HUMAN COMPLEMENT SUBCOMPONENT C2: PURIFICATION AND PROTEOLYTIC CLEAVAGE IN FLUID PHASE BY $C\bar{1}s$, $C\bar{1}r_2$–$C\bar{1}s_2$ AND $C\bar{1}$

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1. Introduction

Activation of the classical pathway of complement proceeds sequentially through activation of C1 and assembly of the C3 and C5 convertases [1]. The C3 convertase is a complex of equimolar amounts of C4b and C2a formed upon limited proteolysis of C4 and C2 by $C\bar{1}$. C2 cleavage by $C\bar{1}$ may be a limiting step in the activation of complement as it occurs when C2 is on or in close proximity to C4b bound to an acceptor [1].

Little is known about the detailed mechanism of $C\bar{1}$–C2 interaction. $C\bar{1}$ active site is located in subcomponent $C\bar{1}s$, which is likely to behave differently in soluble or particulate $C\bar{1}$. The purpose of this paper is to study the cleavage of C2 by isolated $C\bar{1}s$ or by different associations reconstructed from individual subcomponents: $C\bar{1}r_2$–$C\bar{1}s_2$ and two different forms of $C\bar{1}$. Kinetic parameters for these interactions between soluble molecules are reported, as a reference for further studies on particulate components.

For this purpose C2 purification has been reinvestigated and a new protocol established: it is based on the methods in [2–6] with several improvements leading to a simplified preparation and a high yield of purified C2.

Abbreviations: SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TEA, triethanolamine; EDTA, ethylene diamine tetra-acetic acid; DFP, diisopropylphosphorofluoridate; Ig, immunoglobulin; C4bp, C4-binding protein

Nomenclature: components of complement follow that recommended by the World Health Organization (1968); a bar indicates the activated state of a component

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2. Materials and methods

Human citrated plasma was obtained from the Centre de Transfusion Sanguine (Grenoble).

DFP was from Sigma. Sheep erythrocytes were purchased from Bio-Merieux. Hemolysin and antisera to factor B, haemopexin, albumin, C3, C4, C5, IgG were from Behring. Antiserum to human serum was purchased from ICL Scientific. Antisera to human IgM, $C\bar{1}$ Inh, C1q, $C\bar{1}r$, $C\bar{1}s$, C4bp were prepared in our laboratory. Lactoperoxidase (purified grade) was purchased from Calbiochem. $Na^{125}I$ (spec. act. 2 Ci/μmol), and ^{125}I-labelled Bolton and Hunter reagent (spec. act. 2 Ci/μmol) were from the Radiochemical Centre (Amersham). Other chemicals were of analytical grade.

Proteins were estimated according to [7], using bovine serum albumin as a reference. Purified C2 was estimated from its A_{280}, assuming $E^{1\%}_{1\,cm} = 10.0$.

C2 haemolytic activity was measured as in [8] and was expressed in 50% lysis units X no. erythrocytes/ml.

SDS–PAGE and staining of proteins with Coomassie blue were done as in [9,10].

N-Terminal amino acids were identified by dansylation [11] and thin-layer chromatography [12].

Sucrose gradient ultracentrifugation was done as in [13].

C1q, $C\bar{1}r$ and $C\bar{1}s$ were purified as in [14]. Proenzymic C1r was purified as in [15] and proenzymic

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C1s as in [16]. C1 subcomponents were estimated from their A_{280}, using respectively $E^{1\%}_{1\,cm}$ = 11.5 for $\overline{C1s}$ and C1s [17], 9.5 for $\overline{C1r}$ and C1r [17] and 6.85 for C1q [18].

C4 was purified as in [19] and estimated from its A_{280}, using $E^{1\%}_{1\,cm}$ = 10.0 [20]. C4b-like C4 was prepared by incubating purified C4 in 6 M urea, 100 mM NaCl, 100 mM $NaHCO_3$ (pH 8.1) for 15 min at 0°C, followed by overnight dialysis against the same buffer without urea. C4b was obtained by incubation of C4 and $\overline{C1s}$ for 60 min at 37°C at a 50:1 (w/w) ratio, in 150 mM NaCl, 5 mM EDTA, 20 mM phosphate (pH 7.4), followed by treatment with 5 mM DFP for 30 min at 37°C. C4b–Sepharose was prepared by coupling 50 mg C4b to 30 ml CNBr-activated Sepharose 4B [21]. The same protocol was used for the preparation of C4–Sepharose and C4b-like C4–Sepharose.

^{125}I-Labelling of proteins followed [22] or was by lactoperoxidase catalysis as in [20]. The average iodine binding was between 0.04–0.06 mol ^{125}I/mol C2.

C2 cleavage by $\overline{C1s}$ was measured at 37°C using various dilutions of ^{125}I-labelled C2 in a buffer containing 5 mM TEA–HCl, 145 mM NaCl and 1 mg egg albumin/ml (pH 7.4). The amount of $\overline{C1s}$ was chosen in order to cleave 20–40% of C2 in 10 min. At regular time intervals, samples were removed, immediately reduced with 50 mM dithiothreitol for 5 min at 100°C, alkylated by 140 mM iodoacetamide for 20 min at 37°C, then submitted to SDS–PAGE. Gels were cut into 1 mm slices and counted for radioactivity in a MR 480 Kontron γ-counter.

3. Results

3.1. *Purification of C2*

3.1.1. Treatment of plasma with inhibitors

Frozen, citrated plasma was thawed and incubated for 30 min at 20°C with 5 mM DFP, 2 mM EDTA and 0.01% (v/v) 2-mercaptoethanol, then adjusted to pH 6.0 with acetic acid. All subsequent operations were done at 4°C.

3.1.2. $(NH_4)_2SO_4$ precipitation

$(NH_4)_2SO_4$ was added to the plasma to 2.2 M final conc.; the mixture was stirred for 60 min and centrifuged for 30 min at 23 000 × *g*. A second addition of $(NH_4)_2SO_4$ was made to the supernatant to 3.3 M final conc. After 60 min stirring the precipitate was collected by centrifugation for 30 min at 23 000 × *g*. The pellet was then redissolved in 100 ml 50 mM sodium acetate (pH 6.0) containing 2 mM EDTA, 5 mM DFP and 0.01% (v/v) 2-mercaptoethanol, and dialysed overnight against the same buffer.

3.1.3. Chromatography on CM52-cellulose

After dialysis, the fraction was diluted to a conductivity of 2.85 mS (at 4°C) and applied to a column of CM52-cellulose (2.75 × 42 cm) equilibrated in 50 mM sodium acetate buffer (pH 6.0) containing 2 mM EDTA and 0.01% (v/v) 2-mercaptoethanol. The column was washed with this buffer until the A_{280} of the eluate was <0.05. Elution was carried out with a linear NaCl gradient (0–150 mM) in the same buffer (total vol. 1400 ml). C2 was detected in the eluate by haemolytic assay and the active fractions were pooled in the presence of 5 mM DFP. After concentration by ultrafiltration on PM 10 (Amicon), the pH was raised to 8.5 with Tris-base 1 M and the pool was dialysed against 125 mM NaCl, 2 mM $MgCl_2$, 20 mM Tris–HCl (pH 8.5).

3.1.4. Affinity chromatography on C4b–Sepharose

The dialysed fraction was applied to a column of C4b–Sepharose equilibrated in the dialysis buffer containing 0.01% (v/v) 2-mercaptoethanol. The column was washed with 300 ml of this buffer. C2 was eluted with 150 mM NaCl, 10 mM EDTA and 50 mM sodium acetate (pH 6.5). Purified C2 was pooled and concentrated by ultrafiltration on PM 10 in the presence of 5 mM DFP.

A typical purification is illustrated in table 1. The final yield of C2 is ~40% with a purification factor of 5000.

Purified C2 appears as a single polypeptide chain of app. M_r 95 000 on SDS–PAGE under reducing or non-reducing conditions (fig.1). The control by double immunodiffusion in agarose was negative with antisera to factor B, haemopexin, IgG, IgM, albumin, C3, C4, C5, C4-binding protein, $\overline{C1}$ Inh, C1q, $\overline{C1r}$ and $\overline{C1s}$; using antiserum to whole human serum only one precipitin line was detected.

3.2. *Determination of the enzymic constants for the cleavage of C2 by $\overline{C1s}$*

3.2.1. Cleavage of C2 by isolated $\overline{C1s}$

Incubation of C2 with catalytic amounts of $\overline{C1s}$ (C2:$\overline{C1s}$ = 5000:1) (w/w) resulted in cleavage of C2

Table 1
C2 purification

Fraction	Vol. (ml)	Total act. (10^{13} units)	Total protein (mg)	Spec. act. (10^{11} units/mg)	Purification factor	Yield (%)
Plasma	500	10.6	31 500.0	0.034	1.0	100.0
Ammonium sulphate precipitate[a]	585	9.8	13 455.0	0.073	2.2	92.7
CM 52 eluate[b]	44	5.5	43.6	12.700	378.0	52.2
C4b–Sepharose[b] eluate	3	4.2	2.5	168.000	5000.0	39.2

[a] Determinations made after dialysis and dilution of the extract
[b] Determinations on concentrated fractions

into two fragments, C2a and C2b of app. M_r 68 000 and 28 500 (fig.2). No other products were detected on SDS–PAGE after incubation of C2 with $C\bar{1}s$ in a 10:1 (w/w) ratio for ⩽60 min.

N-terminal amino acid analysis of $C\bar{1}s$-cleaved C2 revealed alanine (N-terminal residue of C2b) and lysine (N-terminal residue of C2a) as in [23].

C2 cleavage by $C\bar{1}s$ was followed as in section 2. The decrease of radioactivity in C2 was equal to the amount of radioactivity appearing in C2a and C2b. The initial velocity of the cleavage was calculated for each C2 concentration and the data treated according to [24].

The Lineweaver–Burk plots of the reaction in the

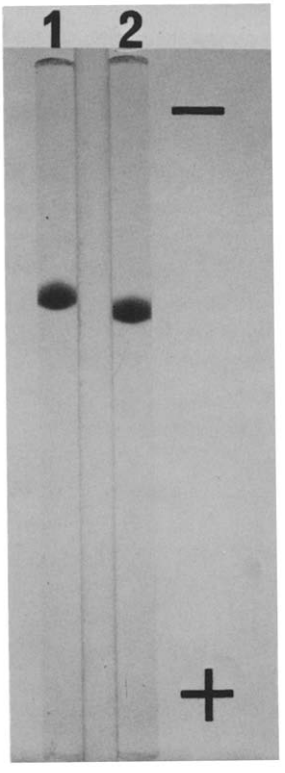

Fig.1. SDS–PAGE of purified C2. Electrophoresis was done as in section 2: 18 μg protein were applied to each gel – (1) unreduced C2; (2) reduced and alkylated C2.

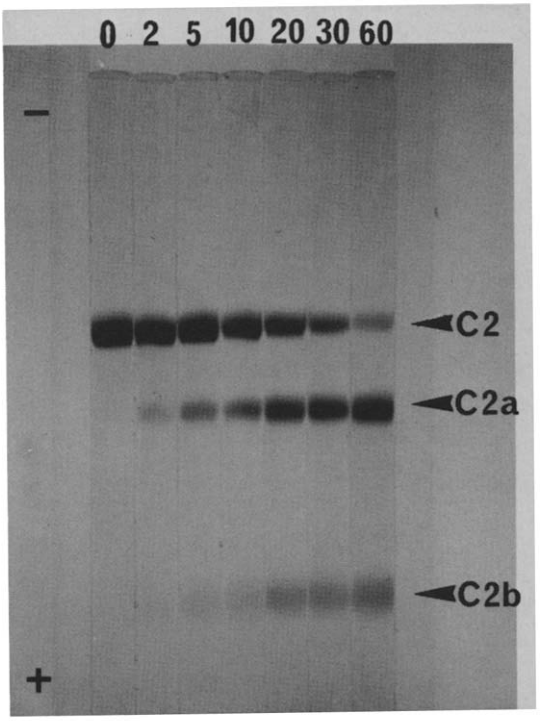

Fig.2. Kinetic analysis by SDS–PAGE of C2 cleavage by $C\bar{1}s$: 140 μg C2 in 200 μl 145 mM NaCl, 5 mM $CaCl_2$, 5 mM TEA–HCl (pH 7.4) were incubated with 0.028 μg $C\bar{1}s$ at 37°C. Aliquots (30 μl) were removed at different times, immediately reduced, alkylated and analysed by SDS–PAGE as in section 2.

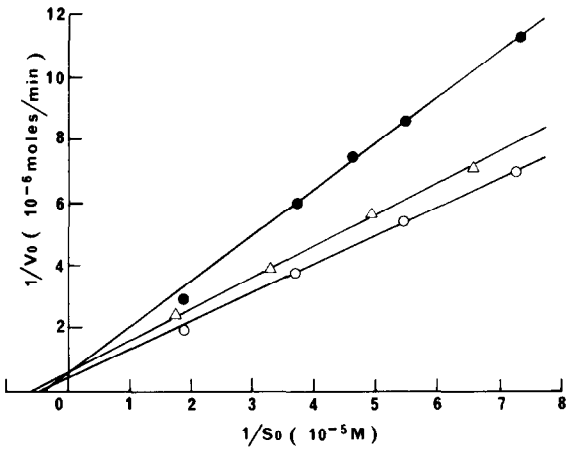

Fig.3. Lineweaver–Burk plot of C2 cleavage by $C\bar{1}s$ and by $C\bar{1}r_2$–$C\bar{1}s_2$: ^{125}I-labelled C2 at 1.5–5.9 X 10^{-6} M was proteolysed either by $C\bar{1}s$ (1.95 X 10^{-9} M) in the presence of 5 mM EDTA (•), 5 mM $CaCl_2$ (○), or by $C\bar{1}r_2$–$C\bar{1}s_2$ ($C\bar{1}r$:$C\bar{1}s$ = 1:1) (w/w) (△). S_0 and V_0 values were obtained as in [24].

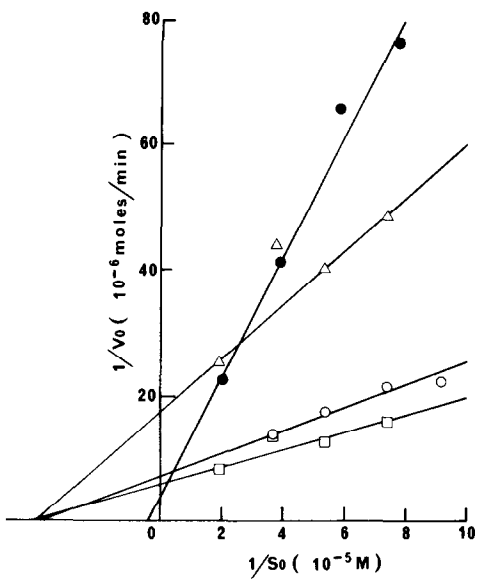

Fig.4. Lineweaver–Burk plot of C2 cleavage by $C\bar{1}$: ^{125}I-labelled C2 at 1.1–5.2 X 10^{-6} M was cleaved by $C\bar{1}s$ (1.95 X 10^{-9} M) in 'hybrid $C\bar{1}$' (•) of by $C\bar{1}s$ in $C\bar{1}$ reconstituted from C1q, C1r, C1s and subsequently activated; $C\bar{1}s$ was: 9.1 X 10^{-9} M (△); 13.6 X 10^{-9} M (○); 18.2 X 10^{-9} M (□). S_0 and V_0 values were obtained as in [24].

presence of 5 mM EDTA or 5 mM $CaCl_2$ are depicted in fig.3 and the kinetic constants of the reactions reported in table 2. They are not significantly influenced by the dimerisation of $C\bar{1}s$ induced by calcium.

3.2.2. Cleavage of C2 by $C\bar{1}s$ in the $C\bar{1}r_2$–$C\bar{1}s_2$ association

The $C\bar{1}r_2$–$C\bar{1}s_2$ association was obtained by incubation of $C\bar{1}r$ and $C\bar{1}s$ in a 1:1 or 2:1 (w/w) ratio for 30 min at 30°C in the presence of 5 mM $CaCl_2$. Control by sucrose gradient ultracentrifugation showed that in both cases $C\bar{1}s$ was entirely incorporated in a $C\bar{1}r_2$–$C\bar{1}s_2$ tetrameric association, as in [25]. The association of $C\bar{1}r$ with $C\bar{1}s$ induces a slight decrease of K_m and V_m of the reaction (fig.3, table 2). Excess of $C\bar{1}r$ was without effect on these parameters.

3.2.3. Cleavage of C2 by $C\bar{1}s$ in $C\bar{1}$ reassembled from C1q, proenzymic C1r and activated $C\bar{1}s$

'Hybrid $C\bar{1}$' [26] was reconstructed in the presence of 5 mM $CaCl_2$ from a mixture of C1q, proenzymic C1r and activated $C\bar{1}s$ in a 2.5:1:1 (by wt) ratio. Control by ultracentrifugation on sucrose gradient showed a major peak at 15.2 S corresponding to $C\bar{1}$ and containing (~90% of the total $C\bar{1}s$ enzymatic activity. No significant difference in the K_m of the reaction, and a 5–6-fold reduction of the V_m was shown in comparison with results reported for isolated $C\bar{1}s$ (fig.4, table 2).

Table 2
Catalytic constants of the cleavage of C2 by $C\bar{1}s$

$C\bar{1}s$ form	[$C\bar{1}s$] (10^{-9} M)	K_m (10^{-6} M)	V_m (10^{-7} mol/min)	Mol C2 cleaved . mol $C\bar{1}s^{-1}$. min^{-1}
$C\bar{1}s$ (5 mM EDTA)	1.95	34.1[a]	21.1[a]	1082[a]
$C\bar{1}s$ (5 mM $CaCl_2$)	1.95	19.7[a]	19.7[a]	1013[a]
$C\bar{1}r_2$–$C\bar{1}s_2$ (5 mM $CaCl)_2$	1.95	15.0[a]	15.4[a]	747[a]
'Hybrid' $C\bar{1}$[b] (5 mM $CaCl_2$)	1.95	23.8	3.1	160
$C\bar{1}$[c] (5 mM $CaCl_2$)	9.1	2.5	0.6	6.5
	13.6	2.7	1.4	10.5
	18.2	2.8	2.0	11.0

[a] Mean values
[b] Reassembled from C1q, proenzymic C1r and activated C1s
[c] Reassembled for C1q, proenzymic C1r and proenzymic C1s, then activated

3.2.4. Cleavage of C2 by $C\bar{1}s$ in $C\bar{1}$ reassembled from C1q, C1r and C1s and subsequently activated

C1 was reconstructed in the presence of 5 mM $CaCl_2$ from a mixture of C1q, proenzymic C1r and proenzymic C1s in a 2.5:1:1 (by wt) ratio, and subsequently activated by incubation for 30 min at 37°C. Control by sucrose gradient ultracentrifugation showed that ~50% of the $C\bar{1}s$ activity was in the 15.2 S peak of $C\bar{1}$. Fig.4 and table 2 indicate a significant decrease of the K_m of the proteolytic reaction (~1:10 of the K_m of 'hybrid $C\bar{1}$'); also no. mol C2 cleaved . mol $C\bar{1}s^{-1}$ min^{-1} is, respectively, 100- and 16-times less than with isolated $C\bar{1}s$ and 'hybrid $C\bar{1}$'.

4. Discussion

This protocol for C2 purification is characterized by a high yield (40% from plasma) and the absence of contaminants (one precipitin line with antiserum to whole human serum); no cleavage of C2 was observed upon incubation for 1 h at 37°C. Purified C2 was very stable and could be stored at 0°C for several months without detectable loss of activity; in contrast freezing and thawing led to significant drop in C2 haemolytic activity.

Particular attention was given to the affinity chromatography step in the purification, due to a specific (Mg^{2+}-dependent) and a non-specific interaction between C2 and C4b–Sepharose; a moderate ionic strength was chosen in order to get rid of a maximum of contaminants perfusing through the column. C4b-like C4 and C4 bound to Sepharose were less efficient than C4b for C2 binding.

Kinetic parameters were established with ^{125}I-labelled C2 prepared with the Bolton and Hunter reagent, as lactoperoxidase-catalyzed iodination of C2 impaired its cleavage by $C\bar{1}s$ and its haemolytic activity. Inocuity of the iodination was established in parallel experiments using unlabelled C2.

The estimate of V_m and K_m for the proteolysis of C2 by $C\bar{1}s$ shows that the calcium-dependent dimerisation of $C\bar{1}s$ does not significantly alter these parameters. When $C\bar{1}s$ is incorporated in a calcium-dependent $C\bar{1}r_2$–$C\bar{1}s_2$ tetrameric association the turnover number of C2 is moderately reduced. The most evident effect is observed when C1s is associated with C1r and C1q in C1 before activation, with a net decrease of the K_m and of the turnover number of C2. When activated $C\bar{1}s$ is incorporated in 'hybrid $C\bar{1}$', together with C1q and proenzymic C1r, only a decrease of the turnover number of C2 is found. These results show that maximum affinity for C2 is exhibited by $C\bar{1}s$ activated inside C1. Studies on C2 proteolysis by $C\bar{1}$ were reported in [27] and similar K_m values for C4 and C2 concluded. No differences in K_m and turnover numbers for the proteolysis of C4 by $C\bar{1}s$ and $C\bar{1}$ were detected in [28]; the value of K_m reported is in the same order of the value we found for $C\bar{1}$ reconstructed in its proenzymic form before activation.

These kinetic data are likely to reveal additional information on C1 structure to results obtained by surface iodination [25,26]. They also indicate that for a physiological concentration of C2 (1.2×10^{-7} M, [28]) cleavage of C2 is probably very inefficient unless C2 is 'presented' to $C\bar{1}$ in an optimal position defined by C4b.

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