml of scintillation fluid consisting of 0.4% (w/v) PPO (2,5-diphenyloxazole) plus 0.005% POPOP [1,4-bis(5-phenyloxazol-2-yl)benzene] in toluene/Triton X-100 (2:1) was added, the contents of the vials mixed thoroughly, and the sample counted for radioactivity on an Intertechnique SL-40 liquid-scintillation spectrometer. Gels to be counted for radioactivity were frozen at –20°C for 2 h and sliced into 1 mm sections. The slices were transferred to scintillation vials, and incubated with 250 μl of Soluene 350 at room temperature in the dark for 2 h. Toluene scintillant (10 ml) was then added, the contents of the vials mixed thoroughly, and counted for radioactivity.

Separation of the chains of subcomponent C1s. The reduced and carboxymethylated chains of [^3H]iPr₂P-labelled subcomponent C1s were separated by ion-exchange chromatography on DEAE-Sepharose CL-6B in the presence of 8 M-urea.

A portion (25 mg) of freeze-dried [^3H]iPr₂P-labelled subcomponent C1s was dissolved in 5 ml of

8M-urea/0.1M-Tris buffer, pH 8.6, and 0.5 ml of a 50 mg/ml solution of EDTA added. Reduction and carboxymethylation were carried out as described by Crestfield *et al.* (1963). The sample was then dialysed against four changes of 300 ml of 5 mM-NaCl/8 M-urea/20 mM-sodium phosphate buffer, pH 7.5, conductivity 4.4 mmho/cm, at room temperature (18–22°C) for 20 h. A column (1.6 cm × 4.5 cm) of DEAE-Sephadex CL-6B equilibrated in 5 mM-NaCl/8 M-urea/20 mM-sodium phosphate buffer, pH 7.5, was packed. The reduced and carboxymethylated [³H]-iPr₂P-F-labelled subcomponent C1s, equilibrated in the same buffer, was loaded on to the column at a flow rate of 16.5 ml/h. The column was washed with 50 ml of the NaCl/urea/phosphate buffer, pH 7.5, before a linear gradient composed of 100 ml of the NaCl/urea/phosphate buffer, pH 7.5, and 100 ml of the NaCl/urea/phosphate buffer, pH 7.5, containing 0.5 M-NaCl was applied. Samples of the column fractions were taken for liquid-scintillation counting and SDS/polyacrylamide-gel electrophoresis. The tubes containing the separated heavy and light chains were pooled, and the samples were dialysed against 3 × 2 litre changes of 1% (w/v) NH₄HCO₃ for 24 h at 4°C. Both pools were then freeze-dried.

N-Terminal determination. Qualitative *N*-terminal analyses of subcomponents C1s and C1s and the separated chains of subcomponent C1s were carried out by the dansyl method described by Weiner *et al.* (1972). The dansyl amino acids were identified by t.l.c. on polyamide sheets (Gray, 1972).

Results

Purification of subcomponent C1s

Table 1 summarizes the purification procedure and gives details of the yields of protein and haemolytic activity.

The first stage was a euglobulin-precipitation step. Euglobulin precipitates can be prepared by dialysis or dilution of serum with low-ionic-strength buffers of varying pH. The method of Tamura & Nelson (1968), with sodium phosphate buffer, pH 7.5, 1.0 M, was

attempted, but this was found to result in substantial activation of subcomponent C1s to C1s. In the method of isolation of human subcomponent C1s reported by Valet & Cooper (1974), a critical factor of the purification schedule is the formation of the euglobulin by dialysis at acid pH and at 0°C. Dialysis of bovine serum against 8.6 mM-NaCl/13.3 mM-sodium phosphate buffer, pH 6.0, strictly at 0°C, resulted in very little activation of subcomponent C1s during formation of the euglobulin. The recovery of C1s in the euglobulin, measured by 'rocket' immunoelectrophoresis, was about 70%.

Slow activation of the zymogen to subcomponent C1s, however, was found during the subsequent steps in the purification schedule. This problem of proteolysis, also encountered during the purification of bovine component C4, was solved by the inclusion of the proteolytic inhibitor benzamidinium hydrochloride (Mares-Guia & Shaw, 1965) in all column buffers.

Chromatography of the euglobulin on DEAE-Sephadex A-50 at pH 7.4 resulted in 48% of the applied protein being eluted with the starting buffer (Fig. 1). The breakthrough peak contained subcomponent C1q haemolytic activity and was used for the further purification of this subcomponent (Campbell *et al.*, 1979). Subcomponent C1s haemolytic activity was retained and later eluted by a NaCl gradient in the conductivity range 20–22 mmho/cm. Subcomponent C1r was also found to elute from the column in this conductivity range.

Further purification of subcomponent C1s was achieved by molecular-sieve chromatography on an Ultrogel AcA 34 column equilibrated in a buffer containing 10 mM-CaCl₂ (Fig. 2). This step was necessary to separate subcomponent C1s from a major contaminating protein that accounted for about 60–70% of the total protein from the DEAE-Sephadex column. Very little separation of subcomponent C1s from this contaminant was achieved by molecular-sieve or ion-exchange chromatography in buffers containing 10 mM-EDTA. However, under the conditions specified in the Materials and Methods section, subcomponent C1s eluted from the Ultrogel

Table 1. Purification of subcomponent C1s from 3 litres of serum

Purification step	Total protein (mg)	10 ⁻⁷ × Total CH ₅₀ units	Specific activity (CH ₅₀ units/mg)	Purification (fold)	Yield (% CH ₅₀)	C1s (mg)*	Yield (% C1s)*
Serum	208 200	ND†	ND	ND	ND	90	100
Euglobulin	9 240	5.53	5 985	1	100	61	68
DEAE-Sephadex A-50	306	4.72	154 250	26	85	41	46
Ultrogel AcA 34 (Ca ²⁺)	31	1.45	468 000	78	26	14.5	16
Ultrogel AcA 34	10.9	1.08	991 000	160	19.5	10	11

* Determined by 'rocket' immunoelectrophoresis.

† 'ND' indicates that the assay of serum for subcomponent C1s haemolytic activity was not reproducible and recovery of activity was therefore calculated with respect to the euglobulin.

Fig. 1. Chromatography of euglobulin on DEAE-Sephadex A-50

The euglobulin (300ml) from 3 litres of serum was loaded on to a column (5cm×30cm) of DEAE-Sephadex A-50 equilibrated in 100mM-NaCl/10mM-EDTA/10mM-benzamidine/2.5mM-iodoacetamide/0.2mM-phenanthroline/25mM-potassium phosphate buffer, pH 7.4. After the first protein peak had eluted, a linear gradient of NaCl (0.1–0.3M) as described in the Materials and Methods section was applied. —, A_{280} ; ----, conductivity (mmho/cm).

Fig. 2. Chromatography of the C1r- and C1s-containing pool from DEAE-Sephadex on Ultrogel AcA 34 in a Ca^{2+} -containing buffer

The C1r and C1s pool (450ml) from the DEAE-Sephadex column was concentrated to 40ml by ultrafiltration and dialysed against 200mM-NaCl/10mM-EDTA/10mM-benzamidine/50mM-sodium acetate buffer, pH 5.5. The sample was then made 10mM in CaCl_2 , dialysed against 200mM-NaCl/10mM- CaCl_2 /10mM-benzamidine/50mM-sodium acetate buffer, pH 5.5, and applied to a column (5cm×139cm) of Ultrogel AcA 34 equilibrated in the same buffer. The column was run at a flow rate of 40ml/h. —, A_{280} .

AcA 34 column in the molecular-weight range 150000–170000, whereas the contaminant eluted in the molecular-weight range 75000–100000 (Fig. 2). This contaminant has recently been identified as a degradation product of bovine component C4 (N. A. Booth, R. D. Campbell & J. E. Fothergill, un-

published work). The low yield of C1s from the column (approx. 30%) can be explained by the fact that some of the subcomponent C1s had formed a complex with subcomponent C1r, the complex eluting from the column with a mol.wt. of about 320000 (Fig. 2). No purification of C1s from the complex has yet been obtained. However, the complex itself has been characterized and has been found to contain 2 molecules of subcomponent C1r (monomer) and 2 molecules of subcomponent C1s (R. D. Campbell, B. M. Anderson & J. E. Fothergill, unpublished work).

Final purification of subcomponent C1s was achieved on a column of Ultrogel AcA 34 equilibrated in a buffer containing 10mM-EDTA (Fig. 3). Approx. 35% of the protein applied to the column was eluted in the mol.wt. range 80000–95000. The recovery of subcomponent C1s haemolytic activity was about 75% (Table 1).

This purification schedule results in the isolation of about 10mg of the proenzyme form of subcomponent C1s from 3 litres of serum (Table 1). Assuming a serum concentration, as measured by 'rocket' immunoelectrophoresis, of 30 $\mu\text{g}/\text{ml}$, this represents a yield of about 11% (Table 1). The material was shown to be homogeneous by analytical ultracentrifugation, and was found to contain one protein-staining band on polyacrylamide-gel electrophoresis in the absence (Fig. 4a) and presence of SDS (Fig. 4b).

Immunochemical characterization

Purified subcomponent C1s gave a single precipitin line in Ouchterlony double-diffusion in agarose gels

Fig. 3. Chromatography of subcomponent C1s on Ultrogel AcA 34 in an EDTA-containing buffer

The subcomponent C1s pool (150ml) from the Ultrogel AcA 34 column run in a Ca^{2+} -containing buffer was made 10mM in EDTA, concentrated to 10ml by ultrafiltration and dialysed against 200mM-NaCl/10mM-EDTA/10mM-benzamidine/50mM-sodium acetate buffer, pH 5.5. The sample was then applied to a column (2.5cm×137cm) of Ultrogel AcA 34 equilibrated in the same buffer, and eluted by upward flow at a rate of 15ml/h. —, A_{280} .

Fig. 4. Polyacrylamide-gel electrophoresis of subcomponents C1s and C1r

(a) Electrophoresis of purified subcomponent C1s on a 5.8% polyacrylamide gel by the method of Davis (1964). (b) Electrophoresis of subcomponents C1s and C1r on 7.5% polyacrylamide gels in the presence of SDS. 1 and 2, Subcomponent C1s run under non-reducing and reducing conditions respectively and stained for protein; 3 and 4, subcomponent C1r run under non-reducing conditions and stained for protein and carbohydrate respectively; 5 and 6, subcomponent C1r run under reducing conditions and stained for protein and carbohydrate respectively.

against a rabbit antiserum raised against a crude subcomponent C1s preparation. No precipitin lines were detected when subcomponent C1s was tested in Ouchterlony double-diffusion against rabbit antisera to bovine immunoglobulin G, subcomponent C1q and components C4 and C3. A single precipitin arc in the α -globulin region was obtained when purified subcomponent C1s was subjected to immunoelectrophoresis and tested against the anti-(crude C1s). No antigenic cross-reactivity was observed between human and bovine subcomponent C1s species.

Enzymic activities of subcomponent C1s

C1s was unable to cause the lysis of sheep erythrocytes in the haemolytic assay unless incubated in the presence of human subcomponents C1q and C1r. Cell-bound activity was also generated in the presence of these subcomponents. Hydrolysis of the ester substrate *N*-Boc-Tyr-ONp and cleavage of component C4 by subcomponent C1s were found to be negligible.

Preincubation of subcomponent C1s with 0.5 mM- i Pr₂P-F or 1 mM-phenylmethanesulphonyl fluoride and removal of the inhibitors by extensive dialysis resulted in no loss of the haemolytic activity of subcomponent C1s when assayed in the presence of subcomponent C1q and C1r, or the esterase activity of subcomponent C1s when subsequently activated by subcomponent C1r.

Activation of bovine subcomponent C1s by human subcomponent C1r

Incubation of bovine subcomponent C1s in 100 mM-NaCl/10 mM-EDTA/25 mM-phosphate buffer, pH 7.4,

Fig. 5. SDS/7.5% polyacrylamide-gel electrophoresis of subcomponent C1r labelled with $[^3\text{H}]i\text{Pr}_2\text{P-F}$

One gel was stained for protein, while an identical gel was sliced into 1 mm segments and taken for liquid-scintillation counting of radioactivity. Full experimental details are given in the text. (a) Sample run under non-reducing conditions; (b) sample run under reducing conditions.

with 2.5% (w/v) human subcomponent C1r for 1 h at 37°C, followed by the addition of a further 2.5% (w/v) human subcomponent C1r and incubation for another hour, resulted in activation to C1s. No subcomponent C1s could be detected if the human

subcomponent C1 $\bar{r}$ was omitted, suggesting that the conversion of subcomponent C1s to C1 $\bar{s}$ was not due to self-activation or to the activity of proteinase contaminants.

No conversion of subcomponent C1s into C1 $\bar{s}$ was observed when C1s was incubated with C1 $\bar{s}$, even with C1s/C1 $\bar{s}$ ratios as high as 1:1, thus showing that C1 $\bar{s}$ could not generate more C1 $\bar{s}$ from C1s. This precludes the autocatalytic conversion of C1s, and suggests that all of the C1 $\bar{s}$ formed when C1s was incubated with human subcomponent C1 $\bar{r}$ was due to the cleavage of the molecule by human subcomponent C1 $\bar{r}$.

The subcomponent C1 $\bar{s}$ thus formed was active in the haemolytic assay in the absence of subcomponents C1q and C1r, was able to cleave and inactivate component C4 (Booth *et al.*, 1979a), to generate the activation product C4a (Booth *et al.*, 1979b), and was found to be active against *N*-Boc-Tyr-ONp, having a specific activity of 20.8 mol/min per mol of protein in the assay used.

Molecular weights and chain structure of subcomponents C1s and C1 $\bar{s}$

The molecular weight of subcomponents C1s and C1 $\bar{s}$ were determined by two methods. In the first, columns of Ultrogel AcA 34 and Sephadex G-200 were equilibrated in buffers containing 10 mM-EDTA and calibrated with proteins of known molecular weight. In both cases subcomponents C1s and C1 $\bar{s}$ eluted with apparent mol.wts. of 88000. If the columns were equilibrated with a buffer containing 10 mM-CaCl₂, subcomponents C1s and C1 $\bar{s}$ eluted in the molecular-weight range 150000–170000, suggesting dimer formation.

Subcomponents C1s and C1 $\bar{s}$ had apparent mol.wts. of 85000 on SDS/7.5% (w/v) polyacrylamide-gel electrophoresis run under non-reducing conditions. On reduction, subcomponent C1s was shown to consist of a single chain of mol.wt. 87000 (Fig. 4b). However, subcomponent C1 $\bar{s}$ under reducing conditions yielded two chains in equimolar ratio with apparent mol.wts. of 65000 and 27000 (Fig. 4b).

Effect of iPr₂P-F and phenylmethanesulphonyl fluoride on subcomponents C1s and C1 $\bar{s}$

iPr₂P-F and phenylmethanesulphonyl fluoride had no effect on the haemolytic, enzymic and C4-cleaving activities of subcomponent C1s if incubated and subsequently removed by dialysis as mentioned previously. However, both compounds inhibited all three activities of subcomponent C1 $\bar{s}$. Incubation of subcomponent C1 $\bar{s}$ with [³H]iPr₂P-F led to the incorporation of 1 mol of [³H]iPr₂P-F mol of subcomponent C1 $\bar{s}$. On SDS/polyacrylamide gels the radioactivity migrated either with the unreduced subcomponent C1 $\bar{s}$ or with the light chain under reducing conditions (Fig. 5). C1s treated under

Fig. 6. Separation of the chains of subcomponent C1 $\bar{s}$. Reduced and carboxymethylated [³H]iPr₂P-labelled subcomponent C1s [15 ml (25 mg)] in 5 mM-NaCl/8 M-urea/20 mM-phosphate buffer, pH 7.5, was loaded on to a column (1.6 cm × 4.5 cm) of DEAE-Sephacrose CL-6B equilibrated in the same buffer. After the column had been washed with 50 ml of starting buffer, a 200 ml linear gradient of NaCl (0.005–0.5 M) in the urea/phosphate buffer was begun. The flow rate of the column was 16.5 ml/h and fractions of about 5 ml were collected. —, A₂₈₀; Δ, radioactivity (c.p.m.). The gels beside each of the protein peaks are of samples from each pool run on SDS/7.5% polyacrylamide-gel electrophoresis.

Table 2. Amino acid composition of bovine and human subcomponents C1s and C1 $\bar{s}$

Samples of subcomponents C1s and C1 $\bar{s}$ were hydrolysed for 24, 48 and 72 h in duplicate. Destruction of the labile amino acids was corrected for by extrapolation to zero hydrolysis time. Valine, leucine and isoleucine values are those determined for the 72 h hydrolysis. Other details are given in the text.

Amino acid	Amino acid composition (residues/100 residues)			
	Bovine		Human*	
	C1s	C1 $\bar{s}$	C1s	C1 $\bar{s}$
Asp	10.25	9.97	11.0	11.1
Thr	4.99	4.97	4.8	5.2
Ser	8.07	7.98	6.6	6.4
Glu	10.82	10.96	11.3	11.1
Pro	7.03	7.16	7.2	7.2
Gly	9.85	9.85	10.9	10.3
Ala	5.20	5.48	6.1	6.0
Cys	2.55	2.56	2.6	2.5
Val	7.70	7.49	7.0	7.3
Met	1.44	1.51	1.7	1.8
Ile	4.30	4.55	3.7	4.0
Leu	5.83	5.61	6.5	6.5
Tyr	4.51	4.37	4.0	4.2
Phe	4.83	4.87	4.7	4.8
His	2.25	2.13	3.0	2.8
Lys	4.81	4.82	4.8	4.7
Arg	4.07	4.26	4.1	4.1
Trp	1.66	1.58	†	†

* From Sim & Porter (1976).

† Not determined.

similar conditions incorporated less than 1% of the [^3H]Pr₂P-F incorporated by C $\bar{\text{I}}$ s.

Separation of the chains of subcomponent C $\bar{\text{I}}$ s

The heavy and light chains of subcomponent C $\bar{\text{I}}$ s were separated by ion-exchange chromatography on DEAE-Sepharose CL-6B in the presence of 8M-urea. After the first protein peak had been eluted, a linear NaCl gradient (0–0.5M) was begun. The elution profile is shown in Fig. 6.

Samples of the column fractions taken for liquid-scintillation counting revealed that all of the applied radioactivity was located in the breakthrough peak (Fig. 6). SDS/7.5% (w/v) polyacrylamide-gel electrophoresis of the column fractions (Fig. 6) showed that the breakthrough peak was composed exclusively of the light chain, whereas the heavy chain bound to the column and was eluted in the NaCl gradient. The yield of radioactivity was 83% of that applied to the

Table 3. *Amino acid composition of the separated heavy and light chains of subcomponent C $\bar{\text{I}}$ s*

Samples of the separated heavy and light chains of subcomponent C $\bar{\text{I}}$ s were hydrolysed for 24, 48 and 72 h in duplicate. Destruction of the labile amino acids was corrected for by extrapolation to zero hydrolysis time, whereas the valine, leucine and isoleucine values were those determined for the 72 h hydrolysis time. Other experimental details are given in the text. Abbreviation used: CmCys, S-carboxymethyl-cysteine.

Amino acid	Amino acid composition (residues/molecule)			C $\bar{\text{I}}$ s
	Heavy chain (H)	Light chain (L)	Sum (H+L)	
CmCys	17	5	22	20
Asp	55	27	82	79
Thr	29	12	41	40
Ser	50	18	68	64
Glu	73	22	95	87
Pro	44	16	60	57
Gly	58	25	83	79
Ala	30	16	46	44
Val	42	25	67	60
Met	11	4	15	12
Ile	24	13	37	36
Leu	32	17	49	45
Tyr	27	9	36	35
Phe	29	11	40	39
His	14	4	18	17
Lys	25	13	38	38
Arg	24	11	35	34
Trp	*	*	*	13
Total	584	248	832	799

* Not determined.

Table 4. *Carbohydrate composition of subcomponent C $\bar{\text{I}}$ s*
Full experimental details are given in the text

Sugar	Content (g/100 g of protein)
Hexose	7.09
Glucosamine	1.85
Galactosamine	0.14
Sialic acid	1.31
Total	10.39

column, suggesting good recovery of the light chain. The recovery of the heavy chain was assessed from the A₂₈₀ and was in the range 60–80%.

Amino acid and carbohydrate analyses

The amino acid analyses of subcomponents C I s and C $\bar{\text{I}}$ s are shown in Table 2; there is very little difference between the two analyses. The analyses for bovine subcomponents C I s and C $\bar{\text{I}}$ s are compared with those of Sim & Porter (1976) for human subcomponents C I s and C $\bar{\text{I}}$ s. Again no significant differences are apparent.

The amino acid analyses of the separated heavy and light chains of C $\bar{\text{I}}$ s are shown in Table 3; the summation of the chains yields a value very close to that of subcomponent C $\bar{\text{I}}$ s. From this and the molecular weight data it appears that there are no large peptides lost during activation of subcomponent C I s to C $\bar{\text{I}}$ s.

The carbohydrate composition of subcomponent C I s is given in Table 4. About 68% of the carbohydrate is in the form of neutral hexose, with approx. 19% hexosamine and 13% sialic acid; 92% of the hexosamine in C I s is glucosamine. When SDS/7.5% polyacrylamide gels of subcomponent C $\bar{\text{I}}$ s run under reducing and non-reducing conditions were stained for carbohydrate by using the periodic acid/Schiff staining procedure of Kapitany & Zebrowski (1973), all of the stain taken up by the intact molecule was found to be associated with the heavy chain after reduction (Fig. 4b).

N-Terminal analyses

The N-terminal amino acid of subcomponent C I s, as determined by the dansyl technique, was found to be lysine. This was also shown to be the N-terminal amino acid of the heavy chain of subcomponent C $\bar{\text{I}}$ s, suggesting that this chain is derived from the N-terminus of the zymogen. The N-terminal amino acid of the light chain of subcomponent C $\bar{\text{I}}$ s was identified as isoleucine. The N-terminal analysis of subcomponent C $\bar{\text{I}}$ s gave a mixture of lysine and isoleucine.

Discussion

Relatively good yields of C I s have been obtained

from bovine plasma by using simple procedures such as euglobulin precipitation and ion-exchange and molecular-sieve chromatography. As with many other complement components, the purification of native zymogen C1s requires the presence of a proteolytic inhibitor from early stages in the purification schedule. Benzamidine has proved satisfactory for this purpose and has the advantages of low cost, easy removal and relatively low toxicity. Our finding, that low temperature and low pH during the euglobulin precipitation stage also appear to contribute to the stability of the zymogens, has been previously reported for the human system (Valet & Cooper, 1974; Ziccardi & Cooper, 1976).

The authenticity of the finally purified C1s was established by haemolytic assay. C1s always required the addition of both C1q and C1r before cell-bound C1 activity could be generated. Preliminary activation of the zymogen to C1s gave the expected activity associated with C1s in the absence of the other two subcomponents, but this could be removed by washing, indicating that the activity was not cell-bound. Since pure bovine C1r was not then available, assays were carried out with purified human C1r. This gave good activation of bovine C1s. The activity of equimolar amounts of human C1r and bovine C1s could not be distinguished from equal amounts of the bovine C1r-C1s complex isolated from the Ca^{2+} -containing Ultrogel column.

In view of the restricted specificity reported for human C1r (Sim *et al.*, 1977), it is noteworthy that it activates bovine C1s quite successfully. C1s produced in this way has an isoleucine residue at the *N*-terminus of the light chain, suggesting that splitting of the zymogen chain takes place in the expected position. This may be important, because of the essential role of the generation of a hydrophobic *N*-terminal residue during the activation of serine proteinases (Sigler *et al.*, 1968; Huber & Bode, 1977; Sawyer *et al.*, 1978).

The molecular-weight value of 88000 for C1s in EDTA-containing solutions is very similar to that obtained for human C1s (Gigli *et al.*, 1976). The presence of Ca^{2+} ions appears to cause dimerization, which has also been suggested for human C1s (Valet & Cooper, 1974; Arlaud *et al.*, 1977). The splitting of the chain on activation gives fragments of 65000 and 27000 mol.wt. that again are similar to the activation products of human C1s (Arlaud *et al.*, 1977; Sim *et al.*, 1977).

Separation of the heavy and light chains of reduced carboxymethylated C1s by ion-exchange chromatography in 8M-urea gives a convenient method for the preparation of relatively large amounts of the two chains. The light chain, which binds an equimolar amount of $\text{iPr}_2\text{P-F}$ is probably of the serine-proteinase type, which again would be similar to that of human C1s (Barkas *et al.*, 1973; Sim & Porter, 1976). Extended amino acid-sequence determination is

possible now that relatively large amounts of material are available.

The characterization of bovine C1s has demonstrated that this molecule is very similar to the corresponding human protein. Together with the similarities of the human and bovine C1q (Campbell *et al.*, 1979) C1r (R. D. Campbell, B. M. Anderson & J. E. Fothergill, unpublished work) and C4 (Booth *et al.*, 1979a), these findings suggest that there is little functional or structural difference between complement proteins in these two species. Bovine plasma can therefore provide the large quantities of material necessary for detailed structural studies of complement proteins that will be generally relevant.

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