Activation of human complement serine-proteinase C1r is down-regulated by a Ca^{2+} -dependent intramolecular control that is released in the C1 complex through a signal transmitted by C1q

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The activation of human C1, a Ca^{2+} -dependent complex proteinase comprising a non-enzymic protein, C1q, and two serine proteinases, C1r and C1s, is based primarily on the intrinsic property of C1r to autoactivate. The aim of the present study was to investigate the mechanisms involved in the regulation of C1r autoactivation, with particular attention to the role of Ca^{2+} ions. Spontaneous activation of proenzyme C1r was observed upon incubation in the presence of EDTA, whereas Ca^{2+} ions reduced markedly the activation process. Several lines of evidence indicated that Ca^{2+} inhibited the intramolecular activation reaction but had little or no effect on the intermolecular activation reaction. C1q caused partial release of this inhibitory effect of Ca^{2+} . Complete stabilization of C1r in its proenzyme form was obtained upon incorporation within the Ca^{2+} -dependent C1s–C1r–C1r–C1s tetramer, and a comparable effect was observed when C1s was replaced by its Ca^{2+} -binding α -fragment.

Both tetramers, C1s–C1r–C1r–C1s and C1s α –C1r–C1r–C1s α , readily associated with C1q to form 16.0 S and 14.7 S complexes respectively in which C1r fully recovered its activation potential. Both complexes showed indistinguishable activation kinetics, indicating that the γ B catalytic region of C1s plays no role in the mechanism that triggers C1r activation in C1. The collagen-like fragments of C1q retained the ability to bind to C1s–C1r–C1r–C1s, but, in contrast with intact C1q, failed to induce C1r activation in the resulting complex at temperatures above 25 °C. On the basis of these observations it is proposed that activation of the serine-proteinase domain of C1r is controlled by a Ca^{2+} -dependent intramolecular mechanism involving the Ca^{2+} -binding α -region, and that this control is released in C1 by a signal originating in C1q and transmitted through the C1q/C1r interface.

INTRODUCTION

The first component of human complement, C1, is a complex proteinase formed from the loose association of two subunits, a non-enzymic protein (C1q), and C1s–C1r–C1r–C1s, a Ca^{2+} -dependent tetramer comprising two distinct, but sequence-related serine proteinases. Binding of C1 to immune complexes or various non-immune activators is mediated by C1q, and triggers conversion of proenzyme C1r into its active form C1r, which in turn activates C1s (reviewed by Cooper, 1985; Arlaud et al., 1987; Schumaker et al., 1987). Activation of both C1r and C1s involves cleavage of a single peptide bond, which converts the proenzymes into active proteinases (C1r, C1s) comprising two disulphide-linked chains. The N-terminal A-chains each comprise five structural units, including one epidermal-growth-factor (EGF)-like motif (II) and two contiguous motifs (IV and V) similar to the complement control protein (CCP) motifs also found in various complement proteins (Reid and Day, 1989). The active site-containing C-terminal B-chains are similar to the serine-proteinase domain of mammalian serine proteinases. Each monomeric proteinase is thought to be organized in various domains distributed in two functionally distinct regions. The α -regions, corresponding to the N-terminal half of the A-chains (including the EGF-like motif II) contain one Ca^{2+} -binding site and mediate the Ca^{2+} -dependent C1r–C1s interactions involved in the assembly of the C1s–C1r–C1r–C1s tetramer (Villiers et al., 1985; Busby and Ingham, 1987, 1990; Thielens et al., 1990); the γ B regions, comprising the C-terminal γ -segment of the A-chains

(i.e. the CCP motifs IV and V) and the catalytic B chains, are responsible for the activation and activity of both proteinases (Villiers et al., 1985; Arlaud et al., 1986; Lacroix et al., 1989; Medved et al., 1989).

Several lines of evidence suggest that C1r activation, the initial step of C1 activation, reflects the intrinsic property of C1r to autoactivate, and that this property is modulated by the various interactions involved in the assembly of C1 and the interactions between C1 and the activators (reviewed by Cooper, 1985). Thus, proenzyme C1r alone activates spontaneously in EDTA-containing buffers, but loses this ability within the Ca^{2+} -dependent C1s–C1r–C1r–C1s tetramer. Again, C1r appears to recover its activation potential in C1, as judged from the ability of C1 to undergo spontaneous activation *in vitro* (Ziccardi, 1982; Villiers et al., 1984; Bianchino et al., 1988). Several models of the C1 complex have been proposed, including S- or 8-shaped models in which the elongated C1s–C1r–C1r–C1s tetramer folds into a more compact conformation, allowing interaction between the γ B regions of C1r and C1s (Colomb et al., 1984; Schumaker et al., 1986; Weiss et al., 1986), and it has been hypothesized that this interaction provides the signal that triggers C1r activation (Weiss et al., 1986; Arlaud et al., 1987). This hypothesis is not consistent with the results reported in the present study, which provide support to an alternative mechanism based on the occurrence of a Ca^{2+} -dependent intramolecular control of C1r autoactivation that appears to be released in C1 through a signal transmitted by C1q.

Abbreviations used: The nomenclature of complement components is that recommended by the World Health Organization; activated components are indicated by an overbar, e.g. C1r. C1 Inh, C1 inhibitor; EGF, epidermal growth factor; iPr₂P-F, di-isopropyl phosphorofluoridate; CCP, complement control protein.

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MATERIALS AND METHODS

Materials

Trypsin [treated with tosylphenylalanylchloromethane ('TPCK')] and porcine gastric pepsin were from Sigma. Lactoperoxidase was obtained from Calbiochem. Sephacryl S-300, Concanavalin A-Sepharose and Sephadex G-50 (fine) were purchased from Pharmacia LKB Biotechnology.

Proteins

C1q, C1r, C1s, proenzyme C1r and C1s, and C1 inhibitor were isolated from human plasma as described previously (Reboul et al., 1977; Arlaud et al., 1979, 1980). The concentrations of purified C1q, C1r (C1r), C1s (C1s) and C1 Inh were determined by using values of $A_{280}^{1\%, 1\text{ cm}}$ at 280 nm of 6.8, 12.4, 14.5, and 4.5 and M_r values of 459 300, 86 300, 78 900 and 100 000 respectively (Reid, 1983; Harpel, 1976; Thielens et al., 1990). The N-terminal α -fragment of C1s was prepared by limited proteolysis of C1s with trypsin as described by Thielens et al. (1990). The purified α -fragment migrated on SDS/PAGE under non-reducing conditions as a doublet of mean M_r 28 000, whereas reduction and alkylation yielded a diffuse band of average M_r 31 000. The concentration of C1s α was determined by using $A_{280}^{1\%, 1\text{ cm}}$ value of 10.0 and an M_r of 24 200 (Thielens et al., 1990). C1s and C1s α were labelled with ^{125}I in the presence of lactoperoxidase as described by Arlaud et al. (1980), and unbound ^{125}I was removed by centrifugation on a Sephadex G-50 (fine) column as described by Penefsky (1977). The C1q collagen-like fragments were prepared by digestion of C1q with pepsin essentially as described by Reid et al. (1977). C1q (2 mg/ml) in 100 mM sodium acetate, pH 4.5, was incubated with 4% (w/w) pepsin for 20 h at 37 °C. The reaction was stopped by addition of Tris to pH 8.0, and the digest was fractionated as described by Sasaki and Yonemasu (1983) on a Sephacryl S-300 column (3 cm $\times$ 115 cm) equilibrated in 150 mM NaCl/20 mM Tris/HCl, pH 7.4. Further purification was achieved by affinity chromatography on a concanavalin A-Sepharose column (1.5 cm $\times$ 25 cm) equilibrated in the same buffer. The column bound specifically uncleaved C1q molecules, owing to the presence of N-linked carbohydrate on their globular regions, but did not retain the collagen-like fragments. No residual C1q could be detected by SDS/PAGE analysis of the purified fragments, which migrated as two bands of M_r 28 000 and 24 500 under non-reducing conditions, and as three bands of M_r 17 500, 17 000 and 15 500 after reduction and alkylation. The concentration of the fragments was determined by using an $A_{280}^{1\%, 1\text{ cm}}$ value of 2.1 (Siegel and Schumaker, 1983) and an M_r of 190 000 (Reid, 1983).

SDS/PAGE analysis

The purified C1s α and C1q collagen-like fragments were analysed on 12.5% polyacrylamide gels as described by Laemmli (1970). Protein staining was performed with Coomassie Blue, and phosphorylase *b* (M_r 94 000), BSA (M_r 67 000), ovalbumin (M_r 43 000), soya-bean trypsin inhibitor (M_r 20 100) and α -lactalbumin (M_r 14 400) were used as M_r markers.

Activation assays

Proenzyme C1 was reconstituted at a concentration of $\sim 1 \mu\text{M}$ by incubating the proenzyme C1s–C1r–C1r–C1s tetramer (assembled from equimolar amounts of C1r and C1s) and C1q (C1q/C1s–C1r–C1r–C1s molar ratio 1:1) for 10 min at 0 °C in 145 mM NaCl/50 mM triethanolamine/HCl/2.5 mM CaCl_2 ,

pH 7.4. Pseudo-C1 complexes in which C1q was replaced by its collagen-like fragments, or C1s was replaced by its α -fragment were reconstituted in the same way. Isolated C1r, or the above-mentioned complexes, were incubated at 30 °C or 37 °C for various periods, and activation was measured by monitoring, by SDS/PAGE analysis on 10% polyacrylamide gels under reducing conditions, the conversion of proenzyme C1r and C1s (or of C1r alone) into their activated two-chain counterparts. This was performed by gel scanning after protein staining with Coomassie Blue, using a Shimadzu CS-9000 densitometer. Data were corrected for initial activation, which was less than 5%.

Sucrose-density-gradient ultracentrifugation

Samples were applied to 5–20% (w/v) linear sucrose gradients prepared in 145 mM NaCl/50 mM triethanolamine/HCl/2.5 mM CaCl_2 , pH 7.4. Runs were performed in a SW 60 rotor in a Beckman L8M ultracentrifuge at 185 000 *g* (r_{av} , 9 cm) for 15 h at 4 °C, or at 292 000 *g* (r_{av} , 9 cm) for 4.5 h at 20 °C. After centrifugation, fractions were collected from the top of the gradients, and protein was determined either by measurement of ^{125}I radioactivity or by the method of Bradford (1976). Yeast alcohol dehydrogenase (7.6 S), ox liver catalase (11.4 S) and horse spleen apoferritin (17.6 S) were used as standards for measurements of $s_{20, w}$.

RESULTS

Ca^{2+} slows down C1r autoactivation through inhibition of the intramolecular activation mechanism

As shown in Figure 1(a), proenzyme C1r activated spontaneously upon incubation in the presence of EDTA. The activation curve was slightly sigmoidal, and activation was nearly complete after incubation for 1 h at 37 °C (Figure 2). In keeping with previous observations (Ziccardi and Cooper, 1976; Arlaud et al., 1980), addition of Ca^{2+} ions to the incubation medium markedly reduced the activation rate, but did not completely inhibit the activation process. Depending on the C1r preparation used, the extent of activation after incubation for 1 h at 37 °C in the presence of 2.5 mM CaCl_2 varied from 25 to about 50%, as illustrated by examples shown in Figure 1(b) and Figure 2 respectively. A comparable inhibitory effect of Ca^{2+} was observed when incubation was performed at 30 °C; after 1 h at this temperature, a C1r preparation undergoing 42% activation the presence of EDTA yielded only 7% activation in the presence of Ca^{2+} .

It has been shown that autoactivation of isolated C1r involves two distinct mechanisms: an intramolecular activation reaction catalysed by the proenzyme, and an intermolecular reaction catalysed by activated C1r generated in the course of the reaction (Villiers et al., 1982a). With a view to evaluating the susceptibility of each mechanism to Ca^{2+} ions, the effect of Ca^{2+} was compared with that produced by $\text{iPr}_2\text{P-F}$, a serine-proteinase inhibitor known to react covalently with the active-site serine residue of C1r (Villiers et al., 1982a), and therefore expected to prevent the intermolecular activation reaction. As shown in Figure 1(c), activation of C1r was reduced in the presence of 10 mM di-isopropyl phosphorfluoridate ($\text{iPr}_2\text{P-F}$), but the resulting curve (Figure 2) was strikingly different from that obtained in the presence of Ca^{2+} . Indeed, the sigmoidal shape of the curve was significantly attenuated by $\text{iPr}_2\text{P-F}$ and, in contrast, markedly increased in the presence of Ca^{2+} , consistent with the hypothesis that the former inhibited the intermolecular mechanism, whereas the latter inhibited the intramolecular mechanism. Also consistent with this hypothesis, C1r activation was nearly completely inhibited when incubation was performed

Figure 1 SDS/PAGE analysis of the effects of Ca²⁺ ions and iPr₂P-F on C1r activation

Proenzyme C1r (3.1 μ M) in 145 mM NaCl/50 mM triethanolamine/HCl, pH 7.4, was incubated for various periods at 37 °C in the presence or absence of 5 mM EDTA, 2.5 mM CaCl₂ or 10 mM iPr₂P-F. Samples were analysed by SDS/PAGE under reducing conditions as described in the Materials and methods section. (a) C1r + EDTA; (b) C1r + Ca²⁺; (c) C1r + EDTA + iPr₂P-F; (d) C1r + Ca²⁺ + iPr₂P-F. Lanes 1–7 of gels (a) and (c) correspond to incubation periods of 0, 5, 10, 20, 30, 45, and 60 min, and lanes 1–6 of gels (b) and (d) correspond to incubation periods of 0, 10, 20, 30, 45, and 60 min. C1r, A and B, indicate the bands corresponding to proenzyme C1r and to the A- and B-chains of C1r respectively. The arrow shows the band corresponding to the γ -fragment of the A-chain, generated from autolytic cleavage of C1r (Arlaud et al., 1980).

in the presence of both iPr₂P-F and Ca²⁺ (Figure 1d and Figure 2), in agreement with previous observations by Dodds et al. (1978). With respect to the reproducibility of the experiments described above, it should be stressed that the only variability observed concerned the extent of inhibition produced by Ca²⁺ ions alone. In contrast, no significant variation was observed for kinetics performed in EDTA alone, EDTA + iPr₂P-F, or Ca²⁺ + iPr₂P-F. A likely explanation for the observed variability of the effect of Ca²⁺ is that, despite the continuous presence of proteinase inhibitors throughout the purification, the trace amounts of activated unblocked C1r initially present in the proenzyme preparation may vary from one batch to another, hence inducing variable intermolecular activation.

Other comparative experiments were performed with C1 inhibitor which, given its ability to react with activated C1r, is also expected to prevent intermolecular activation of C1r (Reboul et al., 1977; Schumaker et al., 1987). At a C1 Inh/C1r molar ratio of 6.5:1, corresponding to the physiological C1 Inh/C1r ratio in serum (Bianchino et al., 1988), C1 Inh reduced C1r activation, yielding activation kinetics comparable with those obtained in

Figure 2 Kinetics of C1r activation: effects of Ca²⁺ ions and iPr₂P-F

Proenzyme C1r (1.68 μ M) in 145 mM NaCl/50 mM triethanolamine/HCl, pH 7.4, was incubated for various periods at 37 °C, in the presence or absence of 5 mM EDTA, 2.5 mM CaCl₂ or 10 mM iPr₂P-F. Samples were analysed by SDS/PAGE as shown in Figure 1, and activation was quantified by gel scanning as described in the Materials and methods section. ●, C1r + EDTA; ■, C1r + Ca²⁺; ○, C1r + EDTA + iPr₂P-F; □, C1r + Ca²⁺ + iPr₂P-F.

the presence of iPr₂P-F (results not shown). In the presence of EDTA, C1 Inh and iPr₂P-F had identical inhibitory effects on incubation of C1r for 1 h at 37 °C, whereas inhibition was increased significantly when both inhibitors were added (Table 1). Again, however, maximal inhibition was observed in the presence of Ca²⁺ and C1 Inh; upon incubation for 1 h at 37 °C, substantial stabilization of C1r in the proenzyme form was achieved in the presence of both effectors, and comparable effects were observed when C1 Inh was replaced by iPr₂P-F, or when both iPr₂P-F and C1 Inh were present (Table 1).

With a view to investigating further the effect of Ca²⁺ on C1r activation, proenzyme C1r was incubated at 37 °C with various amounts of activated C1r in the presence or absence of Ca²⁺. In both cases, increasing the relative amount of C1r increased the activation rate of C1r (Figure 3). In fact, the inhibitory effect of Ca²⁺ progressively decreased when the amount of C1r added increased, and the inhibition became negligible (less than 5%) when proenzyme C1r was incubated with an equimolar amount of C1r. This clearly indicated that Ca²⁺ had little or no effect on the inter-molecular activation reaction, providing further support to the hypothesis that the observed effect was due to inhibition of the intramolecular activation mechanism.

C1q induces partial release of the inhibitory effect of Ca²⁺

In the absence of Ca²⁺, the activation kinetics of C1r were not modified when incubation was performed in the presence of C1q. In contrast, addition of an equimolar amount of C1q significantly increased the activation rate of C1r in the presence of Ca²⁺ (Figure 4), suggesting partial release of the inhibitory effect of Ca²⁺ on C1q–C1r interaction. Various aliphatic diamines, including 1,3-diaminopropane, have been shown to dissociate the C1 complex by disrupting the interaction between C1q and the C1s–C1r–C1s tetramer (Villiers et al., 1984). Although this compound had no effect on the activation of C1r alone, it suppressed the ability of C1q to increase the activation rate of C1r in the presence of Ca²⁺, providing further support to the hypothesis of a C1q–C1r interaction (Figure 4). Increasing the C1q/C1r molar ratio from 0.5 to 3.0:1 significantly increased the activation rate of C1r in the presence of Ca²⁺, although complete release of the inhibitory effect of Ca²⁺ could not be achieved

Table 1 Comparison of the inhibitory effects of iPr_2P -F and C1 Inh on C1r activation

Proenzyme C1r (1.57 μ M) in 145 mM NaCl/50 mM triethanolamine/HCl, pH 7.4, was incubated for 1 h at 37 °C in the presence or absence of 5 mM EDTA, 2.5 mM $CaCl_2$, 10 mM iPr_2P -F or 10.2 μ M C1 Inh. Activation was measured by gel scanning as described in the Materials and methods section. For samples containing C1 Inh, the extent of activation was based on formation of the A-chain only, owing to the formation of a C1 Inh–B-chain complex, and to the fact that C1 Inh and proenzyme C1r co-migrated on the gels. Data were corrected for initial activation, which was 3.9%.

C1r activation (%)							
EDTA				Ca^{2+}			
Buffer	iPr_2P -F	C1 Inh	iPr_2P -F + C1 Inh	Buffer	iPr_2P -F	C1 Inh	iPr_2P -F + C1 Inh
92.1	82.5	81.6	69.0	45.4	10.0	10.8	9.2

Figure 3 Ca^{2+} ions have no effect on intermolecular C1r activation

Proenzyme C1r (0.97 μ M) in 145 mM NaCl/50 mM triethanolamine/HCl, pH 7.4, was incubated for 15 min at 37 °C in the presence or absence of 2.5 mM $CaCl_2$ and in the presence of increasing amounts of activated C1r. Activation was measured by SDS/PAGE analysis. ○, Activation in the presence of Ca^{2+} ; ●, activation in the absence of Ca^{2+} ; □, relative inhibition of C1r activation by Ca^{2+} .

Figure 4 Kinetics of C1r activation: effects of Ca^{2+} ions and C1q

Proenzyme C1r (1.29 μ M) in 145 mM NaCl/50 mM triethanolamine/HCl, pH 7.4, was incubated for various periods at 37 °C, either alone or in the presence of an equimolar amount of C1q, in the presence or absence of 5 mM EDTA, 2.5 mM $CaCl_2$ or 20 mM 1,3-diaminopropane. Activation was measured by SDS/PAGE analysis as described in the Materials and methods section. ○, C1r+EDTA; ●, C1r+ Ca^{2+} ; □, C1r+ Ca^{2+} +C1q; ■, C1r+ Ca^{2+} +C1q+1,3-diaminopropane; △, C1r+ Ca^{2+} +1,3-diaminopropane.

Figure 5 Effect of increasing concentrations of C1q on C1r activation in the presence of Ca^{2+}

Proenzyme C1r (0.69 μ M) in 145 mM NaCl/50 mM triethanolamine/HCl, pH 7.4, was incubated for 30 min at 37 °C in the presence of 5 mM EDTA or 2.5 mM $CaCl_2$, and of increasing amounts of C1q. Activation was measured by SDS/PAGE analysis as described in the Materials and methods section. ●, activation in $CaCl_2$; ○, activation in EDTA.

within this range (Figure 5). C1r activation in the presence of EDTA, in contrast, was insensitive to C1q. Analyses by sucrose-gradient ultracentrifugation performed at 4 or 20 °C, with protein concentrations of 0.5–1.0 μ M, showed no evidence for a C1q–C1r complex in the presence of Ca^{2+} , indicating that the association constant was too low to allow formation of a stable complex under these experimental conditions.

Fragment C1s α stabilizes C1r in the proenzyme form and allows formation of an activatable C1q·C1s α –C1r–C1s α complex

We next studied the effect of the Ca^{2+} -dependent association of C1r with C1s or its N-terminal fragment C1s α on its ability to autoactivate. In agreement with previous reports (Ziccardi and Cooper, 1976; Lin and Fletcher, 1980; Villiers et al., 1984), incorporation of C1r within the Ca^{2+} -dependent C1s–C1r–C1s tetramer induced complete inhibition of its autoactivation potential. As shown in Figure 6, the tetramer remained virtually stable in the proenzyme form after incubation for 60 min at 37 °C. As expected from previous studies using activated C1r (Busby and Ingham, 1990; Thielens et al., 1990), C1s α , the N-terminal Ca^{2+} -binding fragment of C1s, associated to proenzyme C1r in the presence of Ca^{2+} to form C1s α –C1r–C1s complexes which sedimented on sucrose-density-gradient ultracentrifugation with an $S_{20,w}$ of 7.6 S (Figure 7c). These complexes exhibited almost the same stability as

Figure 6 Activation kinetics of C1s-C1r-C1r-C1s and C1: effect of substitution of C1s by its *N*-terminal α -fragment

The Ca²⁺-dependent complexes C1s-C1r-C1r-C1s, C1s α -C1r-C1r-C1s α , C1 and C1q·C1s α -C1r-C1r-C1s α were assembled from appropriate amounts of each constituent protein or fragment as described in the Materials and methods section. Each complex (0.97 μ M) in 145 mM NaCl/2.5 mM CaCl₂/50 mM triethanolamine/HCl, pH 7.4, was incubated for various periods at 37 °C and activation was measured by SDS/PAGE analysis. ○, C1s-C1r-C1r-C1s; ●, C1s α -C1r-C1r-C1s α ; □, C1; ■, C1q·C1s α -C1r-C1r-C1s α ; △, C1q + C1s α -C1r-C1r-C1s α + 20 mM 1,3-diaminopropane.

whole C1s-C1r-C1r-C1s tetramers, although some activation (less than 10%) was detected after incubation for 60 min at 37 °C (Figure 6). This indicated, therefore, that fragment C1s α retained the ability of intact C1s to stabilize C1r in the proenzyme form.

Addition of an equimolar amount of C1q to the proenzyme C1s-C1r-C1r-C1s tetramer resulted in the formation of a C1 (C1q·C1s-C1r-C1r-C1s) complex with a sedimentation coefficient of 16.0 S (Figure 7b). As shown in Figure 6, incubation of this complex at 37 °C induced rapid activation of C1r and C1s. The Ca²⁺-dependent C1s α -C1r-C1r-C1s α tetramer also interacted with C1q to form a C1q·C1s α -C1r-C1r-C1s α complex which sedimented on sucrose density gradients with an *S*_{20,w} of 14.6 S (Figure 7d). Incubation of this pseudo-C1 complex at 37 °C resulted in C1r activation, and the activation curve was exactly superimposable to that obtained for whole C1 (Figure 6). Both types of complexes also showed identical activation kinetics at 30 °C (results not shown). Again, as observed for the C1q-C1r interaction (see Figure 4), 1,3-diaminopropane prevented the interaction between C1q and C1s α -C1r-C1r-C1s α , thereby inhibiting the ability of C1q to induce activation of C1r in the tetramer (Figure 6). The above experiments demonstrated that fragment C1s α was sufficient to promote binding of C1r to C1q, and that the resulting pseudo-C1 complex had autoactivation properties apparently indistinguishable from those of whole C1, indicating that the missing portion of C1s, in particular the C-terminal γ B-region, is not essential for C1r activation to occur.

The collagen-like fragments of C1q associate with C1s-C1r-C1r-C1s, but fail to induce C1r activation

With a view to determine the minimal portion of the C1q molecule able to reconstitute an activatable complex upon interaction with C1s-C1r-C1r-C1s, the collagen-like fragments of C1q were prepared by pepsin digestion. We were especially careful to remove all contaminant uncleaved C1q molecules, which was achieved by further purification of the fragments by affinity chromatography on concanavalin A-Sepharose as de-

Figure 7 Analysis by sucrose-density-gradient ultracentrifugation of various complexes assembled from C1r, C1s, C1s α , C1q and the collagen-like fragments of C1q

(a) C1s-C1r-C1r-C1s; (b) C1; (c) C1s α -C1r-C1r-C1s α ; (d) C1q·C1s α -C1r-C1r-C1s α ; (e) C1q collagen-like fragments·C1s-C1r-C1r-C1s; (f) C1q collagen-like fragments pre-incubated for 30 min at 30 °C·C1s-C1r-C1r-C1s. Runs were performed as described in the Materials and methods section. Protein was determined either by measurement of the radioactivity of ¹²⁵I-labelled C1s (a, b) or C1s α (c, d) or by the method of Bradford (1976).

Figure 8 Activation kinetics of C1: effect of substitution of C1q by its collagen-like fragments

The proenzyme C1s-C1r-C1r-C1s tetramer (0.61 μ M) in 145 mM NaCl/2.5 mM CaCl₂/50 mM triethanolamine/HCl, pH 7.4, was incubated for various periods at 30 °C or 37 °C, either alone or in the presence of an equimolar amount of C1q or of its collagen-like fragments. Activation was measured by SDS/PAGE analysis. ○, C1s-C1r-C1r-C1s + C1q, 30 °C; ●, C1s-C1r-C1r-C1s + C1q, 37 °C; △, C1s-C1r-C1r-C1s + C1q fragments, 30 °C; ▲, C1s-C1r-C1r-C1s + C1q fragments, 37 °C; ■, C1s-C1r-C1r-C1s alone, 37 °C.

Figure 9 (a) Activation of C1s-C1r-C1r-C1s on incubation with C1q or its collagen-like fragments (effect of temperature) and (b) effect of heating of C1q or its collagen-like fragments on their ability to induce activation of C1s-C1r-C1r-C1s

(a) The proenzyme C1s-C1r-C1r-C1s tetramer ($0.61 \mu\text{M}$) in $145 \text{ mM NaCl}/2.5 \text{ mM CaCl}_2/50 \text{ mM triethanolamine/HCl}$, pH 7.4, was incubated with an equimolar amount of C1q (●) or its collagen-like fragments (▲) for 1 h at various temperatures. Activation was measured by SDS/PAGE analysis. (b) C1q (●) or its collagen-like fragments (▲) in $145 \text{ mM NaCl}/50 \text{ mM triethanolamine/HCl}$, pH 7.4, were heated for 1 h at various temperatures, then cooled on ice and each incubated at a concentration of $0.61 \mu\text{M}$ with an equimolar amount of C1s-C1r-C1r-C1s for 1 h at 5°C in the presence of 2.5 mM CaCl_2 . Activation was measured by SDS/PAGE analysis.

scribed in the Materials and methods section. In agreement with previous findings (Reid et al., 1977; Siegel and Schumaker, 1983), the collagen-like fragments retained the ability of native C1q to interact with C1s-C1r-C1r-C1s, forming a complex that sedimented on sucrose density gradients as a relatively broad peak with an $S_{20,w}$ of 11.0 S (Figure 7e). However, in sharp contrast with intact C1, incubation of this complex at 30°C or 37°C only induced very slow activation of the C1s-C1r-C1r-C1s tetramer. As shown in Figure 8, less than 20% activation was measured after incubation for 1 h at 37°C , whereas complete activation of intact C1 was obtained in only about 30 min at this temperature.

We next studied activation of both types of complexes upon incubation for 1 h at various temperatures. As shown in Figure 9(a), increasing the temperature from 21°C to 37°C gradually increased activation of intact C1. Activation of the complex formed from the collagen-like fragments of C1q, in contrast, slightly increased between 21°C and 25°C , then decreased at higher temperatures. To further analyse this unusual effect, C1q and its fragments were first preheated at various temperatures, then tested for their ability to induce activation of C1s-C1r-C1r-C1s at 25°C . As shown in Figure 9b, pre-incubation of C1q for 1 h at temperatures up to 45°C had no effect on its ability to induce

activation of the tetramer. In contrast, this ability was progressively lost by the collagen-like fragments after incubation above 21°C . It became clear that the biphasic curve obtained in Figure 9(a) was the result of two contradictory effects of temperature: (i) a stimulation of the activation reaction, comparable with that observed for complexes containing intact C1q (Figure 9a); (ii) an irreversible inhibitory effect, presumably due to structural modifications occurring within the collagen-like fragments of C1q at temperatures above 21°C . Analysis by sucrose-density-gradient ultracentrifugation showed that the collagen-like fragments pre-heated at 25°C or 30°C retained the ability to bind to C1s-C1r-C1r-C1s, but that the resulting complex sedimented as a sharper peak with an $S_{20,w}$ of 10.2 S , i.e. slightly lower than that of the complexes formed from the unheated fragments (Figure 7f). The same effect was observed when the complexes were assembled first, then incubated at 30°C .

DISCUSSION

The observations presented here lend strong support to the hypothesis that the autoactivation potential of C1r is governed by a Ca^{2+} -dependent intramolecular regulatory mechanism. Thus our data confirm that Ca^{2+} ions inhibit strongly, but not completely, autoactivation of isolated C1r, and provide evidence that this inhibitory effect occurs at the level of the intramolecular activation mechanism. Given that the C1r Ca^{2+} -binding sites are located in its α -regions, it appears therefore that binding of Ca^{2+} to these regions, which are distal in the C1r-C1r dimer, induces inhibition of the activation of the catalytic γB -regions, located in the central part of the dimer. This hypothesis is consistent with previous experiments (Lacroix et al., 1989) indicating that removal of the α -regions of proenzyme C1r yields $(\gamma\text{B})_2$ dimers whose activation is insensitive to Ca^{2+} ions. It is known from previous work by Busby and Ingham (1987) that the α -regions of C1r undergo an irreversible low-temperature transition, with a midpoint varying between 26°C at low ionic strength and 40°C in the presence of 0.5 M NaCl , and that Ca^{2+} shifts the midpoint of the transition upward by more than 20°C . This strongly suggests, therefore, that Ca^{2+} stabilizes the α -region of proenzyme C1r in a conformation that inhibits autoactivation of the catalytic regions. In contrast, incubation of C1r at 37°C , or even at 30°C , in the presence of EDTA would result in thermal unfolding of the α -regions, and thereby release the inhibitory effect induced by Ca^{2+} . The occurrence of such an effect of Ca^{2+} in C1r implies transmission of a signal from the α - to the γB -regions, either through the polypeptide backbone, or by direct contact between these regions. The latter possibility appears plausible in light of studies performed on recombinant tissue plasminogen activator, indicating that the finger and/or EGF domains interact with the serine-proteinase module located at the opposite end of the molecule (Novokhatny et al., 1991). Other studies suggest that Ca^{2+} -binding to the EGF-similar region of protein C induces a conformational change in the serine-proteinase domain of the molecule (Öhlin and Stenflo, 1987; Steiner et al., 1980).

Incorporation of proenzyme C1r in the Ca^{2+} -dependent C1s-C1r-C1r-C1s tetramer results in complete inhibition of its autoactivation potential, and a comparable effect is obtained when C1s is replaced by its N-terminal α -fragment, i.e. upon formation of the C1s α -C1r-C1r-C1s α complex. Assembly of both complexes involves interaction between the α -regions of C1r and the corresponding α -region (or fragment) of C1s, as well as binding of one Ca^{2+} atom to each of the four α -regions (Thielens et al., 1990). The enhanced stabilization of C1r in the proenzyme state observed in both cases appears therefore as a

direct consequence of this interaction. This suggests that C1s α strengthens the inhibition induced by Ca²⁺ alone by further stabilizing C1r α in the conformation appropriate for inducing complete inhibition of the autoactivation ability of the (γ B)₂ regions.

In agreement with previous data obtained with activated C1r (Busby and Ingham, 1990), we show that C1s α facilitates binding of proenzyme C1r to C1q. As underlined by those authors, this does not necessarily imply that the C1s α region itself participates in the interaction with C1q. In fact, in agreement with previous findings by Lakatos (1987), our data provide indirect evidence that proenzyme C1r alone interacts with C1q in the presence of Ca²⁺. However, this occurs with an affinity that is probably lower than that estimated by Lakatos (1987) (who reported a K_D of 6×10^{-9} M for C1q–C1r interaction at 20 °C, while at this temperature we obtained no evidence for a C1q–C1r complex at protein concentrations of 0.5–1.0 μ M) and, in any case, lower than that observed for C1s α –C1r–C1r–C1s α complex. That diaminopropane, which is known to dissociate C1 into its two subunits, also prevents the C1q·C1s α –C1r–C1r–C1s α and C1q–C1r interactions strongly supports the hypothesis that C1 assembly involves a major, diamine-sensitive, interaction between C1q and C1r. Other experiments based on the chemical modification of the C1s–C1r–C1r–C1s tetramer are also consistent with this hypothesis (Illy et al., 1993). Our data suggest that C1q binds C1r through its α -region, and that the conformational change induced in C1r α by interaction with C1s α enhances its affinity for C1q. The possibility cannot be excluded, however, that C1s α contains an accessory binding site for C1q that would also be sensitive to diaminopropane, or that residues in the catalytic γ B region of C1r also participate in the interaction. The latter possibility should be considered seriously, given that the affinity of C1s–C1r–C1r–C1s for C1q is apparently not modified upon activation of C1s alone, but decreases significantly when both C1r and C1s, or C1r alone, are activated (Villiers et al., 1982b; Siegel and Schumaker, 1983; Lakatos, 1987). If the α - and γ B-regions of C1r interact with each other in the presence of Ca²⁺, then a possible explanation is that both contribute to the interaction with C1q.

It is remarkable that the complex formed from the association between C1q and C1s α –C1r–C1r–C1s α exhibits autoactivation kinetics that are indistinguishable from those of whole C1. It may be concluded from this observation that the removed portion of C1s, in particular the catalytic γ B-region, has no significant role in the mechanism that initiates C1r activation in C1. This rules out, therefore, the hypothesis that C1r activation is triggered by interaction of the C1s γ B-region with the corresponding region of C1r (Weiss et al., 1986; Arlaud et al., 1987). It should be mentioned, however, that this does not invalidate S- or 8-shaped C1 models featuring this interaction (Colomb et al., 1984; Weiss et al., 1986; Schumaker et al., 1986), considering that such a contact must obviously occur for C1s activation by C1r to proceed.

The observation that C1q restores the activation potential of C1r in C1s–C1r–C1r–C1s and C1s α –C1r–C1r–C1s α , and also partially in C1r alone, supports the hypothesis that, in each case, the signal that triggers C1r activation originates from C1q itself and is transmitted through the C1q–C1r interaction. It is generally accepted that C1q possesses a 'semi-flexible joint' in the central part of its collagen-like region, at the point where the six arms associate to each other to form the fibril-like stem of the molecule (Schumaker et al., 1981; Kilchherr et al., 1985). Fluorescence-spectroscopy measurements suggest that this flexibility allows slow 'wagging' motions of the individual arm-head segments, and it has been speculated that these motions play a

key-role in triggering C1 activation (Hanson et al., 1985). Other experiments using monoclonal antibodies also support this hypothesis (Kilchherr et al., 1986; Hoekzema et al., 1988). If the C1q $\leftrightarrow$ C1s–C1r–C1r–C1s interaction involves a site, or sites, located in the collagen-like arms of C1q, as suggested by electron-microscopic studies (Strang et al., 1982), and a site on C1r contributed by C1r α or shared by the α - and γ B-regions, then it may be hypothesized that such motions of the C1q arms alter, by means of a mechanical effect, the conformation of the C1r α regions, and/or disrupt the C1r α –C1r γ B contact, thereby releasing the Ca²⁺-dependent inhibition of C1r activation and allowing spontaneous C1 activation. Binding of C1 to an immune activator through the globular heads of C1q, or to any multivalent substance able to bend the C1q arms in the appropriate manner, would amplify, or accelerate these motions of the arm-head segments and thereby accelerate C1 activation.

Our data confirm previous experiments (Reid et al., 1977; Siegel and Schumaker, 1983) indicating that the collagen-like regions of C1q retain the ability of native C1q to form a complex with the C1s–C1r–C1r–C1s tetramer. Unexpectedly, we show, however, that in contrast with intact C1, the resulting complex fails to undergo spontaneous activation at temperatures above 25 °C, owing to temperature-dependent modifications occurring in the collagen-like fragments. These modifications take place at low temperature (around 20 °C), and appear irreversible, which at first sight seems inconsistent with the fact that these fragments were prepared by incubation for 20 h at 37 °C (see the Materials and methods section). It may be hypothesized, however, that the very acidic conditions used for pepsin cleavage prevent, at least in part, these modifications. It is tempting to speculate that subsequent incubation of the C1q collagen-like fragments at neutral pH results in a decrease of their segmental flexibility and, thereby, of their ability to induce activation of C1s–C1r–C1r–C1s. This hypothesis appears consistent with electron-microscopic studies (Brodsky-Doyle et al., 1976) indicating that C1q and its collagen-like fragments exhibit an average angle between adjacent arms of $41^\circ \pm 19^\circ$ and $23^\circ \pm 6^\circ$ respectively, suggesting that removal of the C1q heads not only 'closes' significantly the cone defined by the C1q arms, but also reduces the amplitude of their motion. The results of Hanson et al. (1985), showing no evidence for a motion of the arms within the collagen-like fragments of C1q, may also be interpreted by a decreased segmental flexibility of the C1q molecule upon removal of the C1q heads. If this is true, this would suggest that steric constraints, or perhaps repulsive forces imposed by the C1q heads, play a crucial role in the conformation and the dynamics of the C1q molecule. The possibility cannot be excluded, however, that the drastic conditions used for the preparation of the collagen-like fragments alter a structural element that is essential for their segmental flexibility.

Taken together, the observations reported in here provide a complex, but coherent, scheme of the regulation of C1r activation in the C1 complex. Further work will be required to test this model, and to understand the molecular mechanisms involved at the different stages of this regulation. The role of Ca²⁺, in particular, appears to be crucial, not only at the structural level, but also in the control of the autoactivation potential of C1r.

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