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Primary structure of bovine complement activation fragment C4a, the third anaphylatoxin

Purification and complete amino acid sequence

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Purification of C4a from heat-activated bovine plasma by elution from CM-Sephadex C-50 at pH 7.4 and gel filtration on Sephadex G-50 gives a 20% yield of pure C4a. The complete amino acid sequence of bovine C4a has been determined by automatic sequencer degradation of CNBr and enzymic fragments, and by carboxypeptidase digestion. The 77-residue bovine sequence shows 12 differences from the human sequence with five of these differences occurring in the *C*-terminal 11 residues. The sequence of C4a confirms earlier suggestions of homology with C3a and C5a; the three sequences show an almost equal number of identities with each other. The six cysteine residues of the 'disulphide knot' are conserved as well as seven other residues including the *C*-terminal arginine.

Activation of the complement system generates active proteolytic enzymes that subsequently liberate a number of relatively small protein fragments from C3, C4 and C5 (reviewed by Fothergill & Anderson, 1978; Reid & Porter, 1981). Component C4 is cleaved by $C\overline{1s}$ to give C4a from the *N*-terminus of the α-chain of C4 (Booth *et al.*, 1979*b*), and C3 and C5 are cleaved by the classical or alternative pathway convertases to yield C3a and C5a from the α-chains of C3 and C5. C3a and C5a are known as anaphylatoxins because of their substantial biological activities such as releasing histamine from mast cells, causing contraction of smooth muscle, and increasing vascular permeability (reviewed by Hugli & Müller-Eberhard, 1978); they show substantial homology of amino acid sequence.

The first preliminary sequence evidence for C4a (Booth *et al.*, 1979*b*) suggested that bovine C4a might be homologous with C3a and C5a. Similar sequence evidence obtained for human C4a (Gorski *et al.*, 1981) has recently been extended to the whole molecule (Moon *et al.*, 1981). Comparisons can now be made between bovine and human C4a, and between C4a, C3a and C5a, showing all of these molecules to be homologous. Human C4a has been shown to possess some anaphylatoxin activity (Gorski *et al.*, 1979). Recent studies of the thiol ester regions of C4b (Campbell *et al.*, 1981; Harrison *et al.*, 1981) and C3b (Tack *et al.*, 1980) have emphasized the homology of C4 to C3 as well as to α_2-macroglobulin (Swenson & Howard, 1980).

Abbreviations used: the nomenclature of complement components and subcomponents is that recommended by the World Health Organisation (1968). Activated components are indicated by a bar, e.g. $C\overline{1s}$. SDS, sodium dodecyl sulphate; h.p.l.c., high-pressure liquid chromatography.

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Materials and methods

Materials

Fresh bovine blood (9 litres) was collected into 0.136 M-trisodium citrate anticoagulant (1 litre) at the slaughterhouse. Centrifugation at 1800 ***g*** for 50 min at 4°C gave clear plasma.

Chemicals were obtained as follows: CM-Sephadex C-50, Pharmacia; Quadrol/trifluoroacetic acid buffer in propanol/water (3:4, v/v), pH 9.0, protected by aminocellulose, Fluka; heptafluorobutyric acid, phenylisothiocyanate, sequencer grade solvents, Rathburn Chemicals, Walkerburn, Peebles-shire, Scotland, U.K. The sources of other chemicals have been described previously (Booth *et al.*, 1979*a*,*b*; Campbell *et al.*, 1979*a*,*b*; Nisbet *et al.*, 1981).

Cleavage of C4

Whole plasma was incubated at 37°C at pH 7.4

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for 2h. Conditions similar to these are known to cause activation of bovine C4 by $C\overline{1r}$ (Booth *et al.*, 1979*a*) and to cause activation of human (Ziccardi & Cooper, 1976) and bovine C1r (Campbell *et al.*, 1979*b*). Activated plasma was then frozen by storage at −20°C for 4h, and allowed to thaw overnight at 4°C. This freezing and thawing caused the aggregation of a sticky precipitate formed during activation, which could be removed conveniently by centrifugation after this process.

Purification of C4a

The purification schedule is outlined in Scheme 1. Thawed, activated plasma (2 litres) was centrifuged at 1800*g* for 50min at 4°C, and the supernatant applied to a column (5cm × 35cm) of CM-Sephadex C-50 equilibrated in 28mM-phosphate buffer, pH7.4, containing 2mM-EDTA and 50mM-NaCl. C4a was eluted with a linear gradient (2 × 1 litre) of 50–300mM-NaCl followed by a wash of 1 litre of 300mM-NaCl (Fig. 1). C4a, detected by electrophoresis in polyacrylamide-gel (10%) containing SDS was pooled and applied to a smaller column (5cm × 4cm) of CM-Sephadex C-50 equilibrated in the same starting buffer. C4a was recovered by stepwise elution with buffer containing 300mM-NaCl. C4a-containing fractions were dialysed against 1% (w/v) NH_4HCO_3 and freeze dried. A final stage of purification was done by molecular sieving on Sephadex G-50. Some C4a was reduced with a 60-fold molar excess of dithiothreitol in 0.4M-Tris/HCl, pH8.6, containing 6M-guanidine hydrochloride and 2mM-EDTA, and carboxymethylated with iodo[2-^{14}C]acetate (Crestfield *et al.*, 1963). Before sequencing this material was further purified by gel permeation h.p.l.c. on a tandem combination of Toyo Soda TSK SW 3000 and SW 2000 columns (0.45cm × 60cm) in 0.1% (v/v) trifluoroacetic acid.

CNBr cleavage of C4a and purification of fragments

Reduced carboxymethylated C4a was treated with a 100-fold molar excess of CNBr in 70% (v/v) formic acid at room temperature for 24h in the dark. After dilution and freeze-drying, the mixture of peptides was dissolved in 0.1% (v/v) trifluoroacetic acid and applied to the tandem arrangement of TSK SW 3000 and SW 2000 columns (0.45cm × 60cm) and eluted at 1ml/min by 0.1% trifluoroacetic acid. Peptides were detected by monitoring at 214nm.

Digestion of C4a by staphylococcal proteinase and purification of peptides

Non-reduced C4a was digested in 1% (w/v) NH_4HCO_3 by staphylococcal proteinase (EC 3.4.21.19) at 1:20 (w/w) enzyme:C4a ratio. The digest was freeze-dried, dissolved in 0.1% trifluoroacetic acid and applied to the TSK h.p.l.c. columns as above.

Amino acid analyses

These were carried out as described by Campbell *et al.* (1979*a*).

Automatic N-terminal sequence determination

A Beckman 890C sequencer equipped with the Beckman cold-trap accessory was operated with a double-cleavage, double-extraction programme developed by Dr. Jean Gagnon from the method of Hunkapiller & Hood (1978) using 0.25M-Quadrol. Polybrene (3mg) was added to the cup before each sample (Klapper *et al.*, 1978). The thiazolinones were extracted from the spinning cup with butyl chloride containing dithioerythritol (10mg/l) and tributylphosphine (100μl/l) (Frank, 1979). Samples were collected into tubes containing 0.2ml of freshly prepared 0.1M-HCl containing 0.1% (v/v) ethanethiol. Solvent was blown off by a stream of nitrogen at room temperature until only the aqueous acid layer remained. Samples were then heated at 80°C for 10min, frozen and dried in a vacuum desiccator at room temperature. The phenylthiohydantoin derivatives were identified by h.p.l.c. on Waters C_{18}-μBondapak columns (0.39cm × 30cm) using an acetate buffer pH3.4/methanol gradient system (Bridgen *et al.*, 1976) and a 254nm detector. Yields of serine and threonine were normally about 30%.

Blood
↓ 9 litre blood + 1 litre trisodium citrate (0.136M); centrifuge at 1800*g*, 4°C, 50min
Plasma
↓ Incubate, 37°C, 2h, pH7.4; freeze, −20°C, 4h; thaw, 4°C, overnight; centrifuge at 1800*g*, 4°C, 50min
Supernatant
↓ Dialyse into 28mM-phosphate (pH7.4)/2mM-EDTA/50mM-NaCl and apply to CM-Sephadex C-50 column (5cm × 35cm) in above buffer; C4a is eluted with a linear gradient (2 × 1 litre) of 50–300mM-NaCl followed by 1 litre of 300mM-NaCl
C-50 pool
↓ Dialyse as above and apply to CM-Sephadex C-50 column (5cm × 4cm) in 28mM-phosphate (pH7.4)/2mM-EDTA/50mM-NaCl; C4a is obtained by stepwise elution with buffer containing 300mM-NaCl
C-50 pool
↓ Dialyse against 0.5% NH_4HCO_3 and freeze-dry; chromatograph on Sephadex G-50 column (2.5cm × 90cm) in 0.5% NH_4HCO_3
G-50 pool
↓ Freeze-dry
Purified C4a

Scheme 1. *Schedule for the purification of C4a*

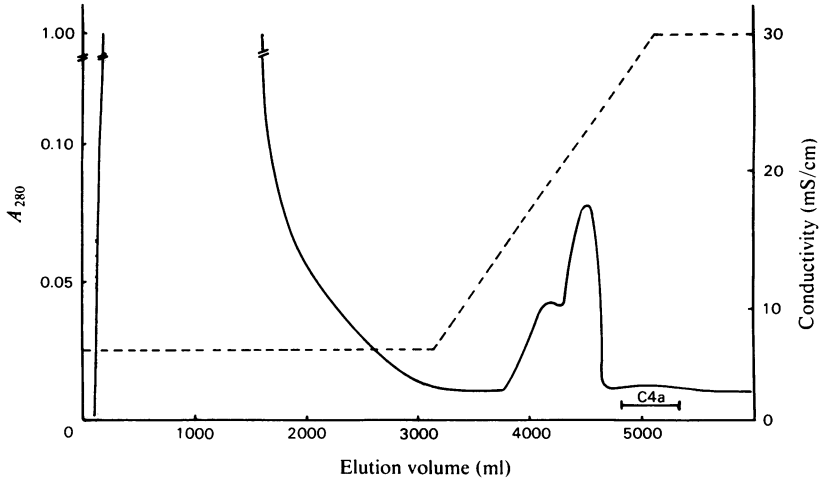

Fig. 1. *Chromatography of C4a on CM-Sephadex C-50*

Activated plasma from 9 litres of blood was applied to a column (5 cm × 35 cm) of CM-Sephadex C-50 in 28 mM-phosphate buffer (pH 7.4)/2 mM-EDTA/50 mM-NaCl. After the first protein peak had eluted a linear gradient (2 × 1 litre) of 50–300 mM-NaCl was applied, followed by buffer containing 300 mM-NaCl. C4a was located by SDS/polyacrylamide-gel electrophoresis and pooled as shown. ——, A_{280}; – – –, conductivity (mS/cm).

Yields of *S*-carboxymethylcysteine were almost theoretical, and were confirmed by radioactive counting. Amino acid analysis after back hydrolysis with HI was used to confirm any doubtful residues (Smithies *et al.*, 1971).

Sequence determination by carboxypeptidase Y

This was done by the method of Kuhn *et al.* (1974).

Results

Purification of C4a

The final yield of C4a was 9 mg from 2 litres of plasma, representing a yield of approx. 20%. Separations were carried out entirely at pH 7.4 suggesting that acidic conditions are not necessary to release C4a from C4b (Booth *et al.* 1979*b*; Budzko & Müller-Eberhard, 1970).

The product of this method was C4a without the *C*-terminal arginine residue. The addition of 6-aminohexanoic acid (1 M), a plasma carboxypeptidase B inhibitor (Vallota & Müller-Eberhard, 1973) at the activation and following steps was not sufficient to prepare material containing the terminal arginine. This was achieved by purifying whole C4 (Booth *et al.*, 1979*a*) followed by activation using $C\overline{1s}$ (Campbell *et al.*, 1979*b*) in the presence of 1 M-6-aminohexanoic acid.

Purification of CNBr fragments

Gel permeation h.p.l.c. in 0.1% trifluoroacetic acid separated undigested material and two fragments, CN-1 and CN-2 (Fig. 2).

Purification of staphylococcal proteinase peptides

Gel permeation h.p.l.c. of non-reduced C4a digested by staphylococcal proteinase showed three main peaks (Fig. 3).

Amino acid analysis

Analyses of reduced carboxymethylated C4a, CNBr fragments and staphylococcal proteinase peptides are shown in Table 1. The single methionine residue of C4a is consistent with the generation of the two CNBr fragments, CN-1 and CN-2. The analyses of the staphylococcal proteinase peptides SP-1, SP-2 and SP-3 suggest that SP-3 is the *N*-terminal decapeptide, SP-2 is the *C*-terminal hexadecapeptide and that SP-1 is mainly the 'disulphide knot' with some undigested material. The 'disulphide knot' probably contains several peptides held together by the disulphide bonds. This digest was intentionally carried out on non-reduced material in order to reduce the complexity of the mixture of peptides to be purified. The amino sugar content was found to be insignificant.

Amino acid sequence determination

The results of automatic sequencer runs on reduced carboxymethylated C4a, CNBr fragment 1 and staphylococcal proteinase peptide 2 are shown in Fig. 4 together with the results of carboxypeptidase Y digestion on the arginine-containing and

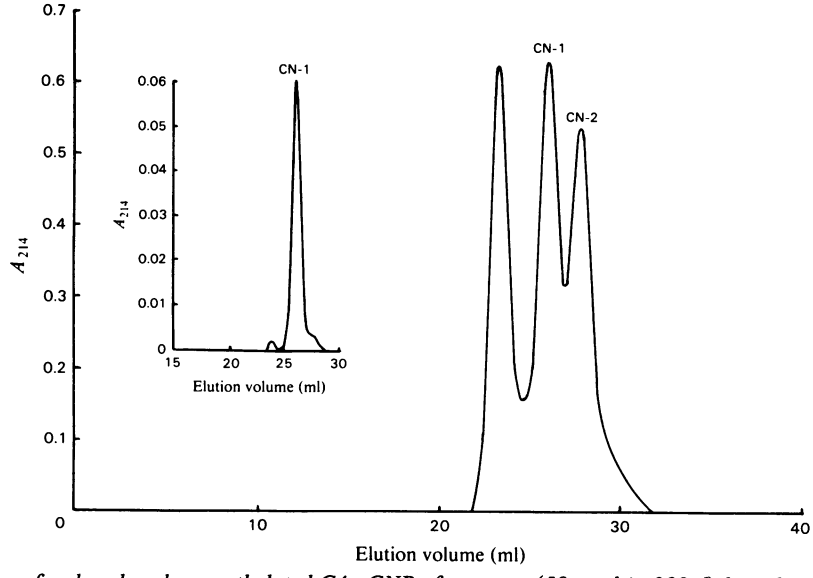

Fig. 2. *Separation of reduced carboxymethylated C4a CNBr fragments (50 nmol in 200 µl) by gel permeation h.p.l.c. in 0.1% (v/v) trifluoroacetic acid*

Columns (0.75 cm × 60 cm) of TSK SW-3000 and SW-2000 were used together in order of decreasing pore size. Flow rate was 1.0 ml/min provided by a Waters 6000A pump. Detection was at 214 nm using a Waters 441 monitor. Inset shows a repeat analytical scale run of peak CN-1 (2 nmol) after pooling several preparative scale runs.

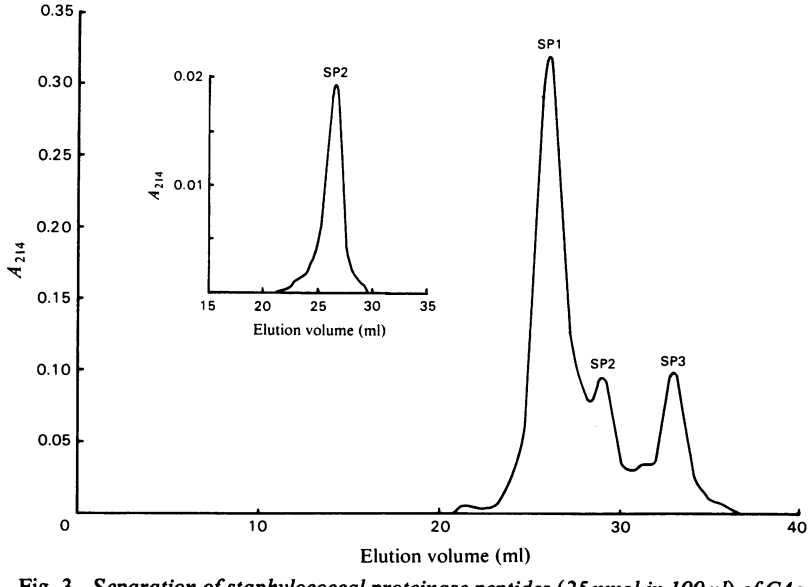

Fig. 3. *Separation of staphylococcal proteinase peptides (25 nmol in 100 µl) of C4a*

Columns and conditions as in Fig. 1. Inset shows a repeat analytical scale run of peak SP-2 (1 nmol) after pooling several preparative scale runs.

arginine-lacking forms of C4a. There is good agreement between the amino acid analyses and the sequences, and the overlaps are thoroughly established. The molecular weight of bovine C4a calculated from the sequence is 8692. There appears to be no glycosylation.

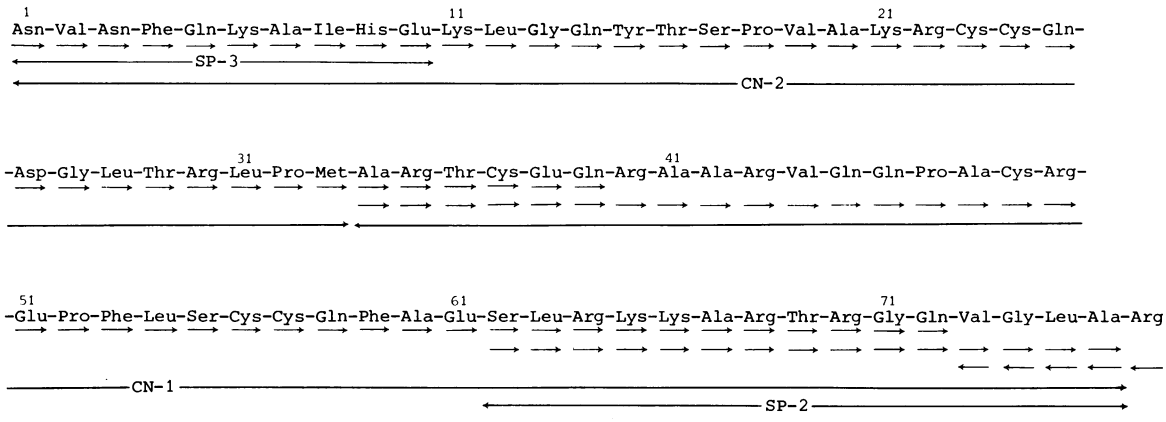

Fig. 4. *Amino acid sequence of bovine C4a*
The sequence is made up from the results of three sequencer runs (→) and of carboxypeptidase experiments (←) on the arginine-lacking and arginine-containing forms. The initial and repetitive yields for the sequencer runs were RCM-C4a, 53% (20nmol), 95.5% (Ala-7–Ala-20); RCM-C4a CNBr fragment 1, CN-1, 44% (26nmol), 94.5% (Ala-34–Ala-48); C4a staphylococcal proteinase peptide 2, SP-2, 44% (18nmol) 96% (Arg-64–Arg-70).

Table 1. *Amino acid composition of C4a and fragments*
Cysteine was determined as *S*-carboxymethylcysteine on reduced and carboxymethylated samples. The results for C4a are derived from samples hydrolysed for 24, 48 and 72h. Values in parentheses are the numbers of residues found in the corresponding sequence. *Indicates that homoserine was detected but not quantified.

Amino acid	Amino acid composition (residues/mol)				
	C4a	CN-1	CN-2	SP-2	SP-3
Cys	7.2 (6)	4.1 (4)	2.4 (2)	—	—
Asp	3.3 (3)	—	2.7 (3)	—	—
Thr	3.8 (4)	2.0 (2)	1.9 (2)	1.0 (1)	1.7 (2)
Ser	2.8 (3)	2.2 (2)	1.1 (1)	0.9 (1)	—
Glu	12.1 (12)	7.5 (8)	4.2 (4)	1.3 (1)	1.9 (2)
Pro	4.1 (4)	2.0 (2)	2.3 (2)	—	—
Gly	4.4 (4)	2.3 (2)	2.3 (2)	1.8 (2)	—
Ala	9.6 (9)	5.7 (7)	2.3 (2)	1.9 (2)	1.0 (1)
Val	3.7 (4)	2.3 (2)	2.0 (2)	1.0 (1)	1.0 (1)
Met	0.9 (1)	—	*	—	—
Ile	0.9 (1)	—	0.8 (1)	—	0.8 (1)
Leu	5.4 (6)	2.7 (3)	2.8 (3)	1.7 (2)	—
Tyr	1.0 (1)	—	1.0 (1)	—	—
Phe	2.9 (3)	2.1 (2)	1.3 (1)	—	0.8 (1)
His	0.9 (1)	—	0.9 (1)	—	0.8 (1)
Lys	4.8 (5)	2.1 (2)	2.9 (3)	1.5 (2)	0.9 (1)
Arg	8.1 (9)	5.0 (7)	1.8 (2)	2.4 (3)	—

Discussion

This purification method for C4a, making use of the unusually high isoelectric point and the unusually small molecular weight, gives micromolar quantities in a relatively short time. This provides adequate material for unambiguous sequence determination, and in combination with h.p.l.c. separation can provide significant quantities of fragments for testing structure–function relationships.

Fig. 5 shows the comparison of the bovine and human C4a sequences. In the human C4a sequence (Moon *et al.*, 1981) the continuity of the two CNBr fragments and the positioning of the second methionine at residue 34 have not been positively established, but comparison with the bovine sequence suggests that the assumed sequence is the most likely. One striking feature of the comparison is that so many of the differences occur in the *C*-terminal region. There are seven replacements in the first 66 residues and five replacements in the *C*-terminal 11 residues. This is particularly surprising in view of the absolute requirement for the C-terminal arginine for biological activity (Gorski *et al.*, 1979). The extent of the amino acid replacement between the bovine and human C4a molecules is 12 in 77 residues or 16%. This is comparable with the replacements between bovine and human sequences of β-globin (17%), α-globin (12%) and cytochrome *c* (10%) (Dayhoff, 1972), suggesting that the C4a sequence is subject to substantial functional constraints greater than would be expected for simple activation peptides of the fibrinopeptide type.

When C4a sequences are compared with those of C3a and C5a (Hugli & Müller-Eberhard, 1978) after introducing the appropriate deletions the homologies

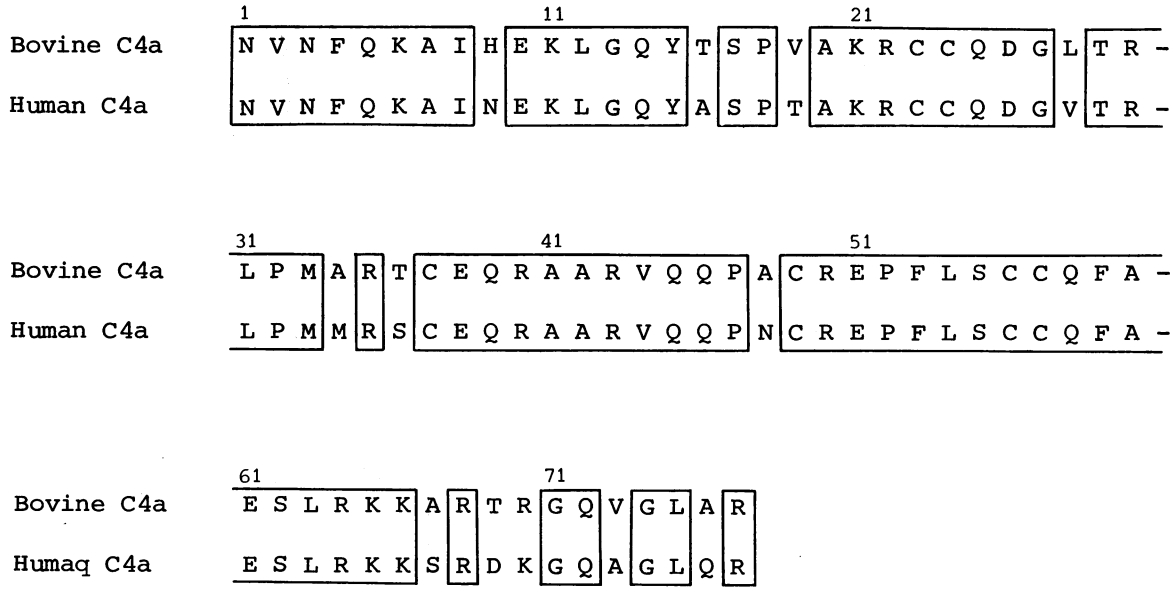

Fig. 5. *Comparison of the amino acid sequence of bovine C4a with that of human C4a (Moon et al., 1981)* Identical residues are boxed. Continuity between residues 33 and 35 and the position of residue 34 in the human sequence have not been positively established.

are obvious (Fig. 6). Over all six sequences there is complete retention of the six cysteine residues, four as double cysteine sequences and two as single cysteine residues, as well as tyrosine at 15, glycine at 27, arginine at 40, phenylalanine at 54, arginine at 65, leucine at 78 and the *C*-terminal arginine. Comparing the residues conserved in both species of C4a with those conserved in both species of C3a and C5a, there are 25 residues conserved between C4a and C5a, and 22 residues conserved between C4a and C3a. A similar comparison of C3a and C5a shows 19 conserved residues. These figures are sufficiently similar to suggest that the gene duplication events that gave rise to separate genes for C3, C4 and C5 occurred at approximately the same stage in evolution.

These comparisons are particularly significant when they are correlated with the X-ray diffraction structure established for C3a (Hüber *et al.*, 1980). The crystal structure allows the assignment of the disulphide bridge arrangement of C3a, and also of C4a and C5a if one assumes that the tertiary structures are homologous. As one might expect, the variability between sequences is mainly located in regions of the chain that forms loops away from the 'disulphide knot'. This is presumably the structural basis for the well defined immunochemical distinction among the anaphylatoxins (Moon *et al.*, 1981). The most surprising finding is that so much variability occurs in the last 15 residues which span between the highly conserved 'disulphide knot' and the highly conserved *C*-terminal arginine residue that is essential for biological activity in all three anaphylatoxins. Moreover, this variability extends to a deletion that must result in the *C*-terminal arginine varying in distance from the 'disulphide knot'. From the conserved arginine at position 65, C3a and C4a have 13 residues to the *C*-terminus, whereas C5a has 12 residues. This has implications for the stereochemistry of binding to the cell surface receptor. In this context the 'disulphide-knot' structure of the anaphylatoxins can be seen as a basic structure that occurs in other biologically active small proteins such as the α-subunits of the glycoprotein hormones (Giudice & Pierce, 1978), the neurophysins (Breslow, 1979) and some snake toxins (Kim & Tamiya, 1981; Walkinshaw *et al.*, 1980).

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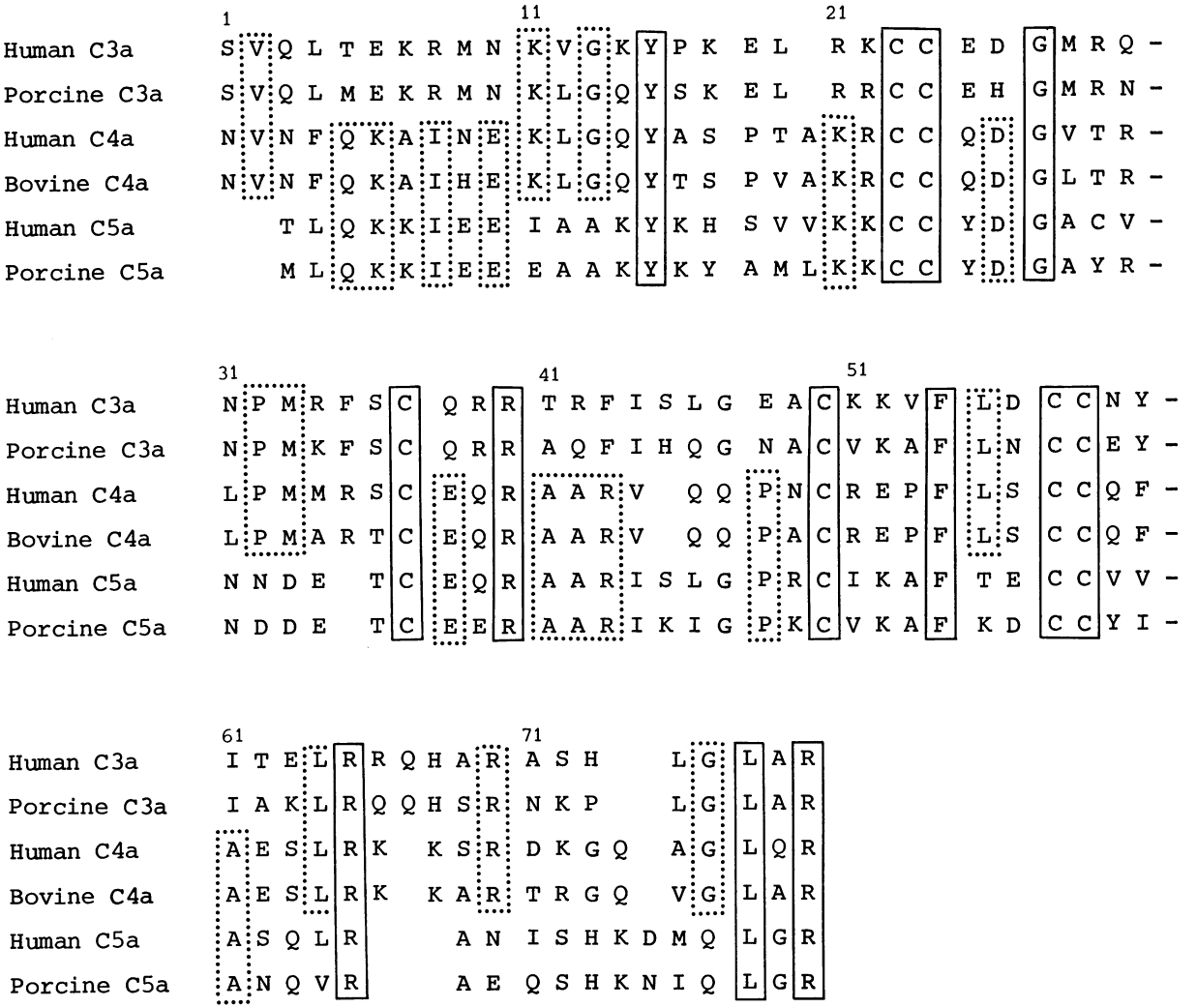

Fig. 6. *Comparison of the amino acid sequences of C3a, C4a and C5a*

The bovine C4a sequence is shown in alignment with the human C4a sequence (Moon *et al.*, 1981), with human and porcine C3a sequences (Hugli & Müller-Eberhard, 1978), and with human (Hugli & Müller-Eberhard, 1978) and porcine (Gerard & Hugli, 1980) C5a sequences. Sequences have been aligned to maximize homology. Residues identical in all six sequences are boxed with solid lines. Residues identical in both C3a and C4a sequences and residues identical in both C5a and both C4a sequences are boxed with broken lines.

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