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A STUDY ON THE STRUCTURE AND INTERACTIONS OF THE C1 SUB-COMPONENTS C1r AND C1s IN THE FLUID PHASE

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Summary

1. Both proenzyme and activated C1r, which are dimers at pH 7.4, dissociated into monomers at pH 5.0 (C1r) and 4.0 ($C\bar{1}r$), as shown by the decrease of apparent molecular weight and of sedimentation coefficient, which was shifted from 7.1 *S* (dimer) to 5.0 *S* (monomer). ^{125}I-labelling of $C\bar{1}r$ in the presence of lactoperoxidase occurred, for the dimer, 16—20% in the A chain and 80—84% in the B chain, whereas the distribution was 67.5% and 32.5%, respectively, for the monomer. It appears likely that the two monomers of $C\bar{1}r$ interact through their A chain and that the A and B chains are relatively independent from each other.

2. ^{125}I-labelling of $C\bar{1}s$ in the presence of lactoperoxidase confirmed the calcium-dependent dimerization of this subcomponent. In the monomer, the B chain appears to be embedded in the A chain, as shown by the ^{125}I distribution in these chains, which was 5% and 95%, respectively. This changed after dimerization to 25% and 75%, respectively, which suggests that interactions occur through the A chain of each monomer and lead to an unfolding of the B chain.

3. $C\bar{1}r$ dimer and $C\bar{1}s$ monomer were found to interact in the absence of calcium to form a $C\bar{1}r_2$-$C\bar{1}s$ complex (7.7 *S*), whereas in the presence of calcium the two sub-components were associated into a $C\bar{1}r_2$-$C\bar{1}s_2$ complex (8.7 *S*). It

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Abbreviations: Tos-Arg-OMe, *p*-toluene sulfonyl-L-arginine methyl ester; IgG, immunoglobulin G. The nomenclature of the components of complement is that recommended by World Health Organization (Bull. W.H.O. (1968) 39, 935—936). An enzymatically active component is indicated by a superscript bar, e.g. $C\bar{1}r$. Enzymatic activities are expressed in nanokatals (nkat) as recommended in Enzyme nomenclature (1973).

appears likely that the formation of this tetrameric complex involves both calcium-dependent, and calcium-independent binding forces, and that $C\bar{1}r$ and $C\bar{1}s$ interact through their respective A chain which, in the case of $C\bar{1}s$, is hidden upon association.

Introduction

The first component of complement, C1, is a macromolecular complex consisting of three types of sub-components C1q, C1r and C1s [1]. Binding of C1 to antibody in immune complexes [2], or in the absence of antibody, to other agents such as lipopolysaccharides [3] and some viruses [4,5] leads to the activation of C1. On immune complexes, C1 binds through its C1q subcomponent, which is believed to undergo conformational changes triggering the sequential activation of C1r and C1s.

Recent findings by Bartholomew and Esser [6] concerning the binding and activation of C1 by retroviruses show that binding of both C1q and C1s to the viral membrane is required for the activation of C1. Also, the activation of C1r in C1 bound to IgG-ovalbumin aggregates requires the presence of C1s [2]. These observations point to a possible direct role of C1s in the activation of C1. From observations based on the release of $C\bar{1}$ sub-components from $C\bar{1}$-(IgG-ovalbumin) aggregates [7], the $C\bar{1}$ complex appears to consist of two separate entities: C1q and a complex containing $C\bar{1}r$ and $C\bar{1}s$. Although one can envisage several activation mechanisms for C1 according to the initiating agent, it appears that a detailed investigation of the structure of $C\bar{1}r$, $C\bar{1}s$ and their association forms is likely to contribute to the understanding of the mechanism of C1 activation.

The peripheral iodination of $C\bar{1}r$ and $C\bar{1}s$ allows a comparative estimate of the organization of the A and B chains in each protease and in the complexes involving $C\bar{1}r$ and $C\bar{1}s$. This approach has enabled us to propose a tentative model for the structure and association of $C\bar{1}r$ and $C\bar{1}s$ which reconciles the other findings on these proteases, such as the catalytic and antigenic properties.

Materials and Methods

Materials. Yeast alcohol dehydrogenase, horse spleen apoferritin, beef liver catalase and lactoperoxidase (purified grade) were obtained from Calbiochem. Hen ovalbumin, bovine serum albumin and di-isopropyl phosphorofluoridate were purchased from Sigma. Soybean trypsin inhibitor was from Worthington and horse cytochrome *c* was from Boehringer. $Na^{125}I$ and [1,3-^{3}H]di-isopropyl phosphorofluoridate were obtained from Amersham.

Components of complement. $C\bar{1}r$ and $C\bar{1}s$ were purified by the method described previously [7], which was modified to obtain proenzymic C1r, as described in the following paper [8]. Isolated $C\bar{1}r$ (C1r) and $C\bar{1}s$ were estimated from their absorbance at 280 nm using $E^{1\%}_{1cm}$ = 11.5 and 9.5, respectively [9].

Iodination. $C\bar{1}r$ (C1r) and $C\bar{1}s$ were labelled by ^{125}I according to a modification of the method originally described for C1q by Heusser et al. [10]. 1 ml

protein solution (0.5—1.0 mg/ml), usually in 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4), or in other buffers as stated in the text, was chilled to 0°C and the following quantities of reagents were added: 50 μl of 20 μM KI, 50 μl of $Na^{125}I$ (0.5 mCi/ml, 1.94 Ci/μmol), 50 μl of 1 mg/ml lactoperoxidase and 20 μl of a 1/20 000 dilution of 30% (v/v) H_2O_2. After incubation for 50 min at 0°C, the reaction was stopped by the addition of 20 μl of 20% (w/v) sodium azide, then free $Na^{125}I$ was removed by three successive dialyses against 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4). The iodinated proteins were stored at 4°C in the presence of 0.02% (w/v) sodium azide.

Labelling of C$\bar{1}$s by [1,3-^{3}H]di-isopropyl phosphorofluoridate. C$\bar{1}$s (1.0 mg/ml) was incubated in 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4) for 30 min at 30°C, in the presence of 1 mM [1,3-^{3}H]di-isopropyl phosphorofluoridate (0.44 Ci/mmol). Excess of reagent was removed by three successive dialyses against the buffer.

Sucrose density gradient ultracentrifugation. Samples were sedimented according to Martin and Ames [11] in 5—20% linear sucrose gradients at 4°C for 15 h at 34 000 rev./min using a TST 54 rotor in a Kontron TGA 50 ultracentrifuge. Yeast alcohol dehydrogenase (7.6 *S*), beef liver catalase (11.4 *S*) and horse spleen apoferritin (17.6 *S*) were added into each sample and used as standards for measurement of $s_{20,w}$.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Unreduced samples were prepared by incubation in 4 M urea/1% (w/v) sodium dodecyl sulphate (SDS)/10 mM iodoacetamide/0.1 M Tris-HCl (pH 8.0) for 1 h at 37°C. Reduced samples were incubated for 1 h at 37°C with 130 mM 2-mercaptoethanol in the same buffer without iodoacetamide, then alkylated with 140 mM iodoacetamide for 20 min at 37°C.

Gels containing 6% (w/v) acrylamide were prepared as described by Fairbanks et al. [12] and run at an intensity of 5 mA per gel. Protein staining with Coomassie blue and de-staining of the gels was done according to Weber and Osborn [13]. Carbohydrate was stained by the method of Kapitany and Zebrowsky [14] and the content of each band was estimated from the scanning of the gels at 550 nm. Gels loaded with radioactive samples were cut into 1 mm slices which were counted either directly in a CG 2000 Intertechnique γ counter, or with scintillation fluid in a SL 3000 Intertechnique scintillation counter.

Gel filtration. ^{125}I-labelled C1r was layered on a 1.8 × 55 cm column of Sephadex G-200 equilibrated with 150 mM NaCl/20 mM glycine/30 mM sodium acetate (pH adjusted to 5.0 or to 7.4 with Tris). Yeast alcohol dehydrogenase (150 000), bovine serum albumin (68 000), hen ovalbumin (42 000), soybean trypsin inhibitor (21 000) and horse cytochrome *c* (13 500) were used to calibrate the column.

Esterase activity. C$\bar{1}$s esterase activity was measured at 25°C with 1.5 mM Tos-Arg-OMe as substrate in 150 mM NaCl/1 mM EDTA/50 mM Tris-HCl (pH 7.6) using a spectrophotometric assay at 247 nm.

Results

Dissociation of the dimeric structure of C1r and C̄1r at acid pH

Sucrose gradient ultracentrifugation of proenzymic or activated C1r at pH 7.4, in the presence or absence of calcium, indicated an $s_{20,w}$ of 7.1 ± 0.1 *S*, corresponding to the values obtained previously [15,16]. Lowering the pH to 5.0 and 4.0, respectively decreased the sedimentation coefficient of C1r and C̄1r to 5.0 ± 0.1 *S*, suggesting a disruption of the dimeric structure of C1r and C̄1r into monomers. At pH 10.0, the dimeric form (7.1 *S*) of C1r was retained.

The effect of pH was studied by gel filtration on Sephadex G-200 (Fig. 1). At pH 7.4, the apparent molecular weight of C1r was found to be 250 000, which is a higher value than that obtained by Ziccardi and Cooper [16] and by Chapuis et al. [17] using the same technique but with a different chemical support medium. Nevertheless, this result is consistent with a dimeric structure of C1r at neutral pH. At pH 5.0, C1r eluted from the Sephadex column with an apparent molecular weight of 120 000 (Fig. 1), confirming the sedimentation results and clearly indicating that acid pH leads to dissociation of the C1r dimer into its monomeric sub-units.

Labelling of C̄1r

At pH 7.4, labelling of C̄1r by $Na^{125}I$ in the presence of lactoperoxidase occurred 16—20% in the A chain and 80—84% in the B chain (Fig. 2). A similar but less sharp result was obtained, using a different iodination technique (chloramine T), by Ziccardi and Cooper [18]. It is evident from the aminoacid content of the C̄1r chains [18,9], that the distribution of ^{125}I-labelling cannot be explained by differences in the tyrosine content. It is, therefore, likely to be an indication of the relative area of each chain accessible to the solvent. Thus,

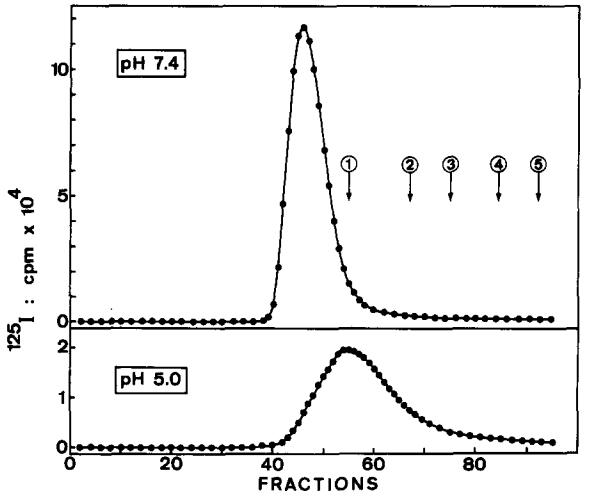

Fig. 1. Gel filtration of proenzymic C1r. ^{125}I-labelled C1r (48 or 18 μg, respectively) was gel-filtered on Sephadex G-200 at pH 7.4 or 5.0, and the radioactivity was measured in each fraction as described in Materials and Methods. M_r standards used: 1, alcohol dehydrogenase; 2, bovine serum albumin; 3, ovalbumin; 4, soybean trypsin inhibitor; 5, cytochrome c.

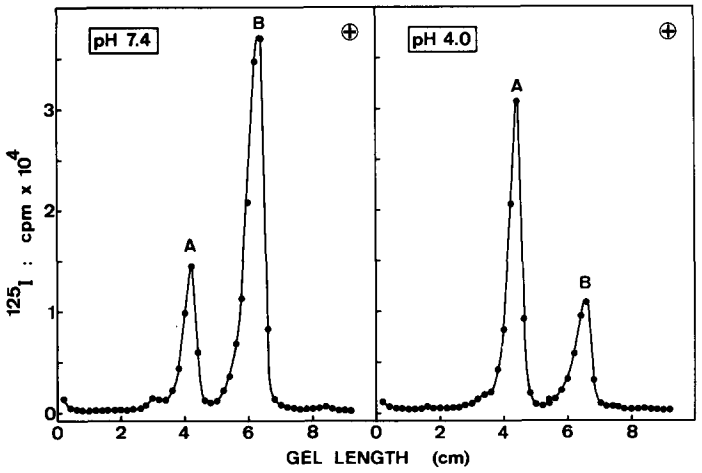

Fig. 2. Labelling of C$\bar{1}$r by ^{125}I. Effect of pH. C$\bar{1}$r (0.5 mg/ml) was labelled by ^{125}I in the presence of lactoperoxidase in 0.15 M NaCl/20 mM glycine/30 mM sodium acetate (pH adjusted to 7.4 or 4.0 with Tris). After reduction and alkylation, ^{125}I-labelled C$\bar{1}$s was analysed by SDS-polyacrylamide gel electrophoresis.

it is probable that the intramolecular C$\bar{1}$r-C$\bar{1}$r interaction involves, in both monomers, an important part of the A chain which thus escapes iodination. In contrast, the B chain is more accessible.

The distribution of labelling between the C$\bar{1}$r chains was significantly modified when iodination was performed under conditions (pH 4.0) where C$\bar{1}$r is in the monomeric form (Fig. 2). In this case, labelling became heavier in the A chain (67.5%) than in the B chain (32.5%), thus confirming the above findings. These values are close to the theoretical distribution expected for the monomer, calculated from the apparent molecular weight and tyrosine content of each chain [18,9], which indicates that both chains occupy external areas in proportion to their weights.

The calcium-dependent C$\bar{1}$s dimerization

Labelling of C$\bar{1}$s by Na^{125}I in the presence of lactoperoxidase occurred, in the absence of calcium, almost exclusively (95%) in the A chain (Fig. 3). This result is in agreement with those obtained by Cooper and Ziccardi [19] and by Sim et al. [20], and it suggests that, in C$\bar{1}$s monomer, the greater part of the B chain is concealed by the A chain, since both contain equivalent amounts of tyrosine [9]. Staining of reduced and alkylated C$\bar{1}$s by the periodic acid-Schiff reagent on SDS-polyacrylamide gels indicated that the A chain contains nearly all the carbohydrate. This result contrasts with the values calculated from the data of Sim et al. [9], but is in agreement with the result obtained by Campbell et al. for bovine C$\bar{1}$s [21]. The concealment of the B chain within the molecule is consistent with the absence of carbohydrate on this chain.

The presence of Ca^{2+} modified ^{125}I-labelling of C$\bar{1}$s, by decreasing the radioactivity incorporated in the A chain (75%) while increasing it to 25% in the B chain (Fig. 3). It appears likely, given a calcium-dependent dimerization of C$\bar{1}$s [15,22], that the interactions involve the A chain of each monomer and lead to

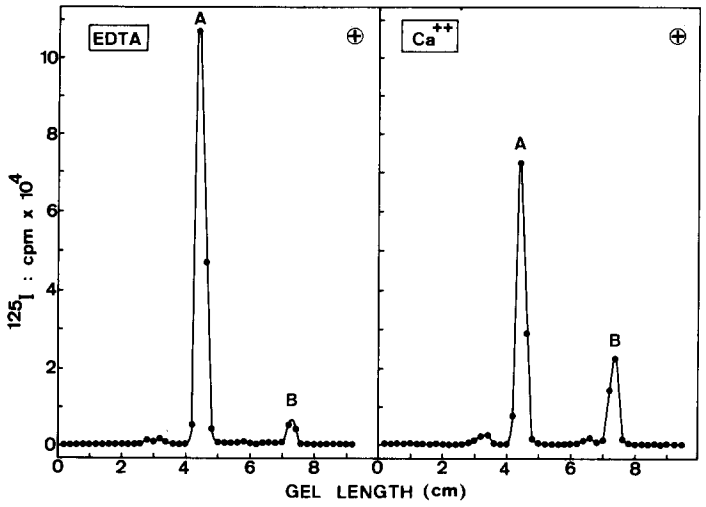

Fig. 3. Labelling of $C\bar{1}s$ by ^{125}I. Effect of calcium. $C\bar{1}s$ (1.0 mg/ml) was labelled by ^{125}I in the presence of lactoperoxidase, in 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4) containing 5 mM EDTA or 5 mM $CaCl_2$. After reduction and alkylation, ^{125}I-labelled $C\bar{1}s$ was analysed by SDS-polyacrylamide gel electrophoresis.

an unfolding of the protein which partially exposes the B chain, which contains the active serine. $C\bar{1}s$ labelled by ^{125}I in the presence of EDTA loses its capacity to dimerize upon addition of calcium and remains as a 4.5 *S* monomer. As the A chain is the most heavily labelled, this favours the hypothesis of a calcium-dependent dimerization involving the A chain of each monomer.

A $C\bar{1}r$-$C\bar{1}s$ interaction independent of calcium

The interactions between $C\bar{1}r$ and $C\bar{1}s$ were studied in the fluid phase by sucrose gradient ultracentrifugation. In the absence of calcium, isolated $C\bar{1}r$ was found to be a dimer (7.1 *S*) while $C\bar{1}s$ was a 4.5 *S* monomer. Under the same conditions, the two proteins, when mixed, associated to form a 7.7 *S* complex, clearly distinct from the $C\bar{1}r$ dimer (Fig. 4). Even in the presence of excess $C\bar{1}r$ ($C\bar{1}r$: $C\bar{1}s$ = 1.3—1.5, w/w), the 7.7 *S* complex formed included all the $C\bar{1}r$, leaving part of $C\bar{1}s$ in its monomeric form (Fig. 4). The most likely hypothesis is therefore that the observed 7.7 *S* complex consists of one $C\bar{1}r$ dimer and one $C\bar{1}s$ monomer bound into a trimeric $C\bar{1}r_2$-$C\bar{1}s$ association. This interaction occurred even at low temperature (4°C) and could explain the observed higher solubility of $C\bar{1}r$ in the presence of $C\bar{1}s$ [7].

The ability of $C\bar{1}s$ to participate in the formation of the complex with $C\bar{1}r$ was diminished after the radioactive labelling. This is demonstrated in Fig. 4a where the complex between $C\bar{1}r$ and ^{125}I-labelled $C\bar{1}s$ is seen to contain only 27% ^{125}I-labelled $C\bar{1}s$. The corresponding value when unlabelled $C\bar{1}s$ was used was 58%. As the labelling occurs almost exclusively in the A chain of $C\bar{1}s$ monomer, it appears likely that this part of the molecule is involved in the binding to $C\bar{1}r$. On the other hand, the iodination of dimeric $C\bar{1}r$ did not appear to alter its capacity of binding to $C\bar{1}s$, suggesting that the $C\bar{1}r$ B chain

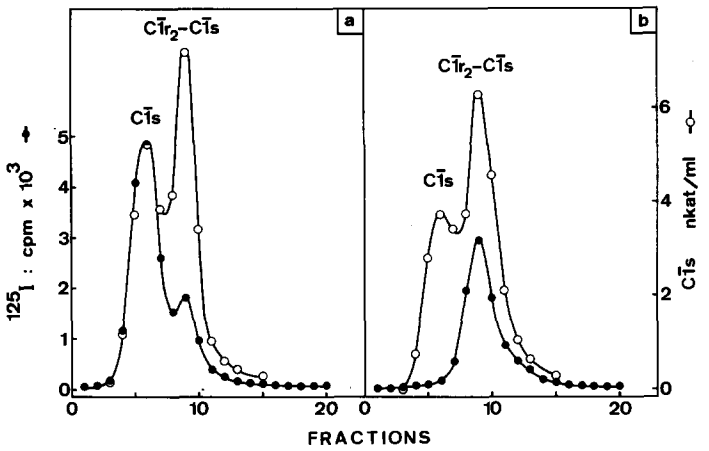

Fig. 4. A calcium-independent interaction between $C\bar{1}r$ and $C\bar{1}s$. A mixture containing $C\bar{1}r$ (1.55 mg/ml), $C\bar{1}s$ (1.15 mg/ml), and either (a) ^{125}I-labelled $C\bar{1}s$ (25 μg/ml) or (b) ^{125}I-labelled $C\bar{1}r$ (24 μg/ml), was incubated for 3 h at 4°C in 5 mM triethanolamine-HCl/145 mM NaCl/5 mM EDTA (pH 7.4), and then analysed by sucrose gradient ultracentrifugation. Fractions were collected from the top of the gradients. $C\bar{1}s$ esterase activity was measured on ToS-Arg-OMe.

(which is the most heavily labelled) does not play an essential role in the interaction with $C\bar{1}s$.

$C\bar{1}s$ labelled by [1,3-^{3}H]di-isopropyl phosphorofluoridate was found to fully retain its capacity to bind to $C\bar{1}r$ and was used as a tracer in mixtures containing native $C\bar{1}r$ and $C\bar{1}s$. As shown in Fig. 5, decreasing the protein concentration led to a progressive decrease of the $C\bar{1}r_2$-$C\bar{1}s$ complex formation at low temperature (4°C). When the concentration of the $C\bar{1}r$ + $C\bar{1}s$ mixture fell down below a value estimated to be about 0.3 mg/ml, all the $C\bar{1}s$ remained in its monomeric (4.5 *S*) form (Fig. 5c).

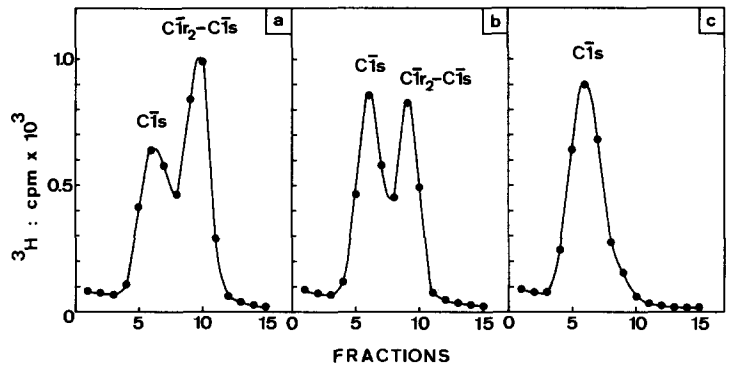

Fig. 5. Effect of protein concentration on the calcium-independent interaction between $C\bar{1}r$ and $C\bar{1}s$. Mixtures containing $C\bar{1}r$ and $C\bar{1}s$ ($C\bar{1}r$: $C\bar{1}s$ = 1.4 : 1.0, w/w) at a total concentration of 2.0, 1.0, and 0.2 mg/ml, were incubated in 5 mM triethanolamine-HCl/145 mM NaCl/5 mM EDTA (pH 7.4) for 4 h at 4°C in the presence of $C\bar{1}s$ labelled by [1,3-^{3}H]-di-isopropyl phosphorofluoridate (40 μg/ml). Samples were analysed by sucrose gradient ultracentrifugation and fractions were collected from the top of the gradients. (a) $C\bar{1}r$ + $C\bar{1}s$ = 2.0 mg/ml; (b) $C\bar{1}r$ + $C\bar{1}s$ = 1.0 mg/ml; (c) $C\bar{1}r$ + $C\bar{1}s$ = 0.2 mg/ml.

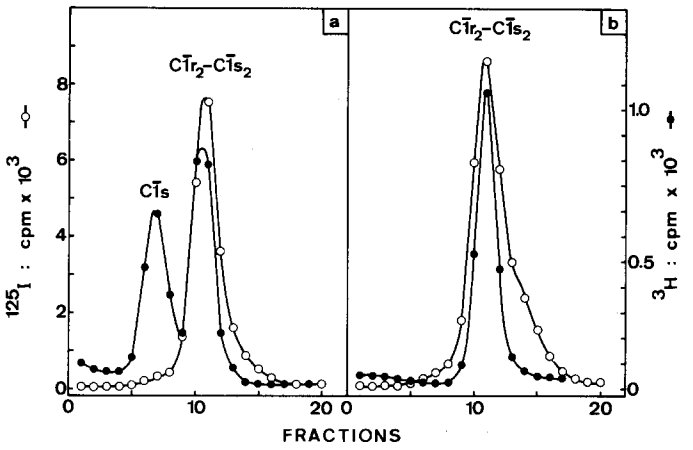

Fig. 6. Stoichiometry of the calcium-dependent $C\bar{1}r_2$-$C\bar{1}s_2$ complex. Mixtures containing ^{125}I-labelled $C\bar{1}r$ and $C\bar{1}s$ labelled by [1,3-^{3}H]di-isopropyl phosphorofluoridate at a total concentration of 140 μg/ml and in ratios $C\bar{1}s$: $C\bar{1}r$ = 1.5 : 1.0, w/w (Fig. 3a) or $C\bar{1}r$: $C\bar{1}s$ = 1.5 : 1.0, w/w (Fig. 3b), were incubated for 30 min at 30°C in 5 mM triethanolamine-HCl/145 mM NaCl/5 mM $CaCl_2$ (pH 7.4). Samples were analysed by sucrose gradient ultracentrifugation and fractions were collected from the top of the gradients.

The $C\bar{1}r_2$-$C\bar{1}s_2$ calcium-dependent complex

In the presence of calcium, $C\bar{1}r$ and $C\bar{1}s$ were found associated on sucrose gradients as a 8.7 *S* complex heavier than the $C\bar{1}r_2$-$C\bar{1}s$ trimer. The stoichiometry of this complex was determined by varying the relative amounts of the two sub-components (Fig. 6): when the $C\bar{1}s$: $C\bar{1}r$ ratio was 1.5 : 1.0 (w/w), all the $C\bar{1}r$ was incorporated in the complex, while part of $C\bar{1}s$ remained as its dimeric (6.0 *S*) form (Fig. 6a). Conversely, for a $C\bar{1}r$: $C\bar{1}s$ ratio of 1.5 : 1.0 (w/w), all the $C\bar{1}s$ was bound to $C\bar{1}r$, whereas excess $C\bar{1}r$ molecules appeared to be associated as soluble aggregates (Fig. 6b). $C\bar{1}r$ and $C\bar{1}s$ mixed in a 1.0 : 1.0 (w/w) ratio were entirely recovered under the 8.7 *S* peak. Therefore, the calcium-dependent complex contains equal amounts of the two sub-components and is likely to be a $C\bar{1}r_2$-$C\bar{1}s_2$ tetrameric association.

Pre-labelling of $C\bar{1}s$ by ^{125}I inhibited the formation of the $C\bar{1}r_2$-$C\bar{1}s_2$ complex, whereas labelling of $C\bar{1}r$ had no effect. The assembly of the complex occurred even at low temperature (4°C) and at low concentrations of each subcomponent (about 50 μg/ml). Nagasawa et al. [23], and Ziccardi and Cooper [16] have also described a calcium-dependent complex containing equal amounts of $C\bar{1}r$ and $C\bar{1}s$.

Labelling of the $C\bar{1}r$-$C\bar{1}s$ associations by ^{125}I

When labelled separately by ^{125}I in the presence of lactoperoxidase, $C\bar{1}r$ and $C\bar{1}s$ each incorporated equivalent amounts of radioactivity. This was not the case when labelling was done with mixtures containing equal amounts of $C\bar{1}r$ and $C\bar{1}s$, as shown by electrophoretic analysis (Fig. 7). Indeed, $C\bar{1}s$ incorporated 25% of total radioactivity in EDTA (Fig. 7a) and only 9% in the presence of calcium (Fig. 7b). These results provide additional evidence that $C\bar{1}r$ and $C\bar{1}s$ assemble as complexes either in the presence or absence of calcium, they also confirm the above observations which suggest that the formation of these com-

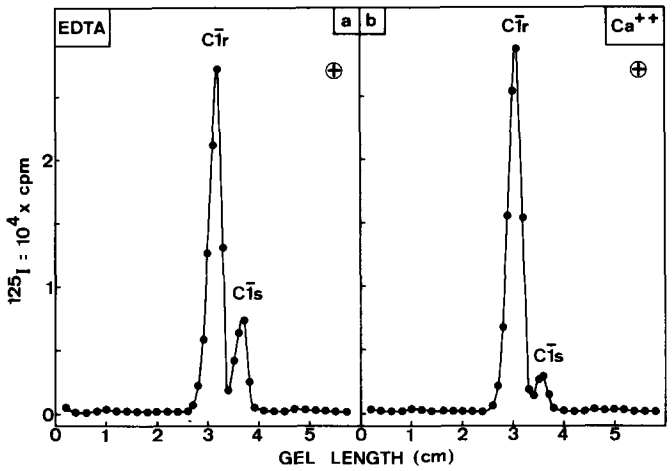

Fig. 7. Labelling of C1r-C1s complexes by ^{125}I. C1r (0.58 mg/ml) + C1s (0.58 mg/ml) were labelled by ^{125}I in the presence of lactoperoxidase, in 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4), containing 5 mM EDTA (Fig. 4a) or 5 mM $CaCl_2$ (Fig. 4b). Unreduced samples were analysed by SDS-polyacrylamide gel electrophoresis.

plexes involves in both cases the A chain of each sub-component. In the case of C1r, labelling of the dimeric form is known to occur principally on the B chain and is, therefore, little decreased by the binding to C1s. In the case of C1s, on the other hand, the A chain is normally the most heavily labelled, either in the monomeric or in the dimeric form, and is concealed by the interaction with C1r, which explains the observed low labelling intensity.

Discussion

The ^{125}I-labelling experiments presented here point out that C1r and C1s are characterized by very different structural features. Basically, if one considers the monomeric tridimensional structure of each sub-component, it appears that, with respect to their molecular weights, both A and B chains of C1r are equally accessible to the solvent, whereas in the case of C1s, the B chain seems to be embedded within the A chain as schematically illustrated in Fig. 8. The localization of carbohydrates favours these pictures. In C1s, the A chain was found to contain all the carbohydrate, while the A and B chains of C1r appeared to bear 51.5% and 48.5%, respectively, of the total carbohydrate, in agreement with previous findings [18,9]. These differences in carbohydrate content could explain why the C1r and C1s B chains, though having identical molecular weights on gel filtration [9], migrate separately on SDS-polyacrylamide gel electrophoresis [9,7], and also why C1r B chain stains poorly with Coomassie blue, compared with C1s B chain. The observed structural differences between C1r and C1s might have important functional consequences, particularly with regard to the B chain, bearing the active serine: in the case of C1r, this is likely to be relatively independent from the A chain and thus accessible and, perhaps movable, whereas the two chains of C1s appear to be more interdependent.

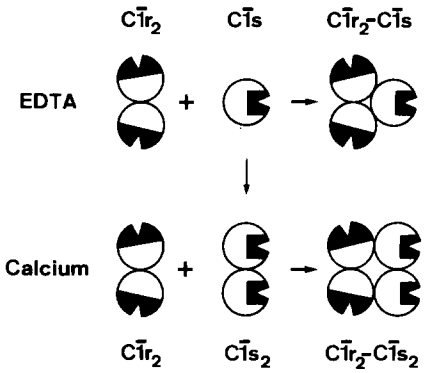

Fig. 8. A schematic diagram of the structure and interactions of $C\bar{1}r$ and $C\bar{1}s$ in the fluid phase. The light B chains bearing the active serine are shaded.

C1r and $C\bar{1}r$ are dimers both in the presence and absence of calcium. As the inter-monomer binding forces are disrupted at acid pH (4.0—5.0) but not at alkaline pH (10.0), it appears likely that the bonds involve aspartic and/or glutamic acids, but their precise nature remains to be elucidated. It is worthy of note that disruption of the dimer requires a lower pH in the case of $C\bar{1}r$ than in that of C1r, which suggests that C1r activation leads to structural changes, which modify the pK of the interacting groups or release new ones, at least in the A chain which is involved in the interaction.

The addition of calcium was shown to increase the sedimentation coefficient of $C\bar{1}s$ [15,22] and to double its apparent molecular weight [15]. Also, calcium was found to influence some of the physicochemical properties of $C\bar{1}s$ [22], and to bind specifically to $C\bar{1}s$ [24]. The experiments reported here provide further evidence for a calcium-dependent dimerization of $C\bar{1}s$. This involves part of the A chain of each monomer, while it apparently leads to an unfolding of the B chain: such a mechanism would be well adapted to the protease function of $C\bar{1}s$, as it can facilitate the active site-substrate interaction.

The interaction between $C\bar{1}r$ dimer and $C\bar{1}s$ monomer in the absence of calcium points out the existence, between the two sub-components, of calcium-independent binding forces which are probably also involved in the building of the tetrameric $C\bar{1}r_2$-$C\bar{1}s_2$ complex, observed in the presence of calcium. The question remains however whether calcium bonds are involved only between the two monomers of $C\bar{1}s$, or whether they also take part in the interaction between $C\bar{1}r_2$ and $C\bar{1}s_2$. Within the $C\bar{1}r_2$-$C\bar{1}s_2$ complex, the two sub-components appear to be intimately bound to each other through their respective A chain which, in the case of $C\bar{1}s$, is likely to be concealed by $C\bar{1}r$. As previously suggested [25], it is likely that the $C\bar{1}r_2$-$C\bar{1}s_2$ complex is one distinct part of the $C\bar{1}$ molecule, which weakly interacts with the other part, C1q, and can be released from it at extreme pH [26,7] or at high ionic strength [7] and also upon interaction of $C\bar{1}$ with $C\bar{1}$ inhibitor [27,28].

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