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The effects of iodine and thiol-blocking reagents on complement component C2 and on the assembly of the classical-pathway C3 convertase

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I_2 can react with complement component C2 in a two-stage process. In the first stage, a form of C2 with enhanced haemolytic activity is produced. This form of C2 is cleaved to C2a and C2b by $C\bar{1}s$ at the same rate as native C2. The enhanced C2 haemolytic activity correlates with the ability to form a stable fluid-phase C3 convertase on addition of the C2 to C4b and $C\bar{1}s$. It reflects an increased affinity for C4b of C2a formed from I_2-treated C2, although the affinity for C4b of I_2-treated C2 itself is not markedly increased. The specific activity of C3 convertase formed from I_2-treated C2 is the same as that formed from native C2. The second stage of the reaction with I_2, which is favoured at high pH or in the presence of excess I_2, inactivates C2 on production of a species that cannot be cleaved by $C\bar{1}s$. The presence of a single free thiol group in C2, which is the site of modification by I_2, was confirmed by titration with *p*-chloromercuribenzoate, iodoacetamide and 5,5′-dithiobis-(2-nitrobenzoic acid). A single thiol group is also present in Factor B, and the cysteine residue, like that in C2, requires denaturation of the protein before reaction with idoacetamide and 5,5′-dithiobis-(2-nitrobenzoic acid) but not *p*-chloromercuribenzoate.

The cleavage of C3 is the central event of the complement system, which leads to the generation of the many biological activities of C3 (for reviews see Muller-Eberhard & Schreiber, 1980; Reid & Porter, 1981; Kerr, 1981). The two proteolytic enzymes that cleave C3, the classical-pathway and alternative-pathway C3 convertases, are similar in structure and function. The classical-pathway C3 convertase is an Mg^{2+}-dependent complex of C2 and C4 activated by the serine proteinase $C\bar{1}s$ subcomponent of the $C\bar{1}$ complex. The alternative-pathway C3 convertase is an Mg^{2+}-dependent complex of C3 and Factor B activated by the serine proteinase Factor D. The active sites of the C3 convertases are in the larger of the two fragments generated on cleavage of C2 and Factor B. C2 and Factor B are homologous proteins; both are serine proteinases with an unusual mode of activation (Christie *et al.*, 1980). The C3 convertases are also unusual in that, once activated, the enzyme activity decays rapidly, with the release of the active-site-containing fragments, C2a and Bb, from the complexes. This decay is probably an important control process in the complement system.

Studies (Kerr, 1980) have shown that in the fluid phase the half-life of the classical C3 convertase is less than 1 min. Study of this enzyme would therefore have been very difficult but for the observation by Polley & Muller-Eberhard (1967) that treatment of human C2 with low concentrations of I_2 allowed the formation of stable C3 convertase when the C2 was added to C4 and $C\bar{1}s$. The stabilization of the C3 convertase results in a marked increase in the haemolytic activity of the C2. The same authors (Polley & Muller-Eberhard,

Abbreviations used: the nomenclature of the complement components is that recommended by the World Health Organisation (1968, 1981); EAC14 cells are sheep erythrocytes sensitized with rabbit anti-(sheep erythrocyte) antibody and coated with C1 (guinea pig) and C4 (human) (Nelson *et al.*, 1966); pCMB, *p*-chloromercuribenzoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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1967; Cooper *et al.*, 1970) reported that reaction of I_2 with C2 caused oxidation of reactive thiol groups and that this augmented three different functions: the binding of C2 to C4b, the catalytic function of C2 in the C3 convertase and the stability of the enzyme (Muller-Eberhard, 1975). 'Oxidized' C2 has since been used extensively as a reagent in the study of the complement system.

With the availability of milligram amounts of purified human C2 it was possible to isolate the stable C3 convertase formed from C4b, $C\bar{1}s$ and I_2-treated C2 and hence to identify the subunit structure of the enzyme (Kerr, 1980). When the C3 convertase was isolated by gel filtration on Sephadex G-200 in Veronal buffer, pH8.5, containing Mg^{2+} the enzyme comprised C4b, C2a and C2b. When isolated from the same column run in Veronal buffer, pH8.5, containing EDTA, the enzyme comprised C4b and C2a only. In the absence of I_2 treatment no C3 convertase could be isolated and no C4b–C2a complex could be detected. It was therefore concluded that the major effect of I_2 treatment of C2 is to increase the affinity for C4b of the C2a produced on cleavage by $C\bar{1}s$, and this results in stabilization of C3 convertase activity. The interaction of C4b and C2b can be detected without I_2 treatment in Mg^{2+}-containing buffer but not in EDTA-containing buffer (Nagasawa & Stroud, 1977; Kerr, 1980). I_2 treatment does not appear to enhance greatly this affinity of C2b for C4b or the affinity of C2b for C2a.

The effects of I_2 treatment are apparently due to modification of one or more groups in C2a. Since the amounts of I_2 incorporated into C2 by I_2 treatment are only around 0.05 g-atom of I/mol of C2 (Polley & Muller-Eberhard, 1967) the effect has been assumed to be due to oxidation of free thiol group(s) in C2. Muller-Eberhard (1975) reported the presence of two thiol groups in C2, which were oxidized by I_2 to a disulphide bond. However, subsequent studies (Parkes *et al.*, 1983) identified, by pCMB titration, a single thiol group in C2, which is the site of reaction of I_2. The results suggested that the reaction of I_2 with C2 causes an intramolecular, covalent, but not disulphide, bond in C2a. The increased affinity of C2a for C4b is unlikely to be due to an intermolecular covalent bond formation, since the subunits can be separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in reducing or non-reducing conditions (Kerr, 1980).

In view of the importance of this chemical modification in future study of the structure of the classical-pathway C3 convertase, we have investigated in more detail the effects of I_2 treatment of C2 on the assembly and activity of the convertase, and by use of a variety of thiol-blocking reagents confirmed the presence of a single thiol group in C2 and Factor B.

Materials and methods

Materials

Outdated human plasma was obtained from the John Radcliffe Hospital, Oxford, U.K. Iodoacetamide and DTNB were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Iodoacetamide was freshly recrystallized from hot ethanol before use. Iodo[1-^{14}C]acetamide was from The Radiochemical Centre, Amersham, U.K. *p*-Chloromercuri[^{14}C]benzoate ([^{14}C]pCMB) was obtained from the Commissariat a l'Energie Atomique, Gif-sur-Yvette, France.

Solutions

A stock I_2/KI solution (5mM-I_2 in 50mM-KI) was made by dissolving 12.7mg of I_2 and 83mg of KI in 1ml of distilled water and diluting to 10ml with distilled water. Lower concentrations were made by diluting this stock with buffer or buffer containing 50mM-KI. The following buffers were used throughout this work: Veronal buffer, pH7.5 (5mM-Veronal buffer, pH7.5, containing 150mM-NaCl, 2mM-$MgCl_2$ and 0.5mM-$CaCl_2$); Veronal buffer, pH8.5 (5mM-Veronal buffer, pH8.5, containing 40mM-NaCl, 2mM-$MgCl_2$ and 0.5mM-$CaCl_2$); Veronal buffer/EDTA (5mM-Veronal buffer, pH7.5, containing 150mM-NaCl and 10mM-EDTA).

Protein purification

C2 and Factor B were purified from outdated human plasma by the methods of Kerr (1982*a*,*b*). C3 and C4 were prepared as described in Kerr (1980). $C\bar{1}s$ was purified by the method of Gigli *et al.* (1976). The methods of haemolytic assay were those described previously (Kerr, 1980). The concentration of protein solution was calculated from absorbance at 280nm by using the absorption coefficient $A^{1\%}_{1cm} = 12.7$ for Factor B (Curman *et al.*, 1977). The absorption coefficient for C2 was assumed to be 10.0.

Reaction of C2 and Factor B with pCMB

A 500μl volume of 1mM-[^{14}C]pCMB was added to 5ml of C2 or Factor B (0.4mg/ml) in Veronal buffer, pH8.5, and incubated at 30°C for 1h. The excess pCMB was then removed by gel filtration on Sephadex G-25 in 0.1M-NH_4HCO_3, and the proteins were assayed for haemolytic activity and specific radioactivity.

Reaction of C2 and Factor B with iodoacetamide

Incorporation of iodo[^{14}*C*]*acetamide.* C2 or Factor B (200μg of freeze-dried sample) was dissolved in 0.5ml of 0.3M-Tris/HCl buffer, pH8.2, containing

0.15mM-EDTA in the presence or in the absence of 5.25M-guanidinium chloride. Then 5μl of iodo-[^{14}C]acetamide (160mM) was added and the mixture was incubated at room temperature for 2h before being desalted on Sephadex G-25 equilibrated in 0.1M-NH_4HCO_3 for the determination of specific radioactivity.

Amino acid analysis. C2 or Factor B (500μg) was dissolved in 0.75ml of 0.3M-Tris/HCl buffer, pH8.2, containing 1.5mM-EDTA in the presence or in the absence of 5.25M-guanidinium chloride. Then 30μl of 100mM-iodoacetamide was added, and after incubation at room temperature for 2h excess reagent was removed by gel filtration. To determine the total cysteine content of C2 and Factor B, 500μg samples were dissolved in 0.75ml of 0.3M-Tris/HCl buffer, pH8.2, containing 1.5mM-EDTA, 5.25M-guanidinium chloride and 8mM-dithiothreitol. After incubation of the mixture, at room temperature for 2h, under N_2, iodoacetamide was added to a final concentration of 20mM, and after further incubation for 2h the excess reagents were removed by gel filtration. Amino acid analyses were carried out with a Durrum D500 analyser on samples hydrolysed *in vacuo* at 110°C for 24h in freshly prepared constant-boiling HCl containing 5mM-phenol and 0.5% (v/v) 2-mercaptoethanol.

Reaction of C2 and Factor B with DTNB

C2 or Factor B (400μg of freeze-dried sample) was dissolved in 1ml of 0.2M-Tris/HCl buffer, pH8.2, containing 5.25M-guanidinium chloride. Then 30μl of 10mM-DTNB was added, and the increase in absorbance at 412nm was followed spectrophotometrically. Free thiol groups were determined by using $\varepsilon = 13600 M^{-1}.cm^{-1}$ at 412nm for 5-mercapto-2-nitrobenzoic acid (Vanaman & Stark, 1970).

Results

Effect of I_2 on C2 haemolytic activity

The results of a typical experiment showing the effect of different dilutions of the stock I_2/KI solution on the haemolytic activity of human C2 in 0.1M-sodium phosphate buffer, pH6.0, are given in Fig. 1(*a*). When I_2-treated C2 was assayed by the conventional method by incubation with EAC14 cells for 5min (the $t_{max.}$) before addition of C3–C9 in 0.1M-EDTA, a marked enhancement of haemolytic activity was observed in those samples treated with 1–10μM-I_2 whereas higher I_2 concentrations were inhibitory. When the same C2 samples were assayed by incubation with EAC14 cells for 60min before the addition of C3–C9 no haemolytic activity was detected in the untreated C2 sample, owing to decay of the unstable EAC142 intermediate. C2 preincubated with 1–10μM-I_2 still showed markedly enhanced haemolytic activity. Because of the poor solubility of I_2 under these conditions the apparent concentration that produced the highest activity varied slightly from experiment to experiment, as did the enhancement of activity, but under optimal conditions a 16–20-fold enhancement of activity was observed. In contrast, when dilutions of the stock I_2/KI solution were made in 0.1M-sodium phosphate buffer, pH6.0,

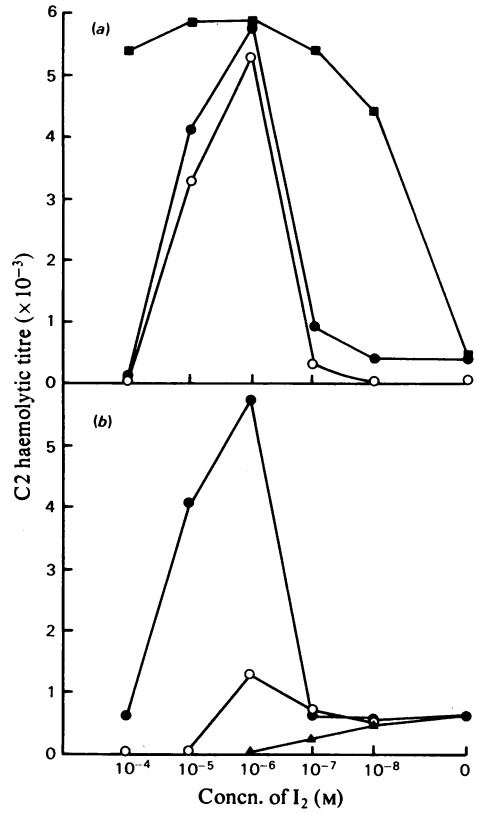


Fig. 1. *Effect of I_2 treatment on C2 haemolytic activity*
(*a*) Purified C2 (5μg/ml; 50nM) in 0.1M-sodium phosphate buffer, pH6.0 (● and ○), or 0.1M-sodium phosphate buffer, pH6.0, containing 50mM-KI (■) was incubated with I_2/KI solutions of different concentrations for 15min at 4°C, and then haemolytic activity was determined by incubation with EAC14 cells for 5min (● and ■) or 60min (○) before addition of guinea-pig C3–C9 in EDTA as described in the Materials and methods section. (*b*) Purified C2 (5μg/ml) was incubated with I_2/KI solutions of different concentrations for 15min at 4°C in 0.1M-sodium phosphate buffer, pH6.0 (●), Veronal buffer, pH7.5 (○), or Veronal buffer, pH8.5 (▲), before assay for haemolytic acitivity by incubation with EAC14 cells for 5min followed by addition of guinea-pig C3–C9 in EDTA as described in the Materials and methods section.

containing 50mM-KI this 16–20-fold enhancement of C2 activity was observed over a wide range of I_2 concentrations down to that where C2 and I_2 were equimolar.

In agreement with the results obtained by Polley & Muller-Eberhard (1967), we have also found a marked buffer- and pH-dependence of the effect of I_2 on C2. In the presence of excess I_2 a maximum enhancement of haemolytic activity was observed in 0.1 M-phosphate buffer at pH 6.0–7.5. Much less enhancement was observed in Veronal buffer, pH 7.5, and treatment in Veronal buffer, pH 8.5, inactivated C2 (Fig. 1*b*). If C2 was treated with I_2 under optimal conditions at pH 6.0 and then the pH raised to 8.5 either in the presence of I_2 or after its removal by gel filtration over Sephadex G-25 in 0.1 M-sodium phosphate buffer, pH 6.0, then complete inactivation of C2 occurred. After inactivation of I_2-treated C2 at pH 8.5, lowering of the pH to 6·0 did not restore haemolytic activity, nor did a second treatment with I_2 at pH 6.0.

Three species of C2 can therefore be present in samples treated with I_2. Untreated C2, which forms a labile C3 convertase not detectable on assay with EAC14 cells after 1 h incubation, I_2-treated C2, which forms a stable C3 convertase that is detectable on assay with EAC14 cells after 1 h incubation, and a third, haemolytically inactive, species. The percentage of each species depends on the I_2 and I^- concentration and the composition and pH of the buffer used. In addition to the inactivation of I_2-treated C2 at higher pH, we have also observed complete inactivation of I_2-treated C2 by addition of NaN_3 (3mM) or KCN (3mM) (Parkes *et al.*, 1983).

Effect of I_2 treatment on C2 cleavage by $C\bar{1}s$

C2 treated with I_2 under optimal conditions (10 μM-I_2, pH 6.0) and then gel-filtered into 5mM-Veronal buffer, pH 7.5, retained 30–50% of the enhanced haemolytic activity (Table 1). The haemolytic titre was the same on incubation with EAC14 cells at either 5 or 60 min, confirming that most of the active C2 had been converted into the species that is able to form stable C3 convertase. When this C2 was incubated with purified $C\bar{1}s$, there was loss of C2 haemolytic activity, owing to C2 cleavage. After complete loss of haemolytic activity, approx. 30% of the C2 had been cleaved to C2a and C2b, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The products were identical with those produced on cleavage of untreated C2 by $C\bar{1}s$ when tested by gel electrophoresis or by *N*-terminal analysis, which showed lysine and alanine. In a series of experiments, correlation was observed between haemolytic activity and the amount of C2 that would be cleaved by $C\bar{1}s$. The rate of cleavage of I_2-treated C2 by $C\bar{1}s$ at three concentrations was identical with that of native C2. $C\bar{1}s$-treated C2 that had been made haemolytically inactive by exposure to high pH, excess I_2 or NaN_3 (3mM) was not cleaved by $C\bar{1}s$ (Table 1).

Table 1. *Effects of I_2 treatment on the cleavage of C2 by $C\bar{1}s$*

C2 (200 μg in 2 ml of 0.1 M-sodium phosphate buffer, pH 6.0) was treated with 10 μM-I_2/50mM-KI or with 50mM-KI alone and then gel-filtered into Veronal buffer, pH 7.5. The C2 pools were then assayed for haemolytic activity: untreated C2 by incubation with EAC14 cells for 5 min, I_2-treated C2 by incubation with EAC14 cells for 60 min. On incubation with different amounts of $C\bar{1}s$, the rates of cleavage of C2 were estimated from the loss of haemolytic activity over 30 min. On complete loss of haemolytic activity the C2 samples were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the percentage cleavage of C2 was determined densitometrically (Kerr, 1980). N.D., Not determined.

Sample	10^{-4} × C2 titre	$C\bar{1}s$ added (μg)	Apparent first-order rate constant for cleavage of C2 (min^{-1})	C2 cleaved on loss of all haemolytic activity (%)
Untreated	1.3	0.2	0.01	95
		1.0	0.05	N.D.
		5.0	0.27	N.D.
	1.1	1.0	0.05	90
	1.5	1.0	0.06	100
I_2-treated C2	8.0	0.2	0.01	35
		1.0	0.05	35
		5.0	0.25	
	6.0	1.0	0.05	20
	4.7	1.0	0.05	15

Formation of stable fluid-phase C3 convertase from I_2-treated C2

To investigate the effect of I_2 treatment of C2 on the ability to form fluid-phase C3 convertase, C2 (25μg) in 250μl of 0.1M-sodium phosphate buffer, pH6.0, was treated with different concentrations of I_2 for 15min at 4°C and then gel-filtered into Veronal buffer, pH7.5. C4b (50μg) and $C\bar{1}s$ (500ng) in the same buffer were added, and samples were removed after 5min and 60min and assayed for C3 convertase activity by incubation with C3 (50μg in 50μl of Veronal buffer/EDTA). The cleavage of C2 at 60min was also determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The results (Fig. 2) showed correlation between the ability of I_2-treated C2 to form stable fluid-phase convertase, the cleavage of the C2 and the haemolytic activity (ability to form cell-bound C3 convertase) shown in Fig. 1.

Under these assay conditions the fluid-phase C3 convertase showed apparent first-order kinetics for C3 cleavage over at least 5 half-lives (Fig. 3*a*). We were therefore, for convenience, able to define 1 unit of C3 convertase activity as that amount of enzyme that causes a 50% loss of C3 haemolytic activity in 1min. By using this semi-quantitative assay optimal conditions for the formation of fluid-phase C3 convertase were investigated in more detail.

C2 was treated with 1μM-I_2 at pH6.0 and then gel-filtered on Sephadex G-25 into 0.1M-sodium phosphate buffer, pH6.0, or 5mM-Veronal buffer, pH7.5 or 8.5. C3 convertase was then made by incubation with equimolar C4b and catalytic amounts of $C\bar{1}s$. Optimum C3 convertase activity was obtained on incubation of I_2-treated C2 with C4b and $C\bar{1}s$ at pH7.5 (Table 2). At higher pH or in the presence of NaN_3 the loss of C2 haemolytic activity correlated with the loss of ability to form fluid-phase C3 convertase. However, C3 convertase that had been assembled at pH7.5 was not inactivated by subsequently increasing the pH to 8.5 or by the addition of NaN_3 (3mM). Although I_2-treated C2 at pH6.0 shows greatest haemolytic activity (the C2 haemolytic activity assay is carried out after dilution in Veronal buffer at pH7.4), no fluid-phase convertase activity can be generated by incubation of I_2-treated C2, C4b and $C\bar{1}s$ at pH6.0 because of the lack of interaction of C2 and C4b under these conditions.

Specific activity of C3 convertase from I_2-treated and untreated C2

Because of the necessary compromise in the use of pH for the two processes, even under optimal conditions only a part of the C2 could be made into C3 convertase. However, C3 convertase formed under these conditions was stable at 4°C over several days and could be purified by gel filtration and concentrated by ultrafiltration without loss of activity (Fig. 3*a*). The amount of C2a associated with C3 convertase could be accurately determined from the small amount of radioactivity incorporated into C2 when $^{125}I_2$ was used during the I_2 treatment (the incorporation was 0.05 and 0.04mol of I_2/mol of C2 in two experiments).

It has previously been shown (Kerr, 1980) that, for short periods, apparently stable C3 convertase could be produced from untreated C2, C4b and $C\bar{1}s$ at 22°C in the presence of excess substrate. The kinetics suggested the C3 convertase to be a

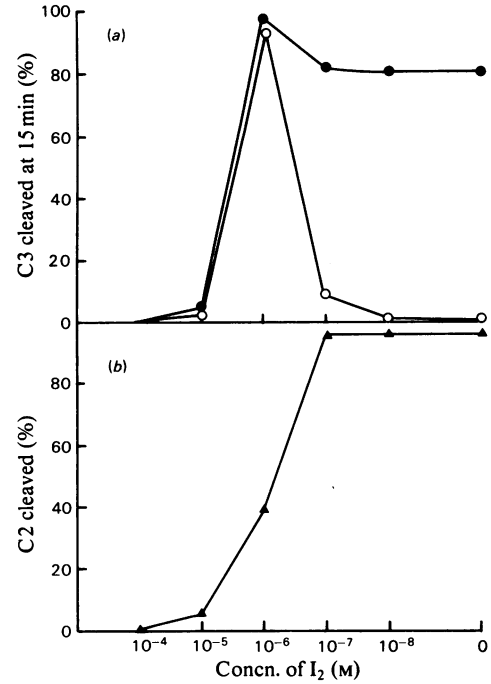


Fig. 2. *Effect of I_2 treatment of C2 on the generation of fluid-phase C3 convertase*

C2 (25μg in 250μl of 0.1M-sodium phosphate buffer, pH6.0) was incubated with I_2/KI solutions of different concentrations for 15min at 4°C. The samples were then gel-filtered into Veronal buffer, pH7.5, and then C4 (50μg) and $C\bar{1}s$ (0.5μg) in the same buffer were added. Samples (5μl) were removed after 5min (●) and 60min (○) and assayed for C3 convertase activity by incubation with C3 (50μg) in 50μl of Veronal buffer/EDTA for 15min at 37°C. Samples were then subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and C3 cleavage was quantified by densitometric scanning (Kerr, 1980). Samples from the first incubation were also removed at 60min for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and the cleavage of C2 was monitored by densitometric scanning (▲).

Table 2. *Effects of I_2 treatment of C2 on haemolytic activity and the generation of fluid-phase C3 convertase*

C2 samples (100 μg in 1 ml of 0.1 M-sodium phosphate buffer, pH 6.0) were treated with 10 μM-I_2/50 mM-KI for 10 min at 4°C. The samples were then gel-filtered in columns of Sephadex G-25 equilibrated in 0.1 M-sodium phosphate buffer, pH 6.0, or 5 mM-Veronal buffers, pH 7.5 and 8.5. Samples were assayed for C2 haemolytic activity. C3 convertase was made by incubation of I_2-treated C2 (50 μg) with C4 (100 μg) and $\overline{C1s}$ (1 μg) for 30 min at 37°C. C3 convertase was assayed by using standard conditions. N.D., Not determined.

Sample	C2 haemolytic units	Fluid-phase C3 convertase units
C2	42000	0
I_2-treated C2	740000	N.D.
After gel filtration into 0.1 M-sodium phosphate buffer, pH 6.0	700000	N.D.
After gel filtration into 5 mM-Veronal buffer, pH 7.5	245000	12.0
After gel filtration into 5 mM-Veronal buffer, pH 8.5	10300	0.6
After gel filtration into 5 mM-Veronal buffer, pH 7.5, containing 3 mM-NaN_3	100	0
After gel filtration into 5 mM-Veronal buffer, pH 7.5; NaN_3 (3 mM) added 15 min after C4 and $\overline{C1s}$	N.D.	11.9

1:1 complex of C4b and cleaved C2. Under these conditions it is therefore possible to compare the specific C3 convertase activity of the enzyme formed from untreated C2 with the stable C3 convertase formed from I_2-treated C2. The results (Fig. 3*b*) show that C3 convertases formed from the two C2 species have the same specific activity. The effect of I_2 treatment on C2 is therefore to increase the stability of the otherwise labile C3 convertase. I_2 treatment will therefore increase the amount of C3 convertase formed from a mixture of C4b, C2 and $\overline{C1s}$; it does not increase the activity of the C3 convertase enzyme that is formed.

Effect of I_2 treatment of C2 on its interaction with C4b

The interaction of C2 with C4b has previously been studied by gel filtration of mixtures of the two proteins on Sephadex G-200 and identification

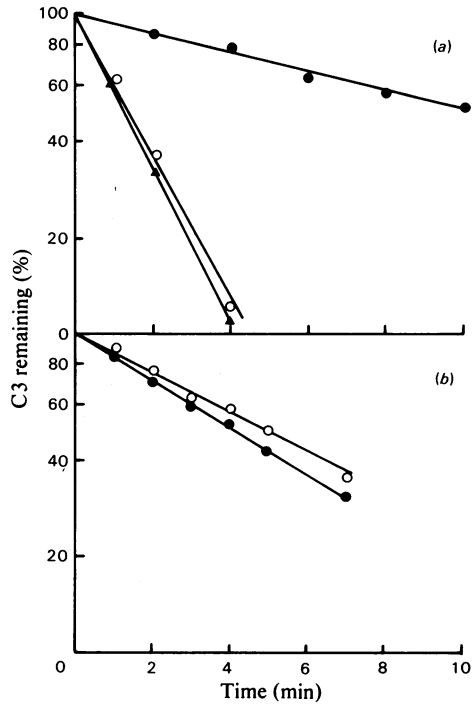


Fig. 3. *Assay of fluid-phase C3 convertase prepared from I_2-treated C2*

(*a*) C4b (45 μg), I_2-treated C2 (25 μg) and $\overline{C1s}$ (200 μg) in 0.5 ml of Veronal buffer, pH 7.5, were incubated at 37°C for 30 min. After removal of 10 μl for C3 convertase assay (▲), the mixture was chromatographed on a Sephadex G-200 column (100 cm × 0.6 cm) in the same buffer. The active fractions were pooled (5 ml) and then concentrated to 0.5 ml by ultrafiltration. C3 convertase activity before (●) and after (○) concentration was determined by incubation of 10 μl samples with 10 μl of C3 (1 mg/ml) in Veronal buffer/EDTA at 37°C. At different times samples (1 μl) were removed and added to 1 ml of ice-cold 'gelatin/Veronal-/Ca^{++}/Mg^{++}' (Nelson *et al.*, 1966) before assay for C3 haemolytic activity. (*b*) A sample (50 μl) of C3 convertase after concentration was assayed for activity by incubation with 50 μl of C3 (1 mg/ml) in Veronal buffer, pH 7.5, at 22°C (●). This C3 convertase contained C2a from 0.1 μg of C2. The C3 convertase activity was compared with that detected on incubation of untreated C2 (0.1 μg), C4b (0.2 μg), $\overline{C1s}$ (0.1 μg) and C3 (50 μg) in the same buffer at 22°C (○).

of the composition of the fractions obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Kerr, 1980).

When an equimolar mixture of C2 and C4b was chromatographed on Sephadex G-200 in Veronal buffer, pH 8.5, both proteins were eluted in the same fractions, suggesting complex-formation. When the C2 and C4b were run separately or when

a mixture of the two was run on the same column equilibrated in 5mM-Veronal buffer, pH8.5, containing 10mM-EDTA, C2 was eluted after C4b, as would be expected from their relative molecular masses. Both proteins were eluted later than the C4b–C2 complex. In all these respects C2 that had been treated with 10μM-I_2 at pH6.0 and desalted into Veronal buffer, pH7.5, behaved the same as untreated C2. I_2 treatment does not apparently markedly increase the affinity of C2 for C4b.

When C4b, I_2-treated C2 and $C\bar{1}s$ were incubated at pH7.5 and then the mixture was subjected to gel filtration on Sephadex G-200 in Veronal buffer/EDTA, C3 convertase activity could be recovered and C4b and C2a could be identified in these active fractions. However, if I_2-treated C2 in Veronal buffer, pH7.5, was cleaved by $C\bar{1}s$ and then added to C4b, no C3 convertase was formed and no evidence for interaction between C4b and C2a could be detected by gel filtration. The enhanced stability of the C4b–C2 complex requires the cleavage of I_2-treated C2 in the presence of C4b. The stable C4b–C2a complex (C3 convertase) formed under these conditions is no longer sensitive to inactivation by increasing pH to 8.5 or to the effect of azide (Table 2).

Reaction of C2 with thiol-blocking reagents

To explain in more detail the unique reactivity of the free thiol group in C2 to I_2 treatment, we have studied the effect of other thiol-blocking reagents on C2. Incubation of C2 with pCMB has previously been shown to cause complete loss of haemolytic activity (Parkes *et al.*, 1983). When pCMB-treated C2 (2.5μg) was incubated with C4 (5.0μg) and $C\bar{1}s$ (50μg) in 100μl of Veronal buffer, pH7.5, no C3 convertase activity was generated. However, the pCMB–C2 is cleaved by $C\bar{1}s$ at the same rate as untreated C2 (results not shown).

In contrast with I_2 and pCMB, iodoacetamide did not react appreciably with C2 in the absence of denaturing agents (Table 3). There was little effect on haemolytic activity when freshly recrystallized iodoacetamide was used. However, in the presence of 5M-guanidinium chloride a single thiol group was reactive. The reaction of iodoacetamide was followed both by incorporation of ^{14}C label or from the formation of carboxamidocysteine detected by amino acid hydrolysis of the product. The detection of 29 thiol groups in a totally reduced C2 sample is consistent with the value obtained earlier by performic oxidation (Kerr & Porter, 1979). DTNB was also only slowly reactive with C2 in the absence of denaturing agents, and in the presence of 5M-guanidinium chloride a single thiol group was titrated.

When Factor B was titrated with iodoacetamide or DTNB, a single free thiol group was detected that required denaturation of the protein before reaction. As has been shown previously (Parkes *et al.*, 1983), the group is reactive to I_2 and pCMB without denaturation. However, neither I_2 nor pCMB had significant effect on the haemolytic activity of Factor B, and pCMB-treated Factor B was cleaved by Factor D in the presence of C3b at the same rate as was untreated Factor B and formed fluid-phase C3 convertase.

Discussion

We have now demonstrated with a variety of reagents the presence of a single free thiol group in both C2 and Factor B. This confirms our results identifying a single thiol group by titration with [^{14}C]pCMB (Parkes *et al.*, 1983), in contrast with the results obtained by Lesavre *et al.* (1979) and Muller-Eberhard (1975), which suggested the presence of two thiol groups in Factor B and C2.

Table 3. *Titration of C2 and Factor B with thiol-blocking reagents under different conditions*
Data for pCMB are the averages for three determinations. N.D., Not determined.

Titrant	Thiol groups titrated (mol/mol of protein) C2	Factor B
pCMB	1.13	1.13
Iodoacetamide (proteins not denatured)	0.17*	0.16*
	0.13†	0.13†
Iodoacetamide (proteins denatured)	1.20*	1.19*
	1.40*	1.22*
	1.14†	N.D.
Iodoacetamide (proteins reduced and denatured)	29.0	25.0
DTNB (protein not denatured)	Reacts slowly	Reacts slowly
DTNB (protein denatured)	0.91	0.95

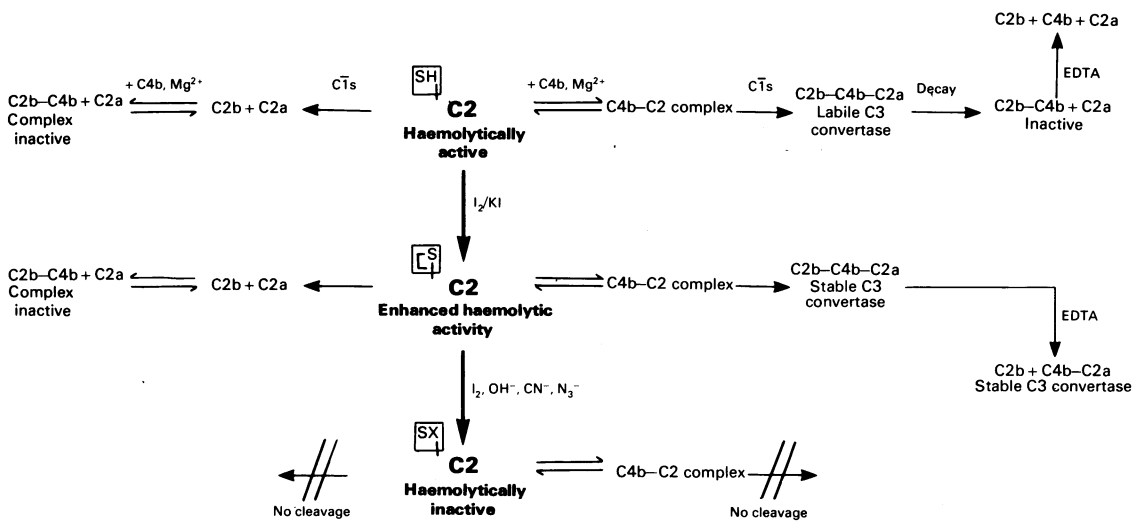
* Data from incorporation of iodo[^{14}C]acetamide.
† Data, from amino acid analysis, of carboxymethyl-cysteine.

Our present results suggest that the residue in both proteins is in a rather unusual environment. The thiol group is accessible to I_2 and to pCMB in the native proteins, but it is inaccessible to iodoacetamide and to DTNB without denaturation. The similar accessibility of the thiol group in C2 and in Factor B is interesting in view of our previous results showing the thiol groups to be at different positions in the amino acid sequences of the two fragments C2a and Bb. Although the fragments C2 and Bb show extensive sequence homology in this part of the molecule, the free thiol group in C2a is at position 18, which is glycine in Bb; the free thiol group of Bb is at residue 33. We have previously suggested the marked effect of I_2 on C2 haemolytic activity but lack of effect on Factor B activity might reflect a difference in the environment of the free thiol group in the two proteins. The similarity of reaction of these other thiol-blocking reagents suggests, however, that, although separated in the linear sequence, these thiol groups might occupy a similar position in the tertiary structure of the two proteins. Although our results still do not allow us to determine the actual mechanism of reaction of I_2 with this thiol group, we have previously suggested that I_2 may react to form a sulphenyl iodide, which on subsequent nucleophilic attack by another amino acid side chain displacing the iodide would form an intramolecular covalent bond. It may be the ability to undergo the second reaction that distinguishes the thiol group in C2 from that in Factor B.

Modification of the thiol group of C2 by I_2 has now been shown to occur in a two-stage process. In the first stage the group is modified without changing the susceptibility of the C2 molecule to $C\bar{1}s$ to a form with enhanced haemolytic activity. In a further reaction the C2 is inactivated by a reaction that renders the molecule uncleavable by $C\bar{1}s$. This further reaction occurs more readily in the presence of excess I_2 and at higher pH. A similar modification is brought about by azide or cyanide. By careful control of the conditions of the I_2 treatment it is, however, possible to limit the extent of this second reaction, and further discussion below is limited to the initial reaction, which potentiates haemolytic activity.

This initial reaction with I_2 has no effect on the rate of cleavage of C2 by $C\bar{1}s$ or on the size of the products C2a and C2b. The effect of I_2 treatment of C2 is to stabilize the subsequent C3 convertase formed on interaction with C4b and $C\bar{1}s$ by increasing the affinity of C2a and C4b. The convertase formed with I_2-treated C2 cleaves C3 at the same rate as does the convertase formed from untreated C2. It is apparently only C2a that is formed by cleavage of C2 bound to C4b that is modified in this way, since a mixture of C2a and C2b produced by cleavage of I_2-treated C2 by $C\bar{1}s$ when added to C4b does not form C3 convertase, and no C2a–C4b complex can be detected by gel filtration. I_2 treatment of C2 does not apparently greatly increase the affinity of the intact C2 molecule for C4b.

Since the stabilized C3 convertase formed from C4b, I_2-treated C2 and $C\bar{1}s$ is no longer sensitive to



Scheme 1. *Interactions of C2 and I_2-treated C2 with C4b and $C\bar{1}s$*

Details are discussed in the text. The proposed scheme emphasizes the role of C2b in the assembly, but not the activity, of the C3 convertase. I_2 stabilizes the binding of C2a to C4b only when the C2a is generated from C2 already bound to C4b.

inactivation by high pH or azide, it seems likely that the thiol group modified by I_2 treatment is shielded from the aqueous environment. It is possible, however less likely, that the group is still reactive but that reaction does not affect the stability of the complex. The close contact between this part of C2a and C4b in the convertase is further suggested by the fact that modification of the group by pCMB inactivates C2 although it does not affect the cleavage of C2 by $C\bar{1}s$. The effects of I_2 on C2 and on the interaction of C2 with C4b and $C\bar{1}s$ are summarized in Scheme 1. Taken together, the data show clearly the importance of formation of the C4b–C2 complex before cleavage of C2 in the assembly of C3 convertase, even though C2 can be cleaved by $C\bar{1}s$ in the absence of C4b. There are therefore similarities to and differences from the alternative-pathway C3 convertase where complex-formation between C3b and Factor B is a necessary pre-requisite both for the cleavage of Factor B by Factor D and for the formation of convertase.

The results confirm our earlier observations that the amount of C3 convertase formed in the fluid phase under normal conditions is very low and that the observed decay rate of the convertase is a mixture of the true decay rate of the enzyme and the rate at which the enzyme is assembled (Kerr, 1980). Recent studies on the formation of C3 convertase from human C4b and guinea-pig C2 (Kerr & Gagnon, 1982), which is more active than the enzyme formed from human C4b and C2, also suggested that this increased activity was due to greater stability of the enzyme, which is the result of higher affinity of guinea-pig C2a for human C4b.

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