Comparative study of the fluid-phase proteolytic cleavage of human complement subcomponents C4 and C2 by $C\bar{1}s$ and $C\bar{1}r_2$–$C\bar{1}s_2$

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The C3 convertase of the classical pathway of complement is composed of fragments C4b and C2a resulting from cleavage of C4 and C2 by activated $C\bar{1}$. The limited proteolysis of these two different substrates by the same protease, $C\bar{1}s$, has been studied in the fluid phase using purified proteins. The turnover numbers of C2 and C4 cleavage by $C\bar{1}s$ were affected to different extents, depending on whether $C\bar{1}s$ was alone or associated with $C\bar{1}r$ or with monoclonal antibodies to $C\bar{1}s$. The binding of C2 to C4 favours the proteolysis of C2 by $C\bar{1}s$, as revealed by the use of I_2-treated C2.

Complement *Protease* *Monoclonal antibody* *C2* *C4* *C1s*

1. INTRODUCTION

Activation of the complement system proceeds via the classical or the alternative pathway through an initial cascade of limited proteolytic reactions [1]. The first activation step of the classical pathway concerns C1, a calcium-dependent complex composed of C1q, two subunits of C1r and two subunits of C1s. Activated C1 contributes through its subcomponent C1s to the formation of the C3 convertase, a bimolecular complex of fragments C4b and C2a resulting from the limited proteolysis of C4 and C2 by $C\bar{1}s$. The proteolytic activity of $C\bar{1}s$ is thus exerted on two different substrates which are normally present in serum in a 1:10 (C2:C4) molar ratio [2]. Reported K_m values for the proteolysis of C2 and C4 [2,3] do not account for a privileged cleavage of C2.

We examine here in detail the differences between the proteolysis of C4 and C2 by $C\bar{1}s$, the influence of modulators of $C\bar{1}s$ activity, such as $C\bar{1}r$ [4] or monoclonal antibodies to $C\bar{1}s$ and also the incidence of the C4–C2 interaction in the proteolysis of C2. The results indicate that the same serine active site in $C\bar{1}s$ is able to cleave C4 and C2, but that there are net differences in the kinetics of proteolysis of both C4 and C2; they also show that binding of C2 to C4 favours the cleavage of C2 by $C\bar{1}s$, reducing the limiting effect due to the low physiological concentration of C2.

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Abbreviations: SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; the nomenclature of the components of complement is that recommended by the World Health Organization (1968); an over bar indicates the activated state of a component

2. MATERIALS AND METHODS

Human citrated plamsa was obtained from the Centre de Transfusion Sanguine (Grenoble). Sheep erythrocytes were purchased from Bio-Merieux. Hemolysin was from Behring. I_2 (Suprapur) was from Merck. Lactoperoxidase (purified grade) was

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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, France). Other chemicals were of analytical grade.

Monoclonal antibodies against $C\bar{1}s$, prepared from mouse hybridomas, were kindly provided by Dr Jane Skok (Imperial Cancer Research Fund Laboratory, Lincoln's Inn Fields, London).

$C\bar{1}r$ and $C\bar{1}s$ were purified as in [5] and estimated from their A_{280} using respectively $A^{1\%}_{1\,cm} = 11.5$ [6] and 9.5 [6]. M_r values were taken as 85 000 for $C\bar{1}r$ and 85 000 for $C\bar{1}s$.

$C\bar{1}s$ esterase activity was measured from the hydrolysis of *p*-tosyl-L-arginine methyl ester as in [7].

The $C\bar{1}r_2$–$C\bar{1}s_2$ complex was obtained by incubation of $C\bar{1}r$ and $C\bar{1}s$ in a 1:1 (w/w) ratio for 30 min at 30°C in the presence of 5 mM $CaCl_2$.

The $C\bar{1}s$-monoclonal anti-$C\bar{1}s$ immune complexes were prepared by incubation of $C\bar{1}s$ with the antibodies at 4°C for 3 h.

C4 and C2 were purified as in [3,8] and estimated from their A_{280} using $A^{1\%}_{1\,cm} = 10$. Iodine-treated C2 was prepared by reaction of C2 with iodine as in [9]. Briefly, C2 was incubated in 0.1 M sodium phosphate, 4 mM KI (pH 6.0) in the presence of a 2-fold molar excess of I_2 for 10 min at 4°C. Free iodine was eliminated by dialysis against 0.1 M sodium phosphate (pH 7.0).

C2 haemolytic activity was measured as in [10]. Treatment of C2 with iodine resulted in a 17-fold enhancement of its haemolytic activity.

Surface ^{125}I labelling of proteins was performed by lactoperoxidase catalysis as in [8] for C4 and according to [11] for C2 and iodine-treated C2. The average iodine binding was between 0.05 and 0.1 mol ^{125}I/mol protein. ^{125}I-labelled protein was estimated by Coomassie brilliant blue G250 staining as in [12], taking the unlabelled protein as reference. M_r values were taken as 200 000 for C4 and 100 000 for C2 and I_2-treated C2.

SDS–PAGE was done as in [13]. Sucrose gradient ultracentrifugation was performed as in [14].

For kinetic analysis, each experiment was performed with 4 different concentrations of the ^{125}I-labelled C2 or ^{125}I-labelled C4. The $C\bar{1}s$ concentration was chosen to give 20–30% substrate cleavage in 10 min. During incubation, at regular time intervals, samples were removed, immediately reduced with 50 mM dithiothreitol for 60 min at 37°C, alkylated with 140 mM iodoacetamide for 20 min at 37°C, then submitted to SDS–PAGE. Gels were cut into 1 mm slices and their radioactivity was counted in an MR 480 Kontron γ Counter. The rate of cleavage was estimated from the distribution of radioactivity between C2, C2a and C2b or between α and α' chains of C4 and C4b, for the proteolysis of C2 and C4, respectively. The residual substrate concentration was plotted against time on a semilog scale and the initial velocity calculated from the slope of this curve [15]. Initial velocities and initial substrate concentrations were plotted according to Lineweaver and Burk. Michaelis constants (K_m) and maximum velocities (V_m) were calculated from this plot by linear regression analysis using a Hewlett Packard (HP 41 CV) calculator. k_{cat} (or alternatively turnover) was calculated from the ratio V_m/E (E, enzyme concentration) and expressed as mol substrate cleaved/mol enzyme per s (or per min).

3. RESULTS

3.1. *Kinitic and thermodynamic parameters of cleavage of C2 and C4 by $C\bar{1}s$ and by $C\bar{1}r_2$–$C\bar{1}s_2$*

3.1.1. Cleavage of C2

Table 1 lists the kinetic parameters of C2 cleavage, determined at 5 different temperatures, with $C\bar{1}s$, alone or in the $C\bar{1}r_2$–$C\bar{1}s_2$ complex; the enzyme concentrations varied from 7.8×10^{-9} M at 10°C to 1.95×10^{-9} M at 37°C.

In comparison with results obtained for isolated $C\bar{1}s$, results for $C\bar{1}r_2$–$C\bar{1}s_2$ show an about 2-fold decrease in the turnover number, whereas no significant difference appears in the K_m of the reaction. The activation energy and Q_{10}, calculated from the Arrhenius plot (fig.1), are very similar, with respective values of 10.1 kcal/mol and 1.84 for $C\bar{1}s$ and 10.5 kcal/mol and 1.89 for $C\bar{1}r_2$–$C\bar{1}s_2$.

3.1.2. Cleavage of C4

Table 2 lists the kinetic parameters of C4 cleavage, determined at 5 different temperatures, with $C\bar{1}s$ alone or in the $C\bar{1}r_2$–$C\bar{1}s_2$ complex; the enzyme concentrations varied from 7.8×10^{-10} M at 10°C to 1.95×10^{-10} M at 37°C.

The activation energy and Q_{10} values of C4 cleavage are lower for $C\bar{1}r_2$–$C\bar{1}s_2$ than for $C\bar{1}s$:

Table 1

Kinetic parameters of C2 cleavage by $C\bar{1}s$ and $C\bar{1}r_2$–$C\bar{1}s_2$

		Temperature (°C)				
		37	31	24	17	10
$C\bar{1}s$	K_m (10^{-5} M)	3.0	3.2	3.3	4.0	4.0
	Turnover number	1025	747	454	321	213
	k_{cat}	17.1	12.4	7.6	5.3	3.5
$C\bar{1}r_2$–$C\bar{1}s_2$	K_m (10^{-6} M)	2.5	1.4	1.4	2.8	3.1
	Turnover number	629	380	258	190	111
	k_{cat}	10.5	6.3	4.3	3.2	1.8

Results are mean values of 2 experiments. For each temperature, ^{125}I-labelled C2 concentration varied from 1.0×10^{-6} to 4.0×10^{-6} M in 145 mM NaCl, 5 mM triethanolamine (pH 7.4) and 1 mg/ml egg albumin. Incubation with $C\bar{1}s$ was in the presence of 5 mM EDTA; incubation with $C\bar{1}r_2$–$C\bar{1}s_2$ was in the presence of 5 mM $CaCl_2$. K_m, turnover number and k_{cat} values were obtained as described in section 2.

they are, respectively, 8.6 kcal/mol and 1.63 for $C\bar{1}s$ and 7.5 kcal/mol and 1.53 for $C\bar{1}r_2$–$C\bar{1}s_2$. In the case of C4 only, the turnover values measured at 10 and 37°C diverge considerably from linearity, and were not taken into account for calculation.

Similarly to C2, there is a decrease in the turnover number of C4 proteolysis when $C\bar{1}s$ is in the $C\bar{1}r_2$–$C\bar{1}s_2$ complex, and also no significant difference in the K_m values.

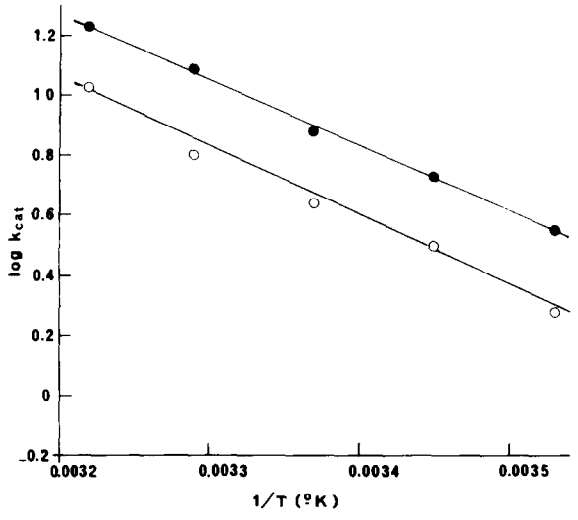

Fig.1. Arrhenius plot of C2 cleavage by $C\bar{1}s$ and by $C\bar{1}r_2$–$C\bar{1}s_2$. The values used for the plot were taken from table 1. (○) $C\bar{1}s$, (●) $C\bar{1}r_2$–$C\bar{1}s_2$.

The turnover numbers of C2 and C4 cleavages are of the same order of magnitude whereas the K_m for C4 cleavage is 10-times less than the K_m for C2 cleavage.

3.2. *Effect of anti-$C\bar{1}s$ monoclonal antibodies on the cleavage of C4 and C2 by $C\bar{1}s$*

As the affinity of the monoclonal antibodies to $C\bar{1}s$ was not very high, $C\bar{1}s$ was used at 3.15×10^{-9} M for cleavage of both C2 and C4: under these conditions the antigen–antibody complex was not dissociated, as estimated from sucrose gradient ultracentrifugation.

As depicted in table 3, the turnover of C2 cleavage by $C\bar{1}s$ is increased in the presence of monoclonal antibodies added to an enzyme/antibody (w/w) ratio of 1:1 and 1:5.

In contrast, the presence of monoclonal antibodies to $C\bar{1}s$ does not influence significantly the turnover of C4 cleavage by $C\bar{1}s$ for enzyme/antibody ratios of 1:1, 1:2.5 or 1:5. The K_m values for the cleavage of C2 and C4 are not grossly altered by the addition of monoclonal antibodies to $C\bar{1}s$ and remain in the 10^{-6} M and 10^{-7} M range, respectively.

3.3. *Influence of C4 on the cleavage of C2 and iodine-treated C2*

As seen in fig.3,4 and table 4, the rate of cleavage of C2 is decreased 2-fold after treatment of C2

Table 2

Kinetic parameters of C4 cleavage by $C\bar{1}s$ and $C\bar{1}r_2$–$C\bar{1}s_2$

		Temperature (°C)				
		37	31	24	17	10
$C\bar{1}s$	K_m (10^{-6} M)	1.0	1,5	0.8	0.9	0.7
	Turnover number	520	441	310	221	80
	k_{cat}	8.7	7.3	5.2	3.7	1.3
$C\bar{1}r_2$–$C\bar{1}s_2$	K_m (10^{-6} M)	0.5	0.9	2.9	1.4	1.4
	Turnover number	120	285	229	156	43
	k_{cat}	2.0	4.7	3.8	2.6	0.7

Results are means of 3 experiments for $C\bar{1}s$ and of 2 experiments for $C\bar{1}r_2$–$C\bar{1}s_2$. For each temperature, ^{125}I-labelled C4 concentration varied from 1.0×10^{-6} to 4.0×10^{-6} M in 145 mM NaCl, 5 mM triethanolamine (pH 7.4) and 1 mg/ml egg albumin. Incubation with $C\bar{1}s$ was in the presence of 5 mM EDTA; incubation with $C\bar{1}r_2$–$C\bar{1}s_2$ was in the presence of 5 mM $CaCl_2$. K_m, turnover number and k_{cat} values were obtained as described in section 2.

with iodine. In the presence of C4 (3.8×10^{-6} to 5.5×10^{-6} M) there is no significant variation in the K_m but a 2-fold decrease in the rate of cleavage of C2 and a 2-fold increase in the rate of cleavage of iodine-treated C2.

The reference turnover values reported in table 4 are lower than corresponding values given, for instance in table 1, for $C\bar{1}s$; this may be explained by the presence of phosphate buffer necessary for the preservation of the I_2-treated C2 haemolytic activity.

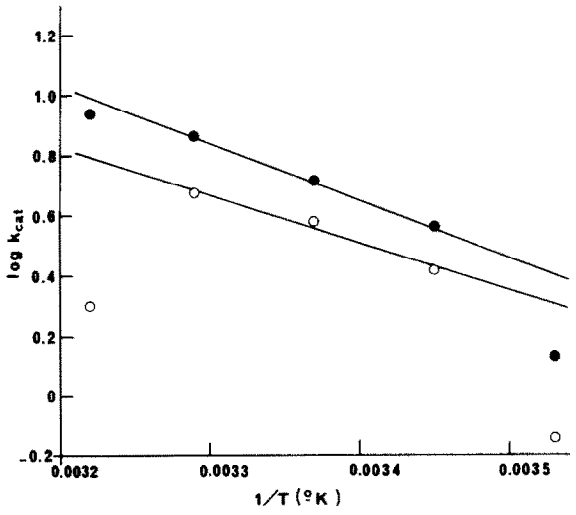

Fig.2. Arrhenius plot of C4 cleavage by $C\bar{1}s$ and by $C\bar{1}r_2$–$C\bar{1}s_2$. The values used for the plot were taken from table 2. (○) $C\bar{1}s$, (●) $C\bar{1}r_2$–$C\bar{1}s_2$.

4. DISCUSSION

The differential behaviour of C2 and C4 as substrates for $C\bar{1}s$ is revealed by differences in the activation energy of their cleavage either by isolated $C\bar{1}s$ or by $C\bar{1}s$ in the calcium-dependent $C\bar{1}r_2$–$C\bar{1}s_2$

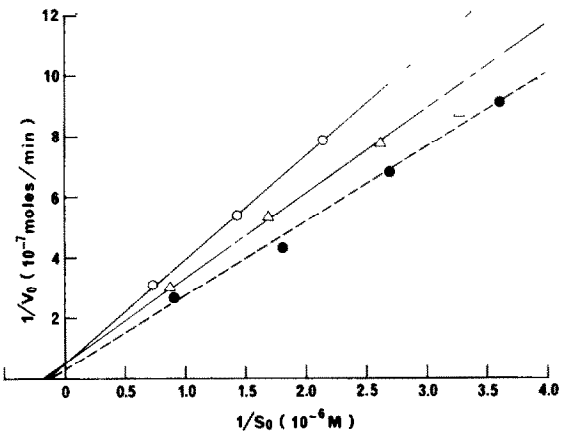

Fig.3. Lineweaver-Burk plot of C2 cleavage by $C\bar{1}s$ in the presence of C4. ^{125}I-labelled C2 at a final concentration varying from 0.3×10^{-6} M to 1.5×10^{-6} M was mixed with C4, at a final concentration of 4.9×10^{-6} M (○) or 3.8×10^{-6} M (△), before addition of $C\bar{1}s$ (7.8×10^{-9} M); control without C4 (●). All proteins were in 0.1 M sodium phosphate (pH 7.0) and 1 mg/ml egg albumin. Incubation was at 25°C; S_0 and V_0 values were obtained as described in section 2.

Table 3

Influence of monoclonal antibodies to $C\bar{1}s$ on kinetic parameters of C2 and C4 cleavage by $C\bar{1}s$

		$C\bar{1}s$/antibody (w/w) ratio			
		1:0	1:1	1:2.5	1:5
C2	K_m (10^{-6} M)	4.3	12.0	N.D.	8.6
	Turnover number	188[a]	384[b]		306[b]
C4	K_m (10^{-7} M)	4.4	8.5	4.2	3.4
	Turnover number	289[b]	234[b]	261[b]	218[b]

N.D., not determined
[a] Mean value of 3 experiments
[b] Mean values of 2 experiments

Experiments were performed at 25°C in 100 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl (pH 8.3) containing 5 mg/ml egg albumin; ^{125}I-labelled C2 and ^{125}I-labelled C4 concentrations varied from 1.0×10^{-6} to 4.0×10^{-6} M. For both substrates, $C\bar{1}s$ concentration, alone or in the $C\bar{1}s$-monoclonal anti-$C\bar{1}s$ complex, was 3.15×10^{-9} M

complex. Furthermore, the activation energy for the proteolysis of C2 by $C\bar{1}s$ or $C\bar{1}r_2$–$C\bar{1}s_2$ is the same, whereas in the case of C4 it is slightly lower for $C\bar{1}r_2$–$C\bar{1}s_2$ than for $C\bar{1}s$. This last effect, due to $C\bar{1}r$–$C\bar{1}s$ interaction, is reminiscent of the case of $C\bar{1}r$: the activation energy of the autocatalytic activation of $C\bar{1}r$ is lower in $C\bar{1}$ than for isolated $C\bar{1}r$ [16,17]. A comparable effect, restricted to C4, has been discussed previously [4].

The turnover of C4 and C2 by C1s was also studied in the presence of monoclonal antibodies to $C\bar{1}s$; here again C4 and C2 behaved differently, as the turnover of C2 was increased upon binding of antibodies to the protease whereas the turnover of C4 was unchanged. This effect is probably exerted at some distance from the active site of $C\bar{1}s$ and the binding of the antibodies most probably involves epitopes located in the A chain of $C\bar{1}s$,

Table 4

Influence of C4 on kinetic parameters of the cleavage by $C\bar{1}s$ of C2 and iodine-treated C2

		C4 concentration (10^{-6} M)			
		0	3.8	4.9	5.5
C2	K_m (10^{-6} M)	9.6	4.0	7.2	11.6
	Turnover number	42[a]	19	27	24

		C4 concentration (10^{-6} M)		
		0	4.2	4.9
I-C_2	K_m (10^{-6} M)	3.8	7.6	5.0
	Turnover number	18[b]	36	32

[a] Mean value of 3 experiments
[b] Mean value of 2 experiments
I-C_2, iodine-treated C_2. K_m and turnover number values were calculated from fig.3,4

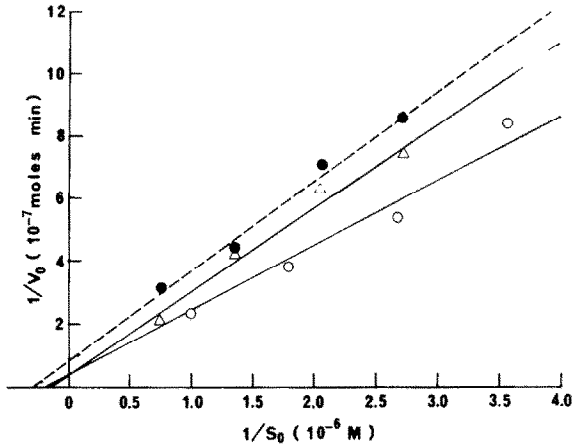

Fig. 4. Lineweaver-Burk plot of the cleavage of I_2-treated C2 by $C\bar{1}s$ in the presence of C4. ^{125}I-labelled I_2-treated C2 at a final concentration varying from 0.3×10^{-6} M to 1.5×10^{-6} M was mixed with C4, at a final concentration of 4.9×10^{-9} M (○) or 4.2×10^{-6} M (△) before addition of $C\bar{1}s$ (7.8×10^{-9} M); control without C4 (●). All proteins were in 0.1 sodium phosphate (pH 7.0) and 1 mg/ml egg albumin. Incubation was at 25°C; S_0 and V_0 values were obtained as described in section 2.

which has been previously shown to be exposed to the solvent [18]. This is also supported by the absence of inhibition of the esterolytic activity of $C\bar{1}s$ upon the binding of monoclonal antibodies to $C\bar{1}s$.

In no case were the K_m values for C4 and C2 significantly altered by the binding either of C1r or of monoclonal antibodies to $C\bar{1}s$; K_m values calculated for C4 (10^{-6}–10^{-7} M) are in good agreement with reported values [2] and differ significantly from the value calculated for C2 (10^{-5}–10^{-6} M).

The presence of two active sites in C1, one for each of the two substrates, C4 and C2, raises the problem of a possible interaction between the substrates. Competition experiments between C2 and C4, I_2-treated C2 and C4 indicate that, if C4 is able to inhibit C2 cleavage by $C\bar{1}s$, in contrast it enhances the cleavage of I_2-treated C2. This effect clearly results from a tight interaction between C4b and C2 due to the treatment of C2 by iodine and shows that binding of C2 and C4b is essential for the proteolysis of C2 by $C\bar{1}s$.

From these results, there is no apparent evidence for distinct subpopulations of $C\bar{1}s$ molecules, one for the proteolysis of C4 and another for the proteolysis of C2. Thus the role of a neighbouring acceptor of C4b appears essential, as bound C4b presents C2 to $C\bar{1}$ very efficiently.

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