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The reaction of iodine and thiol-blocking reagents with human complement components C2 and Factor B

Purification and *N*-terminal amino acid sequence of a peptide from C2a containing a free thiol group

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Human complement components C2 and Factor B each contain one free thiol group/molecule. Reaction with *p*-chloromercuribenzoate destroyed the haemolytic activity of C2 but had no effect on Factor B. Reaction of C2 with I_2 gave a 16-fold enhancement of its haemolytic activity. The pH optimum for the reaction was 7.0. The I_2 reacted at the thiol group in C2 with a stoicheiometry of 1 mol of I_2/mol of C2. The product of the reaction was unaffected by millimolar concentrations of dithiothreitol; however, azide and cyanide were inhibitory. Reaction with azide did not result in re-expression of the thiol group. Mild oxidation of the thiol group with *m*-chloroperbenzoic acid did not enhance the haemolytic activity. The results suggest that reaction with I_2 causes intramolecular covalent, but not disulphide, bond formation. I_2 reacted with Factor B at the free thiol group without affecting the haemolytic activity. A CNBr-cleavage peptide from C2a (obtained by cleavage of C2 by subcomponent C1s) containing the free thiol group was isolated. Automated Edman degradation of the peptide showed that it was the *N*-terminal peptide of C2a. The free thiol group was identified at position 18.

Component C2 and Factor B are two glycoproteins in the human complement system which show both structural and functional homology. Each is composed of a single polypeptide chain; the M_r of C2 is 100000 and that of Factor B is 90000. They are encoded by closely linked genes in the major histocompatibility locus (Barnstaple *et al.*, 1979), and show some amino acid sequence homology (Kerr, 1979; Kerr & Gagnon, 1982). C2 is a component of the classical-pathway C3 convertase. The convertase is formed when C2, bound to C4b in the presence of Mg^{2+}, is cleaved by C1s into the non-disulphide-linked peptides C2a (M_r 70000) and C2b (M_r 30000) (Nagasawa & Stroud, 1977). Similarly, Factor B is a component of the alternative-pathway C3 convertase. The active enzyme is formed when Factor B, bound to C3b in the presence of Mg^{2+}, is cleaved by Factor D into the non-disulphide-linked polypeptides Bb (M_r 60000) and Ba (M_r 30000) (Lesavre *et al.*, 1979). The active sites of these complex proteinases are in the C2a and Bb polypeptides, which are the *C*-terminal fragments of C2 and Factor B respectively. Factor B is a novel type of serine proteinase (Christie *et al.*, 1980; Mole & Niemann, 1980). C2 is inhibited by di-isopropyl phosphorofluoridate, and is also thought to be a serine proteinase (Medicus *et al.*, 1976). The C3 convertases have a half-life of a few minutes at 37°C (Fearon *et al.*, 1973; Kerr, 1980). The decay of activity is due to dissociation of C2a or Bb from the convertase.

Factor B is thought to contain two free thiol groups, one in Ba and one in Bb (Lesavre *et al.*, 1979). The position of one of the free thiol groups in Factor B has been identified by amino acid sequence analysis; it is residue 33 of Bb (Christie & Gagnon, 1982). It has been proposed that C2 also contains two free thiol groups (Polley & Müller-Eberhard,

Abbreviations used: the nomenclature of the complement components is that recommended by the World Health Organisation (1968, 1981); *p*CMB, *p*-chloromercuribenzoate; h.p.l.c., high-pressure liquid chromatography.

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1969). The free thiol groups in C2 appear to be functionally important, since reaction of C2 with *p*CMB abolishes the haemolytic activity (Leon, 1965; Polley & Müller-Eberhard, 1966, 1967). The functional importance of the free thiol groups in Factor B is less certain (Mak *et al.*, 1977; Lesavre *et al.*, 1979).

The enzymic activity of the classical-pathway C3 convertase can be enhanced 7–20-fold by prior reaction of C2 with I_2 (Polley & Müller-Eberhard, 1966, 1967). The increased activity is the result of a tighter binding of C2a to C4b, which prevents dissociation of the C2a and increases the half-life of the convertase to several hours (Kerr, 1980). Polley & Müller-Eberhard (1967) initially suggested that the enhanced activity was due to the oxidation of one or both of the free thiol groups in C2 to sulphenic acid, sulphinic acid or sulphonic acid groups. This conclusion was based on the following observations: with the use of radiolabelled I_2 the uptake was only 0.05 mol of I_2/mol of C2, I_2 treatment prevented subsequent inactivation of C2 by *p*CMB and the reaction was reversed by sodium dithionate, a reagent that is not known to split disulphide bonds (Polley & Müller-Eberhard, 1967). Subsequently (Polley & Müller-Eberhard, 1969) the reaction was found to be reversed by dithiothreitol. It was therefore presumed that the I_2 reaction resulted in disulphide bond formation between the two free thiol groups in C2. Factor B has also been reported to be affected by reaction with I_2 (Mak *et al.*, 1977); however, the change in activity is not nearly as dramatic as that seen with C2.

It appears that the integrity of the free thiol groups in C2 is essential for the normal assembly and decay of the classical-pathway C3 convertase. This constraint does not apply to Factor B in the alternative-pathway C3 convertase. In view of this difference, it was decided to re-examine the reaction of thiol-blocking reagents and I_2 with both C2 and Factor B. It was hoped to determine the exact chemical nature of the I_2 reaction and to discover the reason for the difference in behaviour of the two homologous proteins. Isolation and amino acid sequence analysis of free thiol-containing peptides from C2a were also undertaken, since comparison with the known sequence of Bb might provide an insight into the difference in properties. The results of these investigations are reported in the present paper.

Materials and methods

Materials

Outdated human plasma was obtained from the John Radcliffe Hospital, Oxford, U.K. I_2 was purchased from Reeve Angel Scientific, London E.C.4, U.K. Iodoacetic acid was from Sigma Chemical Co., Poole, Dorset, U.K. Dithiothreitol was obtained from Calbiochem, Hereford, U.K., and CNBr was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Iodo[2-^{3}H]acetic acid (64 Ci/mol) was from The Radiochemical Centre, Amersham, Bucks., U.K. *p*-Chloromercuri[^{14}C]benzoate was obtained from the Commissariat à l'Energie Atomique, Gif-sur-Yvette, France.

Reagents for h.p.l.c. and automated amino acid sequence analysis were obtained as described by Christie & Gagnon (1982). *m*-Chloroperbenzoic acid was kindly given by Dr S. G. Davies, University of Oxford, Oxford, U.K.

Preparation of C2, C2a and Factor B

C2 and Factor B were isolated from human plasma as described by Kerr & Porter (1978) and Kerr (1979). C2a was obtained from C2 by digestion with $C\overline{1s}$ and separation of C2a and C2b on Sephadex G-100 in 0.1 M-ammonium bicarbonate buffer (Kerr, 1979).

Haemolytic assay of C2 and Factor B

C2 was assayed with the use of EAC14 cells by the method of Kerr & Porter (1978). Factor B was assayed with the use of EAC43 cells as described by Parkes *et al.* (1981). The titre of a component is the dilution required to give 63% lysis in the standard assay.

Reaction of C2 and Factor B with I_2

The reaction of C2 and Factor B with I_2 was performed in 0.1 M-sodium phosphate buffer, pH 6.0. In some of the experiments the reaction conditions described by Kerr (1980) were used. The stock iodine solution, 10 mM-I_2 in 24 mM-KI, was used at 1 μl per 0.01–0.10 mg of protein. These conditions are referred to as excess of I_2. In other experiments the stock solution was made up as 1 mM-I_2 in 0.2 M-KI and used at 2-fold molar excess over the protein in each experiment. These conditions are described as equimolar I_2. In both cases the I_2 was allowed to react for 10 min at 4°C. The protein was then desalted on a column (50 cm × 1 cm diam.) of Sephadex G-25 (coarse grade) equilibrated in 0.1 M-sodium phosphate buffer, pH 6.0.

Reaction of C2 and Factor B with [^{14}C]pCMB

C2 or Factor B in 0.1 M-sodium phosphate buffer, pH 6.0, was incubated at 30°C for 1 h with a 50-fold molar excess of [^{14}C]*p*CMB. At the end of the incubation the protein was desalted on a column (50 cm × 1 cm diam.) of Sephadex G-25 (coarse grade) equilibrated in 0.1 M-sodium phosphate buffer, pH 6.0. The stock solution of [^{14}C]*p*CMB (24 000 c.p.m./nmol) was made up by dissolving the solid in 0.1 M-NaOH, neutralizing with Tris/HCl buffer and diluting with water to give a final concentration of 1 mM.

Titration of C2 and Factor B with I_2

The stoicheiometry of the reaction of I_2 with C2 and Factor B was measured by using the spectrophotometric titration method described by Cunningham & Nuenke (1959, 1960). I_2 in the presence of KI exists as I_3^-, which has an absorption maximum at 355 nm. If I_2 is added to an excess of protein in KI no absorption at 355 nm is observed until sufficient I_2 has been added to react completely with the protein, and thus the stoicheiometry can be determined. A Beckman model 35 spectrophotometer fitted with a recorder/controller was used for the titrations. The iodine solution, 0.2mM-I_2 in 0.2M-KI, was added in 5μl portions to the protein solution (3ml, containing approx. 0.3mg of protein/ml, in 0.1M-sodium phosphate buffer, pH 6.0, containing 0.05M-KI). After each addition the protein solution was mixed, and the absorbance at 355 nm was recorded for 1 min in comparison with a cuvette containing only buffer. The titration was also performed in the absence of protein. An accurate value for the concentration of the iodine solution was calculated from mequiv. of I/ml = $7.77 \times 10^{-5} \times A_{355}$ for several dilutions of the stock solution.

Determination of protein concentration

The concentrations of protein solutions were calculated from their absorbance at 280 nm by using the following absorption coefficients ($A_{1cm}^{1\%}$): 12.7 for Factor B (Curman *et al.*, 1977) and 10.0 for C2.

Measurement of radioactivity

Liquid radioactive samples were added to 5 ml of 1,4-dioxan containing 0.5% 2,5-diphenyloxazole and 2% (w/v) naphthalene in scintillation vials and counted for radioactivity for 5 min or 10 min in a LKB Wallac 1210 Ultrobeta counter.

Alkylation of C2a

Freeze-dried C2a (150 nmol) was dissolved in 3.3 ml of 0.5M-Tris/HCl buffer, pH 8.1, containing 2mM-EDTA and 6M-guanidinium chloride. The sample was incubated at 37°C for 1 h and cooled on ice. Iodo[2-^{3}H]acetic acid (250μCi) was added, and the sample was left on ice for 5 min. Iodoacetic acid (100μl of a 1 M solution, pH 8.0) was added, and the incubation on ice was continued for a further 5 min. The sample was then acidified with acetic acid, dialysed against three 1-litre volumes of 0.5M-acetic acid and freeze-dried.

CNBr digestion of alkylated C2a

The alkylated C2a was dissolved in 98% (v/v) formic acid. The sample was diluted to 3 ml, giving a final concentration of 70% (v/v) formic acid. A 4-fold weight excess of CNBr was added, and the mixture was incubated for 24 h at 4°C in the dark. The digest was then diluted 10-fold with water and freeze-dried.

Reduction and alkylation of the CNBr digest of alkylated C2a

The digest was dissolved in 1.5 ml of 0.5M-Tris/HCl buffer, pH 8.1, containing 2mM-EDTA and 6M-guanidinium chloride. Dithiothreitol was added to give a final concentration of 20 mM, and the sample was incubated at 37°C for 3 h. Unlabelled iodoacetic acid (100μl of a 1 M solution, pH 8.0) was then added, and the sample was left on ice for 45 min. The digest was then acidified with acetic acid.

H.p.l.c.

The h.p.l.c. system (Waters Associates) was as described by Christie & Gagnon (1982). Reverse-phase chromatography was performed with a μBondapak C_{18} column (300 mm × 3.9 mm) at a flow rate of 1 ml/min. The peptide mixtures were separated in 0.1% (v/v) trifluoroacetic acid by using a 60 min linear 5–65% (v/v) gradient of methanol/acetonitrile/propan-2-ol (1:1:1, by vol.).

Amino acid analysis

Amino acid analysis of peptides was performed as described by Christie & Gagnon (1982). Samples were analysed on a Durrum D-500 amino acid analyser.

Automated amino acid sequence analysis

Automated Edman degradation was performed in a Beckman 890C sequencer with the 0.3M-Quadrol program of Hunkapiller & Hood (1978) as described by Christie & Gagnon (1982).

Results

Reaction of C2 and Factor B with [^{14}C]pCMB and with I_2

C2 or Factor B was allowed to react with an excess of I_2, with [^{14}C]*p*CMB or with an excess of I_2 followed by [^{14}C]*p*CMB. The results are given in Table 1. The activity of C2 is enhanced by I_2 and inhibited by [^{14}C]*p*CMB. The reagents have little effect on the activity of Factor B. The incorporation of radioactivity into C2 and Factor B after reaction with [^{14}C]*p*CMB suggests that 1 mol of [^{14}C]*p*CMB reacts with 1 mol of protein, and thus that each protein contains only one free thiol group/molecule. Reaction with I_2 abolishes the incorporation of [^{14}C]*p*CMB into C2, suggesting that both reagents interact with the same region of the protein. There is a decrease in the I_2-enhanced activity despite the lack of incorporation of label. This may be due to inactivation of the I_2-treated material by Tris/HCl buffer added with the *p*CMB (see Table 2). The incorporation of [^{14}C]*p*CMB into Factor B is somewhat decreased by prior reaction with I_2; however, there was no change in the haemolytic

Table 1. *Reaction of C2 and Factor B with* [^{14}C]*pCMB and* I_2

C2 or Factor B (0.5–1.0mg) was treated with [^{14}C]*p*CMB, with an excess of I_2 or with an excess of I_2 followed by [^{14}C]*p*CMB in 1–2ml of 0.1M-sodium phosphate buffer, pH6.0. After each reaction the protein was desalted on a column (50cm × 1cm diam.) of Sephadex G-25 (coarse grade) into 0.1M-sodium phosphate buffer, pH6.0, and the protein concentration, incorporation of radioactivity and haemolytic activity of each fraction were determined. In the control experiments no I_2 or [^{14}C]*p*CMB was added. Where a range of values is given it is the maximum and minimum values obtained from four determinations.

	Factor B	C2
Titre after reaction with I_2/titre of control	0.9	16.0
Titre after reaction with [^{14}C]*p*CMB/titre of control	0.9	0.1
Incorporation of [^{14}C]*p*CMB (mol/mol of protein)	0.84–0.97 0.80–0.94*	0.62–0.72
Incorporation of [^{14}C]*p*CMB (mol/mol of I_2-treated protein)	0.32–0.41	0.02
Titre after reaction with I_2 and [^{14}C]*p*CMB/titre of control	1.0	7.5

* Results obtained after reaction of [^{14}C]*p*CMB with Factor B for 20h.

Table 2. *Reaction of* I_2*-treated C2 with various small molecules*

C2 or equimolar I_2-treated C2 (5μg) in 100μl of 0.1M-sodium phosphate buffer, pH6.0, was treated with various reagents for 1h at 37°C. The haemolytic activity was then determined. Control samples incubated in the absence of the reagent were also assayed. The results are expressed as percentages of these control values. The experiment with dithiothreitol was performed at room temperature.

Reagent	Concn. (mM)	C2 activity (% of control)	I_2-treated C2 activity (% of control)
NaN_3	2	88	7
KI	200	108	8
	3	100	97
KBr	3	98	100
KCN	3	83	13
KSCN	3	100	96
Dithiothreitol	0.5	72	68
β-Mercaptoethylamine	1	100	100
Tris/HCl, pH 7.0	10	100	77

activity. The latter is expected, since neither reagent alone affects the activity.

C2 (3μg) was allowed to react with equimolar I_2 in 200μl of 0.1M-sodium phosphate buffer. The pH of the buffer was varied in different experiments in the range pH6–8. The maximum enhancement of C2 haemolytic activity was obtained at pH7.0; at this pH the enhancement was 1.6-fold greater than at pH6.0.

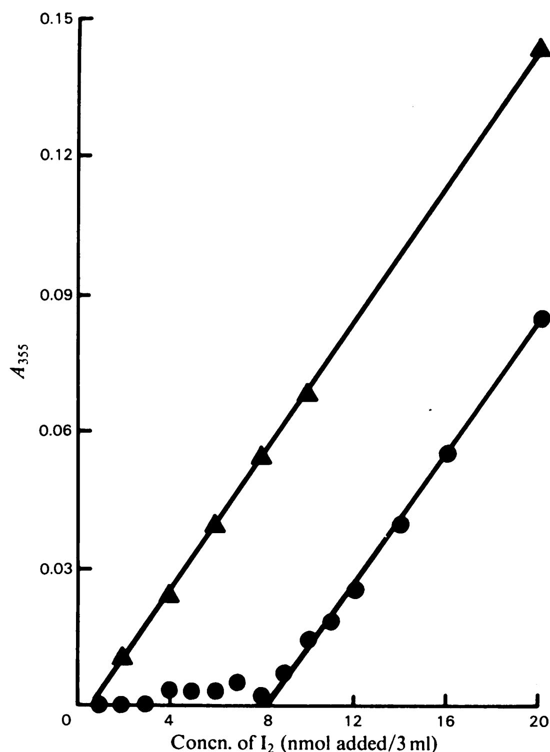

Fig. 1. *Titration of C2 with* I_2

The Figure shows the change in absorption at 355nm on addition of I_2 (0.2mM in 0.2M-KI) to 3ml of 0.05M-KI/0.1M-sodium phosphate buffer, pH6.0 (▲), or 3ml of 0.05M-KI/0.1M-sodium phosphate buffer, pH6.0, containing 7.1nmol of C2 (●). The titration was conducted at room temperature.

Titration of C2 and Factor B with I_2

The result of titrating C2 with I_2 is shown in Fig. 1. The data give an end point at 0.9mol of I_2/mol of C2. The haemolytic activity of C2 was assayed at the end point. The titre was 9.2 times higher than before the titration, showing that enhancement had been achieved. Addition of more I_2 up to 100-fold molar excess did not affect the enhanced activity. An absorption spectrum of the protein (220–300nm)

was recorded before the titration and at the end point. No differences in the spectra were observed.

Factor B was titrated in a similar way to C2. When an amount of I_2 equivalent to 0.25–0.45 mol of I_2/mol of Factor B (the value varied in different titrations) had been added, there was a first-order increase in the absorbance at 355 nm over 5 min. Further additions of I_2 gave step increases in absorbance, as observed after the end point in the C2 titration. The absorption spectrum of Factor B (220–300 nm) was unaffected by the reaction. The Factor B after titration showed decreased incorporation of [^{14}C]*p*CMB, as previously observed (Table 1).

Reaction of C2 with various concentrations of I_2 followed by [^{14}C]pCMB

The results in Table 1 suggest that I_2 reacts with the one free thiol group in C2; 1 mol of I_2/mol of C2 is sufficient for the reaction (Fig. 1). In order to confirm that I_2 reacts with the free thiol group it was necessary to determine whether reaction with 1 mol of I_2/mol is sufficient to block the incorporation of [^{14}C]*p*CMB into the thiol group. C2 was therefore treated with various concentrations of I_2 in the range 0–3 mol of I_2/mol of C2, and the subsequent incorporation of [^{14}C]*p*CMB was determined. The results, given in Fig. 2, show that the increase in haemolytic activity of C2 parallels the blocking of the incorporation of [^{14}C]*p*CMB into C2 as increasing amounts of I_2 are added.

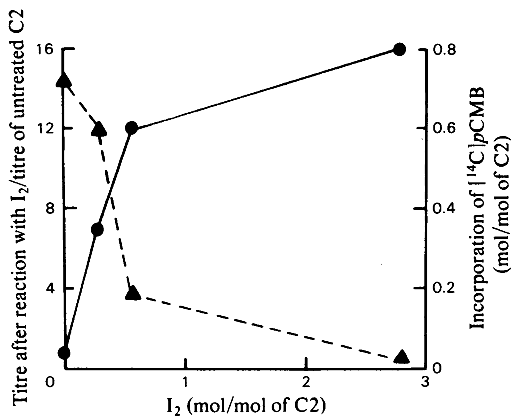

Fig. 2. *Reaction of C2 with varous concentrations of I_2 followed by [^{14}C]pCMB*

Portions of C2 (0.5 ml of 0.22 mg/ml solution in 0.1 M-sodium phosphate buffer, pH 6.0) were treated with various concentrations of I_2 for 10 min at 4°C. The stock iodine solution was 0.2 mM-I_2 in 0.2 M-KI. The titre of the C2 solutions before and after reaction was determined. The I_2-treated C2 was then treated with [^{14}C]*p*CMB, and the radioactivity incorporated was measured. ▲, Incorporation of [^{14}C]*p*CMB (mol/mol of C2). ●, Titre of C2 after reaction with I_2/titre of untreated C2.

Reaction of I_2-treated C2 with various small molecules

In order to gain information about the product of the reaction of C2 with I_2, its stability in the presence of various small molecules was investigated (Table 2). The enhanced activity is lost in the presence of azide or cyanide. Iodide at higher concentrations is also inhibitory. Dithiothreitol and thiocyanate, reagents that would be expected to break disulphide bonds, are not inhibitory under the conditions used. At higher concentrations of dithiothreitol (1 mM), the conditions used by Polley & Müller-Eberhard (1969), considerable inhibition of the activities of C2 and I_2-treated C2 were observed. [^{14}C]*p*CMB could not be incorporated into I_2-treated C2 that had been reacted with azide, showing that the thiol group is not re-exposed (Table 3). The product of the reaction showed diminished activity compared with the control.

Oxidation of the thiol group in C2

m-Chloroperbenzoic acid is an oxidizing agent that will oxidize cysteine, methionine and tryptophan. It has a similar structure to *p*CMB. *p*CMB reacts rapidly with the free thiol group in C2 under non-denaturing conditions. It was therefore hoped that *m*-chloroperbenzoic acid, used at approximately equimolar amount, would preferentially oxidize the free thiol group in C2. C2 (100 μg in 1 ml of 0.1 M-sodium phosphate buffer, pH 6.0) was treated with a 4-fold molar excess of *m*-chloroperbenzoic acid for 1 h at room temperature. The haemolytic activity of the C2 was determined, and, after the mixture had been desalted on a column (50 cm × 1 cm diam.) of Sephadex G-25 (coarse

Table 3. *Reaction of I_2-treated C2 with NaN_3 followed by [^{14}C]pCMB*

C2 or equimolar I_2-treated C2 (1 mg in 1.5 ml of 0.1 M-sodium phosphate buffer, pH 6.0) of known titre was incubated with 7 mM-NaN_3 for 30 min at 30°C. The titre of the solution was found and, after it had been desalted on a column (50 cm × 1 cm diam.) of Sephadex G-25 (coarse grade) in 0.1 M-sodium phosphate buffer, pH 6.0, the ability to incorporate [^{14}C]*p*CMB was assessed.

	Titre	Incorporation of [^{14}C]*p*CMB (mol/mol of C2)
C2	56 100	
I_2-treated C2	1 170 300	
NaN_3-treated C2	54 600	0.73–0.84
NaN_3- and I_2-treated C2	19 000	0.01

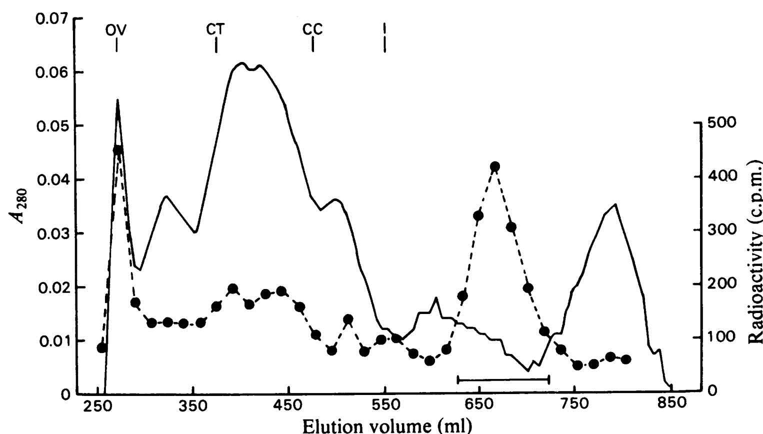

Fig. 3. *Separation of C2a CNBr-cleavage peptides on Sephadex G-75*
C2a (150nmol) was alkylated with iodo[2-^{3}H]acetic acid, digested with CNBr, reduced and alkylated with unlabelled iodoacetic acid and run on a column (90cm x 3.2cm diam.) of Sephadex G-75 in 10% (v/v) acetic acid. ——, A_{280}. ●, Radioactivity of 100μl samples of certain fractions. The position of various M_r markers is indicated: ovalbumin (OV), chymotrypsinogen (CT), cytochrome *c* (CC) and insulin (I). The radioactive fractions were pooled as indicated.

grade) equilibrated in 0.1M-sodium phosphate buffer, pH6.0, the ability to incorporate [^{14}C]*p*CMB was assessed. Reaction with *m*-chloroperbenzoic acid completely abolished the haemolytic activity of the C2 and decreased the incorporation of [^{14}C]*p*CMB to 0.04mol/mol of C2.

Isolation and N-terminal amino acid sequence analysis of a peptide from C2a containing the free thiol group

The results in Table 1 suggest that C2 and Factor B each contain only one free thiol group/molecule. Since the free thiol group in Factor B had been identified as being in the Bb portion of the molecule, it was decided to use C2a as the starting material for the isolation of a free-thiol containing peptide from C2. In the presence of 6M-guanidinium chloride, 1mol of C2a did incorporate 0.75–0.86mol of iodo[2-^{3}H]acetic acid. During the initial attempts to isolate the required peptide, the following observations were made. Peptides derived from C2a tended to aggregate very readily and become insoluble under both acid and basic conditions. CNBr or trypsin digestion of C2a labelled with iodo[^{3}H]-acetic acid under denaturing conditions by conventional procedures resulted in the formation of many labelled peptides. The latter may be due to incomplete digestion, possibly a result of aggregation, or due to the presence of labile disulphide bonds in the molecule. In order to circumvent these problems, C2a was labelled for a short time with iodo[2-^{3}H]acetic acid and the number of freeze-drying steps in the procedure was minimized.

C2a (150nmol) was alkylated with iodo[2-^{3}H]-acetic acid, digested with CNBr and reduced and alkylated with unlabelled iodoacetic acid as described in the Material and methods section. After reduction and alkylation, the digest was acidified with acetic acid and run directly on a column (90cm x 3.2cm diam.) of Sephadex G-75 equilibrated in 10% (v/v) acetic acid. The elution profile of the column is shown in Fig. 3. Two radioactive peaks were observed. The peak at the void volume of the column is probably due to aggregated or incompletely digested material, and was not included in subsequent purification steps. The second radioactive peak was pooled as indicated in the Figure, and freeze-dried. The pool was dissolved in 0.1% (v/v) trifluoroacetic acid and further purified by using h.p.l.c. as described in the Materials and methods section. The column profile is shown in Fig. 4. There are two radioactive peaks, peptide A and peptide B. The peaks are doublets; these probably arise from the homoserine and homoserine lactone forms of the same peptide. Peptide A, which had most of the radioactivity and the higher specific radioactivity, was investigated further. One-twenty-fifth of the pool was used for amino acid analysis (Table 4). The remainder of peptide A was subjected to automated Edman degradation. Amino acid phenylthiohydantoin derivatives were identified for the first 26 cycles and are shown in Fig. 5. *S*-Carboxymethylcysteine was identified at cycle 18, and 80% of the radioactivity recovered from cycles 1–44 was also found at cycle 18. The specific radioactivity of peptide B was one-tenth of that of peptide A.

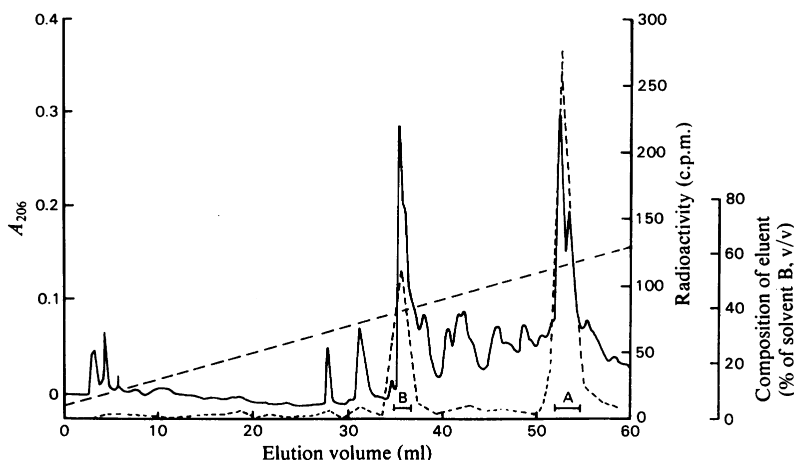

Fig. 4. *Further purification of C2a CNBr-cleavage peptides by h.p.l.c.*
The freeze-dried pool from the Sephadex G-75 column was dissolved in 0.6ml of 0.1% (v/v) trifluoroacetic acid and run on a μBondapak C_{18} column (300mm × 3.9mm) in two portions. The column was initially equilibrated in 0.1% (v/v) trifluoroacetic acid containing 5% (v/v) methanol/acetonitril/propan-2-ol (1:1:1, by vol.) (solvent B). The peptides were eluted at 1ml/min by using a 60min 5–65% (v/v) linear gradient of solvent B. ——, A_{206}. ----, Composition of eluent. ------, Radioactivity of *S*-carboxy[^{3}H]methylcysteine. Pools A and B were made as indicated.

Table 4. *Amino acid composition of peptide A*
The amino acid composition of peptide A was calculated from a 24h HCl hydrolysate. Cysteine and half-cystine were estimated as *S*-carboxymethylcysteine. Homoserine was quantified as the sum of the homoserine and homoserine lactone values. No correction for the destruction of threonine and serine was made. Tryptophan was not determined.

	Amino acid composition (mol of residue/mol)
Asx	3.9
Thr	0.9
Ser	4.0
Hse	0.7
Glx	4.0
Pro	0.0
Gly	3.1
Ala	2.0
Val	2.0
Cys	0.7
Ile	2.5
Leu	4.8
Tyr	0.6
Phe	1.4
His	1.0
Lys	1.8
Arg	1.5

Discussion

C2 and Factor B both incorporate 1mol of [^{14}C]*p*CMB/mol, suggesting that they each contain one free thiol group/molecule (Table 1). A stoicheiometry of one free thiol group/molecule for both C2 and Factor B is also obtained by the use of iodo[2-^{14}C]acetamide and 5,5′-dithiobis-(2-nitrobenzoate under denaturing conditions (results not shown). The result for Factor B is in conflict with that reported by Lesavre *et al.* (1979), who found 2mol of [^{14}C]*p*CMB incorporated/mol. The reason for this difference is uncertain; similar reaction conditions were used, but, however, Lesavre *et al.* (1979) removed the unincorporated [^{14}C]*p*CMB by dialysis rather than by gel filtration. In agreement with published data, reaction of C2 with *p*CMB abolishes the haemolytic activity. Reaction of Factor B with *p*CMB did not affect the haemolytic activity, whereas a 50% decrease in activity had been reported previously (Mak *et al.*, 1977; Lesavre *et al.*, 1979).

C2 reacts rapidly with I_2, giving a 16-fold enhancement in the haemolytic activity (Table 1; Polley & Müller-Eberhard, 1967). The pH optimum for the reaction was found to be pH 7.0. The result of I_2 titration (Fig. 1) shows that 1mol of I_2 reacts with 1mol of C2 to give the enhanced haemolytic activity. The I_2 when it has reacted in equimolar amounts is sufficient to block the incorporation of [^{14}C]*p*CMB at the free thiol group in C2 (Table 1 and Fig. 2). These results clearly show that I_2 enhances the haemolytic activity of C2 by reacting with the free thiol group. The question that remains is what is the product of the reaction? I_2 is not incorporated into C2 during the I_2 reaction (Polley & Müller-Eberhard, 1967; M. A. Kerr, unpublished work). Thus formation of a stable sulphenyl iodide

	1 5 10 15 20 25 30
Peptide A	K I Q I Q R S G H L N L Y L L L D **C** S Q S V S E G D
Human C2a	K I Q I Q S X G X K N L Y
Guinea-pig C2a	K I Q I Q R S G H L N L Y L L L D A S K S $\overset{V}{M}$ S E E D I E
Human Bb	K I V L D P S G S M N I Y L V L D G S D S I G A S - F T G A K K **C** L

Fig. 5. *N-Terminal amino acid sequences of peptide A, human C2a, human Bb and guinea-pig C2a*
The *N*-terminal amino acid sequence found for peptide A from human C2a is shown together with the published *N*-terminal sequences of human C2a (Kerr, 1979; Kerr & Gagnon, 1982), guinea-pig C2a (Kerr & Gagnon, 1982) and human Bb (Christie & Gagnon, 1982). The radioactive cysteine residue found in peptide A is shown in **bold** type, as is the free cysteine residue in Bb. The gap in the sequence at position 26 in Bb results from the presence of an asparagine-linked carbohydrate. Amino acids are in the single-letter notation: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, unknown; Y, Tyr.

group seems unlikely. The I_2 could act by oxidizing the free thiol group. Thus 1 mol of I_2/mol could give oxidation to a sulphenic acid group but not to sulphinic acid or sulphonic acid groups. The experiment with *m*-chloroperbenzoic acid, however, suggests that direct oxidation is not occurring. Under mild oxidation conditions the free thiol group was modified but the C2a haemolytic activity was abolished. Formation of a disulphide bond after reaction with I_2 can also be ruled out, as C2 contains only one free thiol group, no protein dimerization is observed on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of I_2-treated C2 and the product of the I_2 reaction is not affected by dithiothreitol (Table 2). The most likely sequence of events in the reaction of I_2 with C2, which is consistent with the observed results, is formation of a sulphenyl iodide group and subsequent nucleophilic attack by another amino acid side chain displacing the iodide and forming an intramolecular covalent bond. Azide and cyanide, which abolish the I_2-enhanced activity (Table 2), are both strong nucleophiles, and could act by breaking the putative covalent bond, giving $-SN_3$ or $-SCN$. This would be consistent with the results given in Table 3, which show that azide does not simply reverse the effect of I_2. Although the exact nature of the product of the reaction of I_2 with C2 cannot be deduced from the data in the present paper, direct oxidation of the thiol group or disulphide bond formation seems very unlikely.

The results obtained on titration of Factor B with I_2 and the decreased incorporation of [^{14}C]*p*CMB observed after reaction of Factor B with I_2 (Table 1) suggest that I_2 also interacts with the free thiol group in Factor B. The reaction does not affect the haemolytic activity of Factor B. The interaction is clearly different from that observed with C2, and its nature is unknown.

The procedure described for the isolation of a free-thiol-containing CNBr-cleavage peptide from C2a resulted in the purification of two labelled peptides (Fig. 4). Peptide B had a lower specific radioactivity than that of peptide A. Peptide A was therefore assumed to be the free-thiol-containing peptide. The *N*-terminal amino acid sequence of peptide A (Fig. 5) is similar to the *N*-terminal sequence of human C2a (Kerr, 1979; Kerr & Gagnon, 1982) and very closely homologous to the *N*-terminal sequence of guinea-pig C2a (Kerr & Gagnon, 1982). Thus peptide A is presumed to be the *N*-terminal CNBr-cleavage peptide of C2a. The free thiol group was identified at position 18.

The amino acid sequences of peptide A and guinea-pig C2a are identical up to position 18, where the free cysteine occurs in peptide A. The subsequent sequences are homologous, but notably the sequence of guinea-pig C2a up to residue 28 does not contain cysteine. Guinea-pig C2a forms a C3 convertase that has a decay rate about 10 times lower than that of the convertase formed with human C2a (Kerr & Gagnon, 1982). The amino acid sequence difference identified in the *N*-terminal regions of human and guinea-pig C2a may partly explain the difference in stability of the two convertases, since the structure of this area of the molecule is known, from the effects of *p*CMB and I_2, to be critical in the interaction of C2 with C4.

Comparison of the amino acid sequences of peptide A and Bb indicate that C2a and Bb show greater sequence homology than previously suspected (Kerr, 1979). However, the free thiol groups in the two molecules are probably in rather different environments. The free thiol group in Bb is residue 33 and is close to the carbohydrate-attachment site at residue 28. Peptide A is probably not a glycopeptide, as amino acid analysis suggests that it is about 35 residues long and the Sephadex G-75 column gives it an M_r of approx. 3000. Thus the environment of the free thiol groups in C2a and Bb may be related to the difference in reactivity of the two proteins with I_2.

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