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INTERACTION OF ^{125}I-LABELLED COMPLEMENT SUBCOMPONENTS $C\overline{1}r$ AND $C\overline{1}s$ WITH PROTEASE INHIBITORS IN PLASMA

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1. Introduction

Activation of the classical pathway of the complement system is brought about by the binding of the first component of complement, C1, to antibody–antigen complexes. C1 is a glycoprotein complex, containing 3 types of subunits, C1q, C1r and C1s, of which the two latter are serine protease zymogens [1]. The interaction of C1q with antibody in immune complexes leads to the sequential activation of C1r and C1s by limited proteolysis [2]. The proteases $C\overline{1}r$ and $C\overline{1}s$ are both of mol. wt 83 000, and have structures similar to that of plasmin [3]. As with other plasma proteases, the activities of $C\overline{1}r$ and $C\overline{1}s$ are regulated by protease inhibitors. In plasma there are 7 well-characterised protease inhibitors, each capable of inhibiting several serine proteases [7,8]. Additional plasma serine protease inhibitors have been described [7]. Although $C\overline{1}r$ and $C\overline{1}s$ are both known to form stable complexes with $C\overline{1}$ inhibitor ($C\overline{1}$ In) [4,5], little information is available on their activity towards other protease inhibitors. A simple, and widely applicable technique, involving incubation of ^{125}I-labelled $C\overline{1}r$ and $C\overline{1}s$ with plasma, followed by gel filtration, affinity chromatography and SDS–polyacrylamide gel analyses has been used to investigate the interaction of $C\overline{1}r$ and $C\overline{1}s$ with inhibitors in whole plasma. The results demonstrate, consistent with the very restricted proteolytic specificity of $C\overline{1}r$ and $C\overline{1}s$, that $C\overline{1}$ In is the only physiological inhibitor of both proteases. The iodination procedure used does not detectably alter any of the activities of the two enzymes.

Abbreviations SDS, sodium dodecyl sulphate, BSA, bovine serum albumin, EDTA, ethylene diamine tetraacetic acid, The nomenclature of complement components is that recommended by the World Health Organization (1968)

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2. Materials and methods

2.1. *Proteins*

Human citrated plasma was obtained from the Centre de Transfusion Sanguine, Grenoble. Preparation of serum [9] and the isolation of $C\overline{1}$ In [4], $C\overline{1}r$ and $C\overline{1}s$ [1,10] were as previously described. Purified proteins were quantified from their specific A_{280}, using $E^{1\%}_{1\,cm}$ = 4.5, 11.5 and 9.4, respectively, for $C\overline{1}$ In, C1r and C1s [3,11].

2.2. *Anti-$C\overline{1}$ in antibody linked to Sepharose 6B*

The total immunoglobulin fraction was precipitated from 5 ml rabbit anti $C\overline{1}$ In antiserum (Behring) and coupled to 10 ml (packed volume) of cyanogen bromide-activated Sepharose 6B. Coupling yield was 70%. Procedures were as in [12].

2.3. *Iodination*

Iodination of $C\overline{1}r$ and $C\overline{1}s$ was done by a modification of the C1q-labelling method in [13]. Solutions of $C\overline{1}s$ (500 µg/ml) or $C\overline{1}r$ (250 µg/ml) in 5 mM triethanolamine–HCl, 145 mM NaCl (pH 7.4) were chilled to 0°C, and the following quantities of reagents, made up in the same buffer, were added per

1 ml protein solution 50 μl 20 μM NaI, 50 μl $Na^{125}I$ (Amersham) adjusted to spec. act. 0.5 μCi/ml, 50 μl 2 mg/ml lactoperoxidase (Calbiochem, Grade B) and 20 μl 1/20 000 dilution of 30% H_2O_2. After 50 min incubation at 0°C, free ^{125}I was removed from the mixture by gel filtration on a column (25 × 1 cm) of Sephadex G-50 in 5 mM triethanolamine – HCl, 145 mM NaCl, 0.02% w/v NaN_3 (pH 7.4). The labelled protein was pooled, dialysed against 1 l of the gel filtration buffer, and stored at 4°C. ^{125}I was counted on an Intertechnique CG 2000 gamma counter. The activities of the labelled proteins were verified in the following functional tests C$\bar{1}$s α-*N*-benzyloxycarbonyl-L-lysine *p*-nitrophenol esterase activity [3], C$\bar{1}$r acetyl arginine methyl esterase activity [14], capacity of C$\bar{1}$r to activate proenzymic C$\bar{1}$s [3]; subcomponent C$\bar{1}$r and C$\bar{1}$s haemolytic activity [9], and the specific and sequential binding of C$\bar{1}$r then C$\bar{1}$s to antibody–antigen–C1q complexes [2,15]. In addition, the rates of reaction of labelled and unlabelled C$\bar{1}$r and C$\bar{1}$s with C$\bar{1}$ In were compared (see fig.1).

2.4. *Protease–protease inhibitor Interactions*

The interaction of C$\bar{1}$r of C$\bar{1}$s with protease inhibitors was studied by adding 25 μg or 250 μg C$\bar{1}$r or C$\bar{1}$s, containing in each case 2 × 10^5 cpm ^{125}I-labelled material, to 500 μl citrated plasma or to 500 μl serum in the presence of 5 mM $CaCl_2$ or 5 mM EDTA. The two concentrations of labelled protease used were chosen to be respectively less than, and greater than, the total inhibitory capacity of C$\bar{1}$ In in plasma. Soybean trypsin inhibitor (1 mg/ml) 100 μl was added to limit proteolysis by other proteases present in plasma. Control experiments were performed in which 500 μl 5 mM triethanolamine–HCl, 145 mM NaCl (pH 6.4) containing 150 μg C$\bar{1}$ In + 15 mg BSA was used instead of plasma. Samples were incubated 1 h, 37°C, and the size distribution of the labelled species in each sample was then examined by gel filtration on Ultrogel AcA-34. All eluate fractions containing radioactivity were pooled and passed through a column of anti-C$\bar{1}$ In antibody linked to Sepharose-6B. Radioactive material not binding to this column was pooled, concentrated to 1 ml by ultrafiltration, and the remaining labelled species were reexamined on the Ultrogel AcA-34 column. Peaks of radioactivity eluted from Ultrogel were pooled individually, concentrated to a small volume, and their composition was analysed by SDS–polyacrylamide gel electrophoresis. Material bound to the anti-C$\bar{1}$ In column was eluted with 1 M NH_4SCN, dialysed against the chromatography buffer, and examined on SDS–polyacrylamide gels.

Preparation of samples for SDS–polyacrylamide gel electrophoresis, the electrophoresis system, and gel staining were as in [3]. Gels stained with Coomassie blue were scanned [12] and cut into 2 mm slices for ^{125}I counting.

Determination of hydrodynamic parameters by sucrose density gradient centrifugation and gel filtration was as in [3].

3. Results and discussion

3.1. *Iodination*

Iodination of C$\bar{1}$r and C$\bar{1}$s resulted in 35–40%

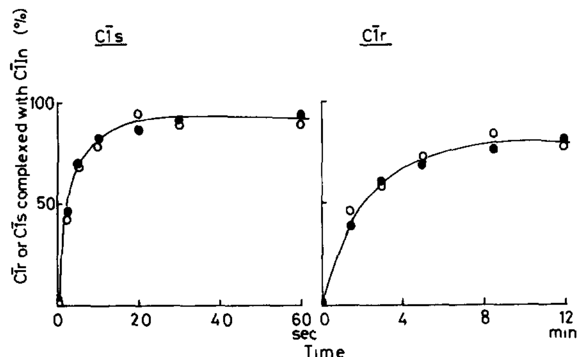

Fig 1 Comparison of the rates of reaction of iodinated and non-iodinated C$\bar{1}$r and C$\bar{1}$s with C$\bar{1}$ In. ^{125}I-labelled C$\bar{1}$r or C$\bar{1}$s were diluted with cold C$\bar{1}$r or C$\bar{1}$s such that the overall labelling represented 5 atoms I/100 molecules C$\bar{1}$r or C$\bar{1}$s. The C$\bar{1}$r (450 μg/ml) or C$\bar{1}$s (440 μg/ml) in 5 mM triethanolamine–HCl, 145 mM NaCl, 5 mM EDTA (pH 7 4) was then incubated with C$\bar{1}$ In (920 μg/ml) at 37°C Reaction was stopped at various times by placing 50 μl of the reaction mixture in 50 μl 0 2 M Tris–HCl, 8 M urea, 2% w/v SDS, 40 mM iodoacetamide (pH 8 0) and incubating 4 min at 100°C Samples were then examined on SDS–polyacrylamide gels, and the gels stained and scanned The rate of uptake of total (i.e., labelled + unlabelled C$\bar{1}$r or C$\bar{1}$s into complexes with C$\bar{1}$ In (•) was estimated from the decrease in intensity of the free C$\bar{1}$r or C$\bar{1}$s peaks on the gels Gels were then sliced and the rate of complexation of labelled C$\bar{1}$r or C$\bar{1}$s with C$\bar{1}$ In (○) calculated from the radioactivity associated with the free protease and C$\bar{1}$ In–protease complex bands

incorporation of the total radioactivity supplied for C$\bar{1}$r, and 40–45% for C$\bar{1}$s, corresponding to final spec. act. 2.9–3.3 μCi/nmol for C$\bar{1}$r and 1.7–1.9 μCi/nmol for C1s. In C$\bar{1}$r, 66–70% of the total labelling is in the light (B) polypeptide chain, while for C$\bar{1}$s, 93–95% is in the heavy (A) chain. None of the binding or enzymic activities of C$\bar{1}$r or C$\bar{1}$s was detectably altered by iodination. The rate of interaction of the labelled proteases with C$\bar{1}$ in (fig.1) was identical to the rate observed with total (i.e., labelled + unlabelled) material showing that iodination is again without affect on this activity. It has been shown [16] that iodination of C$\bar{1}$s may lead to loss of reactivity with C$\bar{1}$ In, and possibly to altered capacity to interact with other C1 subcomponents. The iodination procedure described above is, however, done with considerably lower quantities of oxidising agent than in [16].

The same iodination procedure is applicable to proenzymic C1r and C1s, with which similar iodination yields are obtained.

3.2. *Protease–protease inhibitor interactions*

All of the well-characterised plasma protease inhibitors (α_1 antitrypsin, α_2 macroglobulin, C$\bar{1}$ In, inter-α-trypsin inhibitor, α_2 antiplasmin, antichymotrypsin and antithrombin III) inhibit proteases by forming complexes with them [6,7]. These complexes are stable during gel filtration, and also (with the exception of α_2 macroglobulin–protease complexes) during SDS–polyacrylamide gel electrophoresis. Incubation of ^{125}I-labelled C$\bar{1}$r or C$\bar{1}$s with plasma, followed by gel filtration demonstrates (fig.2) that C$\bar{1}$r and C$\bar{1}$s are taken up into complexes of higher molecular weight during incubation. In this gel filtration system free C$\bar{1}$s behaves as an 83 000 mol. wt monomer while C$\bar{1}$r is a 166 000 mol. wt dimer [3]. With additions of 25 μg C$\bar{1}$r or C$\bar{1}$s to plasma, nearly all of each protease becomes complexed (fig.2b,d) while with 250 μg additions much of the C$\bar{1}$r and C$\bar{1}$s added does not form higher molecular weight complexes. Elution profiles of radioactivity from samples incubated with plasma, with serum in the presence of Ca^{2+} or EDTA, or control samples incubated with C$\bar{1}$ in were all very similar.

Passage of the pool of radioactive fractions from Ultrogel through the column of anti-C$\bar{1}$ In resulted in retention of a percentage of the radioactivity

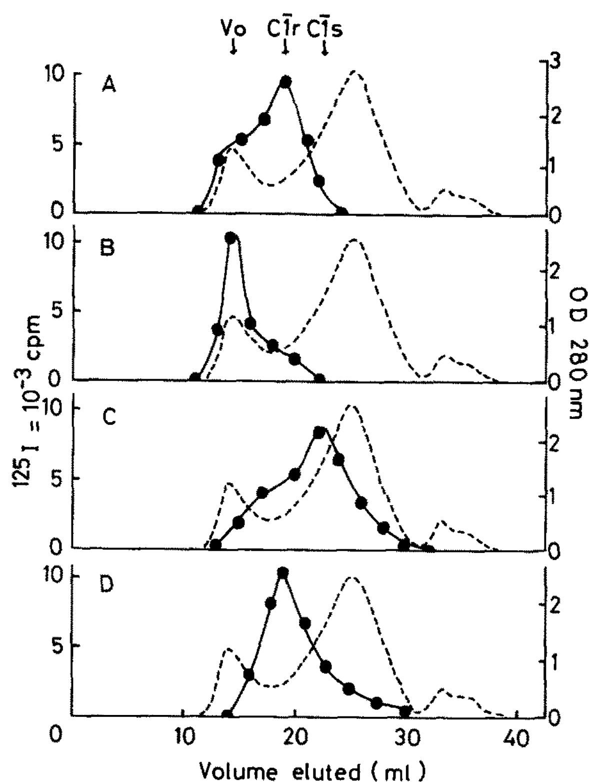

Fig 2. First gel filtration analysis of complex formation Samples were run on a column (48 × 1 cm) of Ultrogel AcA 34 in 5 mM triethanolamine–HCl, 145 mM NaCl, 5 mM EDTA The elution profile of radioactivity (—•—) and of A_{280} (- - -) shown for samples of proteases incubated with plasma. (A) 250 μg C$\bar{1}$r, (B) 25 μg C$\bar{1}$r, (C) 250 μg C$\bar{1}$s, (D) 25 μg C$\bar{1}$s. The elution positions of free labelled C$\bar{1}$r and C$\bar{1}$s, run as standards, are shown by arrows. V_0 = void volume.

Table 1
Percentage retention of ^{125}I-labelled C$\bar{1}$r or ^{125}I-labelled C$\bar{1}$s on Sepharose-anti C$\bar{1}$ In

Sample	% Radioactivity retained
C$\bar{1}$r (25 μg) + plasma	72
C$\bar{1}$r (25 μg) + C$\bar{1}$ In + BSA	77
C$\bar{1}$s (25 μg) + plasma	81
C$\bar{1}$s (25 μg) + C$\bar{1}$ In + BSA	85
C$\bar{1}$r (250 μg) + plasma	26
C$\bar{1}$r (250 μg) + C$\bar{1}$ In + BSA	29
C$\bar{1}$s (250 μg) + plasma	31
C$\bar{1}$s (250 μg) + C$\bar{1}$ In + BSA	35

(table 1) which corresponds closely to the % ^{125}I-labelled protease taken up into higher mol. wt complexes (as seen in fig.2). The specificity of the affinity column for the retention only of material bound to C$\bar{1}$ In was confirmed by showing that all radioactive material retained on this column, when examined on SDS–polyacrylamide gels, behaved as C$\bar{1}$r–C$\bar{1}$ In, or as appropriate, C$\bar{1}$s–C$\bar{1}$ In complexes (c f. fig.3). The molecular weight of C$\bar{1}$ In is sufficiently distinct from those of other well-characteristed protease inhibitors that simple analysis by SDS–polyacrylamide gel electrophoresis is adequate to identify complexes of C$\bar{1}$ In with a known protease. The mobilities of complexes of C$\bar{1}$r and C$\bar{1}$s with C$\bar{1}$ In in this electrophoresis system have been described [5]

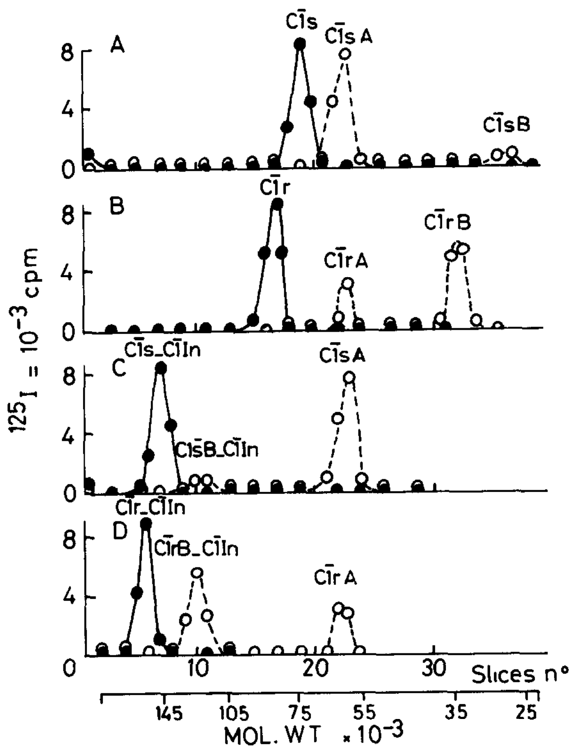

Fig.3 Pattern of migration of ^{125}I-labelled polypeptide chains of C$\bar{1}$r, C$\bar{1}$s and their complexes with C$\bar{1}$ In on SDS–polyacrylamide gels. 15 000–18 000 cpm was loaded onto each gel Alkylated samples (——•——) and reduced and alkylated samples (– –o– –) are shown (A) C$\bar{1}$s, (B) C$\bar{1}$r, (C) C$\bar{1}$s–C$\bar{1}$ In complex, (D) C$\bar{1}$r–C$\bar{1}$ In complex The molecular weight scale refers only to reduced samples C$\bar{1}$sA, C$\bar{1}$rA and C$\bar{1}$sB, C$\bar{1}$rB A chain of C$\bar{1}$r, C$\bar{1}$s and B chain of C$\bar{1}$r, C$\bar{1}$s, respectively.

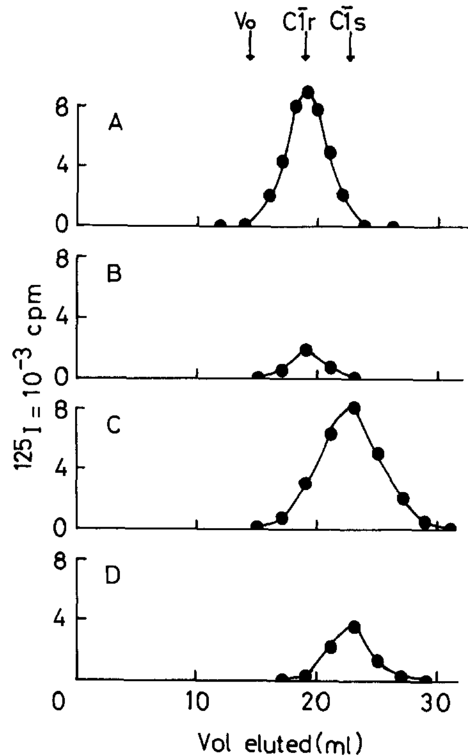

Fig.4 Second gel filtration analysis radioactive material not retained on Sepharose 6B–anti C$\bar{1}$ In The elution profile of radioactivity is shown. Conditions, and identification of samples as in fig.2

Re-examination on Ultrogel AcA 34 of the remaining radioactive material which did not bind to the affinity column showed (fig.4) that all of this material behaved as free C$\bar{1}$r or C$\bar{1}$s. This was confirmed by subsequent analysis on SDS polyacrylamide gels (c.f. fig.3). In some experiments, up to 10% of the remaining free C$\bar{1}$r was seen to have undergone a proteolytic degradation similar to that in [17].

These results demonstrate that C$\bar{1}$ In is the only protease inhibitor in plasma or serum which forms a stable complex with C$\bar{1}$r or C$\bar{1}$s. Incubation of 25 μg additions of these proteases, which do not exceed the inhibitory capacity of C$\bar{1}$ In in plasma, result in uptake of most of the protease into higher molecular weight complexes. When the addition of protease exceeds the capacity of C$\bar{1}$ In, however, much of the added protease remains in the free state. All higher molecular weight complexes formed were removed by

chromatography on anti-C$\overline{1}$ In, leaving only free C$\overline{1}$r and C$\overline{1}$s. Since in these experiments recovery of radioactivity at each step was >95%, non-specific loss of other protease–protease inhibitor complexes is unlikely to influence the results. The method used here, gel filtration before and after affinity chromatography using antibody against the principal known inhibitor, is generally applicable as a means to simplify systems where several proteases and inhibitors may be involved.

C$\overline{1}$ In is likely therefore to be the principal inhibitor of C$\overline{1}$r and C$\overline{1}$s in vivo, although cellular protease inhibitors may also be involved. Evidence has been presented [18] that C$\overline{1}$ In is the major in vivo control of C$\overline{1}$s. C$\overline{1}$s has also been reported to be inhibited by very high concentrations of heparin and antithrombin III [19]. The experiments described above, however, were done in heparin-free conditions. The result obtained here confirms observations that C$\overline{1}$r is not inhibited by α_2 macroglobulin, inter-α-trypsin inhibitor and α_1 antitrypsin [20]. Similarly, C$\overline{1}$s has been reported to be unreactive towards α_1 antitrypsin [19] and α_2 antiplasmin [21]. Thus C$\overline{1}$r and C$\overline{1}$s, unlike the many other plasma proteases are inhibited by only one of the plasma inhibitors. This is compatible with the very restricted proteolytic specificity of these two enzymes [3,9].

An anomaly in the behaviour of C$\overline{1}$r was also noted in these experiments. Since C$\overline{1}$r is a dimer [3] it may be expected to form complexes containing either one or two molecules of C$\overline{1}$ In. The C$\overline{1}$r dimer dissociates on SDS–polyacrylamide gel electrophoresis, and thus complexes of type C$\overline{1}$r$_2$–C$\overline{1}$ In$_1$ should dissociate into free C$\overline{1}$r plus C$\overline{1}$r$_1$–C$\overline{1}$ In$_1$ complexes in SDS. Analysis on SDS–polyacrylamide gels of material retained on the affinity column after incubation of excess C$\overline{1}$r (250 μg) with plasma, showed only C$\overline{1}$r$_1$–C$\overline{1}$ In$_1$ complexes, and no free C$\overline{1}$r. Thus in conditions where C$\overline{1}$r is in excess of C$\overline{1}$ In, which should favour formation of C$\overline{1}$r$_2$–C$\overline{1}$ In$_1$, this type of complex was not detected. Sucrose density gradient and gel filtration analysis of mixtures of excess C$\overline{1}$r + C$\overline{1}$ In also suggest that only complexes of the type C$\overline{1}$r$_2$–C$\overline{1}$ In$_2$ are formed. This result may indicate cooperativity in the reaction of C$\overline{1}$r with C$\overline{1}$ In. The techniques presently available for investigating the kinetics of this reaction (as seen in fig.1b) are not sufficiently rapid to confirm this possibility.

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