Isolation and functional characterization of the proenzyme form of the catalytic domains of human C1r

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The proenzyme form of C1r catalytic domains was generated by limited proteolysis of native C1r with thermolysin in the presence of 4-nitrophenyl-4'-guanidinobenzoate. The final preparation, isolated by high-pressure gel permeation in the presence of 2 M-NaCl, was 70–75 % proenzyme and consisted of a dimeric association of two γ B domains, each resulting from cleavage of peptide bonds at positions 285 and 286 of C1r. Like native C1r, the isolated domains autoactivated upon incubation at 37 °C. Activation was inhibited by 4-nitrophenyl-4'-guanidinobenzoate but was nearly insensitive to di-isopropyl phosphorofluoridate; likewise, compared to pH 7.4, the rate of activation was decreased at pH 5.0, but was not modified at pH 10.0. In contrast, activation of the (γ B)₂ domains was totally insensitive to Ca²⁺. Activation of the catalytic domains, which was correlated with an irreversible increase of intrinsic fluorescence, comparable with that previously observed with native C1r [Villiers, Arlaud & Colomb (1983) *Biochem. J.* **215**, 369–375], was reversibly inhibited at high ionic strength (2 M-NaCl), presumably through stabilization of a non-activatable conformational state. Detailed comparison of the properties of native C1r and its catalytic domains indicates that the latter contain all the structural elements that are necessary for intramolecular activation, but probably lack a regulatory mechanism associated with the N-terminal $\alpha\beta$ region of C1r.

INTRODUCTION

The first component of the classical pathway of complement is a complex proteinase comprising two loosely interacting entities: C1q, the recognition unit, and C1s–C1r–C1s, the catalytic unit, which is a Ca²⁺-dependent association of two serine proteinases, C1r and C1s, that are sequentially activated through limited proteolysis during C1 activation (Reid & Porter, 1981; Cooper, 1985; Arlaud *et al.*, 1987a). The first event of C1 activation is the autoactivation of C1r. Proenzyme C1r is a homodimer comprising two copies of a single-chain polypeptide of *M_r* approx. 90 000, and each monomer is activated through cleavage of a single Arg–Ile bond (Arlaud & Gagnon, 1985), which yields active two-chain proteinases consisting of the disulphide-linked heavy (A) and light (B) chains. Complete sequences of the N-terminal A chain and of the C-terminal B chain have been determined (Arlaud & Gagnon, 1983; Journet & Tosi, 1986; Leytus *et al.*, 1986; Arlaud *et al.*, 1987b). The B chain is homologous to the catalytic chain of other mammalian serine proteinases, whereas the A chain is subdivided into five structural units, referred to as I–V, including one epidermal growth factor-homologous region and two pairs of internal repeats. Each monomer is organized into at least two distinct domains. The catalytic domain, which comprises γ (the C-terminal portion of the A chain) associated with the B chain, can be generated from activated C1r by autolytic cleavage and by extrinsic cleavage with a variety of proteolytic enzymes (Villiers *et al.*, 1985; Arlaud *et al.*, 1986; Weiss *et al.*, 1986), and is considered as a true globular domain.

The interaction region, α , derived from the N-terminal half of the A chain is also obtained by C1r autolytic cleavage, and is most probably divided into a series of small domains which are responsible for specific interaction functions (Arlaud *et al.*, 1987a).

With a view to generating the proenzyme form of C1r catalytic domains, proenzyme C1r was submitted to limited proteolysis by thermolysin, a metallo-endopeptidase, in the presence of 4-nitrophenyl-4'-guanidinobenzoate, a specific inhibitor of C1r activation. The isolated domains, obtained by high-pressure gel permeation, were used as a simplified model for the study of C1r activation, and their properties were compared with those of the native molecule.

MATERIALS AND METHODS

Materials

1,10-Phenanthroline and 4-nitrophenyl-4'-guanidinobenzoate hydrochloride were obtained from Merck. Di-isopropyl phosphorofluoridate (iPr₂P-F) and thermolysin (proteinase type X) were purchased from Sigma. Proenzyme C1r was isolated from human serum as described previously (Arlaud *et al.*, 1980) and its concentration estimated from *A*₂₈₀ using *A*_{1 cm}^{1%} = 11.5 (Sim *et al.*, 1977).

Isolation of the proenzyme form of C1r catalytic domains

Proenzyme C1r was concentrated to 0.5 mg/ml by ultrafiltration (PM10 membrane, Amicon Corp.) and

Abbreviations used: iPr₂P-F, di-isopropyl phosphorofluoridate; the nomenclature of complement components is that recommended by the World Health Organization; activated components are indicated by an overbar, e.g. C1r̄.

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dialysed exhaustively at 0 °C against 0.145 M-NaCl/0.1 mM- i -Pr₂P-F/50 mM-triethanolamine hydrochloride (pH 7.4), to ensure complete removal of EDTA, which is used during C1r purification. C1r was then subjected to limited proteolysis by thermolysin (enzyme/C1r weight ratio = 1:25) for 45 min at 25 °C in the presence of 1 mM-4-nitrophenyl-4'-guanidinobenzoate, and the reaction was terminated by addition of 1 mM-1,10-phenanthroline. After dialysis at 0 °C against 2 M-NaCl/0.1 mM-EDTA/0.5 mM- i -Pr₂P-F/50 mM-triethanolamine hydrochloride (pH 7.4), the digest was fractionated at 4 °C by high-pressure gel permeation on a TSK G3000 SWG column (21.5 mm × 300 mm) (LKB) equilibrated in the same buffer and run at 6.0 ml/min. Fractions containing the (γ B)₂ catalytic domains were pooled, concentrated by ultrafiltration to 0.3–0.5 mg/ml and stored until use at 0 °C in the buffer containing 2 M-NaCl. For activation experiments, C1r catalytic domains were dialysed against 0.145 M-NaCl/50 mM-triethanolamine hydrochloride (pH 7.4). The concentration of the purified (γ B)₂ domains was estimated from A_{280} using $A_{1\text{cm}}^{1\%} = 14.0$, as calculated by the method of Edelhoch (1967) from the number of Trp, Tyr and disulphides (Arlaud & Gagnon, 1983; Arlaud *et al.*, 1987b).

SDS/polyacrylamide-gel electrophoresis

Activation of the (γ B)₂ catalytic domains was monitored by SDS/polyacrylamide-gel electrophoresis after reduction and alkylation. Gels containing 12.5% (v/v) acrylamide were prepared as described by Laemmli (1970) and protein staining was performed with Coomassie Blue. Bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), glyceraldehyde 3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000), bovine trypsinogen (M_r 24000), soya-bean trypsin inhibitor (M_r 20100) and lactalbumin (M_r 14200) were used as molecular mass markers.

Fluorescence studies

Intrinsic-fluorescence-emission spectra of the proenzyme and activated forms of C1r and its catalytic domains were recorded at 15 °C in an SLM 3000 spectrofluorometer in the quantum mode. Excitation was at 283 nm and emission spectra were recorded between 300 and 400 nm, with excitation and emission slits of 2 nm and 4 nm, respectively. Kinetic measurements were performed at 37 °C with excitation at 283 nm and emission at 334 nm.

High-pressure gel permeation

The purified C1r catalytic domains were analysed by high-pressure gel permeation on a TSK G3000 SW column (7.5 mm × 600 mm) (LKB), equilibrated in 50 mM-triethanolamine hydrochloride (pH 7.4) containing 0.145 M- or 2 M-NaCl, or in 0.145 M-NaCl/50 mM-sodium acetate (pH 5.0), and run at 1.0 ml/min.

Sucrose-density-gradient ultracentrifugation

Samples were sedimented at 4 °C according to Martin & Ames (1961) in 5–20% linear sucrose gradients (110000 g; r_{av} 9 cm) for 15 h (buffers containing 0.145 M-NaCl) or 20 h (buffers containing 2 M-NaCl) in a Kontron TST 54 rotor. Yeast alcohol dehydrogenase (7.6 S), ox liver catalase (11.4 S) and horse spleen apoferritin (17.6 S) were used as standards for measurement of

$s_{20,w}$. Proteins were detected by using a Coomassie-Blue-staining method (Bradford, 1976).

N-terminal sequence determination

Automated Edman degradation was performed in an Applied Biosystems model 477 A gas-phase sequencer, and identification of amino acid phenylthiohydantoin derivatives was performed according to the protocol recommended by Applied Biosystems on a model 120 A h.p.l.c. system.

RESULTS

Isolation and analysis of C1r catalytic domains

Proenzyme C1r was subjected to limited proteolysis by thermolysin as described in the Materials and methods section. To minimize C1r activation, incubation was performed at low temperature (25 °C) and in the presence of 4-nitrophenyl-4'-guanidinobenzoate, which is an efficient and reversible inhibitor of C1r activation (Dodds *et al.*, 1978; Arlaud *et al.*, 1980). The digest was fractionated by high-pressure gel permeation in the presence of 2 M-NaCl to prevent activation (see below) and C1r catalytic domains were eluted in the first peak (Fig. 1). Analysis of this material by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions (Fig. 2, lane 1) indicated the presence of a major and a minor band of respective apparent M_r 51000 and 56000, as estimated by comparison with non-reduced standards. Reduction and alkylation (Fig. 2, lane 3) gave a major band of M_r 55000, corresponding to the

Fig. 1. Isolation of C1r catalytic domains by high-pressure gel permeation

Proenzyme C1r was subjected to limited proteolysis by thermolysin, and the digest was fractionated on a preparative TSK G3000 SWG column as described in the Materials and methods section. C1r catalytic domains were contained in the first peak and pooled as indicated (—).

Fig. 2. Analysis of C1r catalytic domains by SDS/polyacrylamide-gel electrophoresis

Lane 1, proenzyme form, unreduced; lane 2, activated form, obtained by incubation for 1 h at 37 °C, unreduced; lane 3, proenzyme form, reduced and alkylated; lane 4, activated form, reduced and alkylated.

proenzyme γ B fragment, with significant amounts of the B chain (M_r 35 500) and of the γ fragment, usually resolved into two bands of M_r 19 700 and 19 500. As estimated from the relative amounts of γ B, B and γ , 70–75% of the γ B molecules were in the proenzyme state.

N-Terminal sequence analysis performed on the purified catalytic domains allowed identification of three sequences. Two major sequences, Thr-Thr-Glu-Ile-Ile-Lys and Thr-Glu-Ile-Ile-Lys(Cys), were obtained in equal amounts, indicating that the Tyr-Thr bond at position 285 of C1r and the Thr-Thr bond at position 286 were both cleaved by thermolysin (Fig. 3), as is the case for activated C1r (Arlaud *et al.*, 1986). An additional sequence was obtained, Ile-Ile-Gly-Gly-Gln-Lys, corresponding to the *N*-terminus of C1r B chain (Arlaud & Gagnon, 1983). Compared with the sum of the *N*-terminal sequences of γ , the *N*-terminal sequence of the B chain represented approx. 25%, which agreed with the estimated amount of proenzyme molecules given above.

Activation of C1r catalytic domains

Incubation of C1r catalytic domains for 1 h at 37 °C at physiological ionic strength (0.145 M-NaCl) led to their activation, as shown by the conversion of the single-chain γ B fragment into the B chain and the γ fragment (Fig. 2, lane 4). Analysis in non-reducing conditions (Fig. 2, lane 2) indicated that activation also resulted in the conversion of the M_r 51 000 species (which is predominant in the proenzyme sample) into the M_r 56 000 species, indicating that the former corresponded to the proenzyme form of the γ B fragment and the latter to the activated

Fig. 3. Schematic representation of the monomeric structure of proenzyme C1r and of the cleavages occurring during limited proteolysis by thermolysin and subsequent activation of the catalytic domains

I–V represent the structural units of the A chain moiety and α , β , γ , the fragments generated from C1r autolytic cleavage (Gagnon & Arlaud, 1985). The positions of the single disulphide bridge connecting the A and B chain moieties, and of the three amino acids (H, D, S) involved in the serine proteinase active site are indicated. S* represents the activated state of the serine residue.

form. A comparable decrease of electrophoretic mobility was also observed upon activation of intact C1r.

Comparative experiments performed with native C1r or its catalytic domains (Fig. 4) indicated that both proteins were activated in a comparable manner upon incubation at 37 °C. Although activation of the catalytic domains proceeded significantly more rapidly, the kinetics of both proteins were slightly sigmoidal, and in each case activation was nearly complete after 1 h. As reported previously (Arlaud *et al.*, 1980; Villiers *et al.*, 1982), the rate of C1r activation was greatly decreased at acid pH (5.0) compared with that observed at pH 7.4 but was not modified at alkaline pH (10.0). Identical results were obtained with C1r catalytic domains: after incubation for 1 h at 37 °C, no inhibition was observed at pH 10.0, whereas 99 % of the molecules remained unactivated at pH 5.0. 4-Nitrophenyl-4'-guanidinobenzoate and iPr_2P -F also exerted parallel effects on the activation of both proteins: at 0.5–1.0 mM, the former induced 90 % inhibition, whereas the activation process was virtually insensitive to iPr_2P -F: concentrations of 0.5–5.0 mM produced no significant inhibition, whereas only restricted effects (10–30 % inhibition) were observed at 10 mM.

As already reported by our group and others (Ziccardi & Cooper, 1976; Arlaud *et al.*, 1980; Villiers *et al.*, 1982), C1r activation was markedly inhibited in the presence of Ca^{2+} ions. Although complete inhibition was never obtained, 60–80 % of the C1r molecules remained in the proenzyme form after incubation for 1 h at 37 °C in the presence of 0.5–2.5 mM- $CaCl_2$. In contrast, activation of C1r catalytic domains was not affected by Ca^{2+} ions, and superimposable kinetics were obtained in the absence of $CaCl_2$ or at concentrations up to 20 mM.

Effect of ionic strength on the activation of C1r catalytic domains

Previous experiments (Villiers *et al.*, 1982) have shown that C1r activation is inhibited at high NaCl concen-

trations. A detailed study performed in the presence of 0.5–2.5 M-NaCl indicated that the inhibition of C1r activation was dependent on the concentration of NaCl and slightly sigmoidal (Fig. 5). Increasing NaCl concentration also inhibited the activation of C1r catalytic domains, although these were more sensitive to ionic strength: 45–50 % inhibition was observed in the presence of 0.5 M-NaCl (compared with only 15–20 % in the case of C1r), and activation of the domains was entirely prevented at concentrations above 1.5 M. This inhibitory effect was also obtained with a variety of other salts, namely KCl, NH_4Cl , $MgCl_2$, $CaCl_2$, Na_2CO_3 , Na_2SO_4 and Na_2HPO_4 . When used at relative concentrations giving a calculated ionic strength value of 1.0 (equivalent to 1 M-NaCl) all of these salts produced comparable inhibition of the activation of C1r catalytic domains upon incubation for 30 min at 37 °C: compared with the value obtained with 1 M-NaCl (85 %), the inhibition ranged from 76 % ($MgCl_2$) to 95 % (NH_4Cl), and no obvious correlation could be established between the relative efficiency of a given salt and its predicted lyotropic behaviour.

Fluid-phase autoactivation of proenzyme C1r has previously been shown to result from two distinct mechanisms: an intramolecular activation catalysed by the proenzyme, and an intermolecular reaction catalysed by activated C1r formed in the process of the reaction (Villiers *et al.*, 1982). The question arose, therefore, of the possible involvement of a comparable two-step mechanism in the activation of the isolated C1r catalytic domains and, in this hypothesis, of the relative susceptibility of each mechanism to inhibition at high ionic strength. As shown in Fig. 6, addition of equimolar amounts of activated C1r catalytic domains significantly accelerated activation of the proenzyme domains at physiological ionic strength, thus demonstrating the ability of C1r catalytic domains to undergo intermolecular activation. When the same experiment was

Fig. 4. Activation kinetics of C1r and C1r catalytic domains

C1r (0.52 mg/ml) and its $(\gamma B)_2$ catalytic domains (0.31 mg/ml) were incubated for 60 min at 37 °C in 0.145 M-NaCl/50 mM-triethanolamine hydrochloride (pH 7.4) and activation was monitored by SDS/polyacrylamide-gel electrophoresis of reduced and alkylated samples as described in the Materials and methods section. ●, C1r; ○, $(\gamma B)_2$.

Fig. 5. Effect of ionic strength on the activation of C1r and C1r catalytic domains

C1r (0.52 mg/ml) and its $(\gamma B)_2$ catalytic domains (0.31 mg/ml) were incubated for 30 min at 37 °C in 50 mM-triethanolamine hydrochloride (pH 7.4) containing 0.5–2.5 M-NaCl, and activation was measured as described in the Materials and methods section. ●, C1r; ○, $(\gamma B)_2$.

Fig. 6. Effect of ionic strength on intramolecular and intermolecular activation of C1r catalytic domains

Proenzyme $(\gamma B)_2$ domains (0.42 mg/ml) were incubated for 45 min at 37 °C in the presence or absence of equimolar amounts of the activated $(\gamma B)_2$ domains, in 50 mM-triethanolamine hydrochloride (pH 7.4) containing either 0.145 M- or 2 M-NaCl. Activation was monitored as described in the Materials and methods section. O, Δ , $(\gamma B)_2$ alone in the presence of 0.145 M or 2 M-NaCl, respectively; ●, ▲, $(\gamma B)_2 + (\gamma B)_2$ in the presence of 0.145 M- and 2 M-NaCl, respectively.

conducted in the presence of 2 M-NaCl, a significant residual activation was observed, whereas activation of the proenzyme domains alone was abolished in these conditions. Thus, these data clearly demonstrated that high ionic strength fully inhibited the intramolecular activation reaction, whereas it had little or no effect on intermolecular activation.

Previous studies (Arlaud *et al.*, 1980) have shown that monomerization of the C1r-C1r dimer, which occurs at pH 5.0, abolishes its ability to autoactivate. The question arose, therefore, of whether the inhibition of the activation of C1r catalytic domains observed at high ionic strength was a consequence of the monomerization of these domains. Analysis by high-pressure gel permeation on a TSK G3000 SW column indicated that, at neutral pH and physiological ionic strength, C1r catalytic domains eluted as a peak of M_r approx. 90000 corresponding to the dimer (elution time 12.6 min). The peak was slightly retarded in the presence of 2 M-NaCl (elution time 12.9 min), probably due to increased hydrophobic interactions with the matrix, whereas at pH 5.0 the protein eluted as a peak corresponding to the monomer (elution time 13.7 min). The absence of monomerization at high ionic strength was confirmed by sucrose-density-gradient ultracentrifugation which indicated that, either at physiological ionic strength or in the presence of 2 M-NaCl, C1r catalytic domains sedimented at neutral pH with a coefficient of 6.0 S, a value which is close to that (6.1 S) previously obtained

Fig. 7. Variation of intrinsic fluorescence during activation of C1r catalytic domains

Proenzyme $(\gamma B)_2$ domains (0.1 mg/ml) were incubated for 45 min at 37 °C in 50 mM-triethanolamine hydrochloride (pH 7.4) containing either 0.145 M- (a) or 2 M-NaCl (b), and variation of intrinsic fluorescence was recorded with excitation at 283 nm and emission at 334 nm. Activation (O) was measured in (a) by removal of aliquots at various times and analysis by SDS/polyacrylamide-gel electrophoresis after reduction and alkylation, as described in the Materials and methods section.

for the dimeric form of activated C1r catalytic domains (Arlaud *et al.*, 1980). In contrast, the sedimentation coefficient decreased to 3.9 S at pH 5.0, in agreement with the values of 3.8 S and 3.5 S obtained respectively for the monomeric form of activated C1r catalytic domains (Arlaud *et al.*, 1980) and for activated C1s catalytic domains.

Variation of intrinsic fluorescence during activation of C1r catalytic domains

The emission fluorescence spectrum of proenzyme C1r catalytic domains showed a maximum at 333 nm, identical to that reported previously for proenzyme C1r (Villiers *et al.*, 1983). After activation for 1 h at 37 °C, the observed maximum was 334 nm, with a 10–15 % increase in fluorescence intensity. The evolution of intrinsic fluorescence of C1r catalytic domains upon incubation at 37 °C is shown in Fig. 7. From a minimal value corresponding to the proenzyme state, the fluorescence increased for a period of 30 min and reached a plateau. Between these limits, the fluorescence increase was closely correlated with activation of the domains, as shown by superimposition of the two curves. These data were closely comparable with those obtained previously with proenzyme C1r (Villiers *et al.*, 1983), and clearly demonstrated that the increase of intrinsic fluorescence

observed during activation of the intact protein occurs within the catalytic domains. When incubation was performed in the presence of 2 M-NaCl (Fig. 7), a slight decrease (4%) in fluorescence was observed, confirming that the increase of intrinsic fluorescence is the specific consequence of a molecular event linked to the activation of the catalytic domains.

DISCUSSION

Limited proteolysis of proenzyme C1r by thermolysin in the presence of 4-nitrophenyl-4'-guanidinobenzoate, a serine proteinase inhibitor that reversibly inhibits C1r activation, allowed us to obtain the proenzyme form of C1r catalytic domains. The purified preparation, isolated by high-pressure gel permeation in the presence of 2 M-NaCl, contained 70–75% of proenzyme molecules and could be stored at high ionic strength for several weeks at 4 °C without significant activation. It is noteworthy that, although the amount of activated fragments at the final stage could not be reduced to below 20–25%, these fragments were expected to be quantitatively inactivated by 4-nitrophenyl-4'-guanidinobenzoate or iPr_2P-F , as these inhibitors, which irreversibly react with the active-site serine of human C1r (Cooper, 1985), were used at several stages of the preparation.

Compared with the native C1r molecule, the isolated catalytic domains displayed similar activation kinetics, and identical inhibition patterns with respect to 4-nitrophenyl-4'-guanidinobenzoate, which almost completely prevented activation, and iPr_2P-F , which had only restricted effects, at relatively high concentration (> 10 mM). Our data thus confirm that C1r autoactivation is poorly sensitive to iPr_2P-F , as previously reported (Sim, 1981; Cooper, 1985). Again, in both cases, the activation reaction was not affected at pH 10.0, but was strongly inhibited at pH 5.0, compared to pH 7.4. In this respect, it should be mentioned that, for unknown reasons, some batches of iPr_2P-F occasionally induced strong acidification of incubation media, thereby producing 'false' inhibition. As previously found in the case of native C1r, fluid-phase activation of C1r catalytic domains was shown to be a two-step mechanism involving intramolecular activation of the proenzyme and intermolecular activation catalysed by the activated domains formed in the process of the reaction. Whereas the former reaction probably represents the basic mechanism of C1r activation within C1, the latter is only observed with the isolated protein or its catalytic domains in solution. The existence of this intermolecular reaction, however, obviously explains the sigmoidal shape of the activation kinetics, and the fact that activation proceeds slightly more rapidly in the case of the isolated domains, given that removal of the *N*-terminal $\alpha\beta$ region of the molecule can facilitate access to the susceptible Arg-Ile bond.

The fact that proenzyme C1r and its catalytic domains exhibit similar autoactivation properties provides further evidence that autoactivation is an intrinsic property of this protein and demonstrates that all the structural elements that are necessary for activation are contained in the $(\gamma B)_2$ domains. In contrast to native C1r, however, activation of C1r catalytic domains was neither inhibited nor retarded by Ca^{2+} ions. This finding rules out the hypothesis of the existence, in addition to the Ca^{2+} -binding site located in the *N*-terminal α region, of a

Ca^{2+} -dependent regulatory site in the $(\gamma B)_2$ domain, which was proposed earlier to explain the inhibitory effect of Ca^{2+} on C1r activation (Arlaud *et al.*, 1987a). If this effect could partly be explained, in the case of isolated C1r, by the increased propensity of the protein to aggregate in the presence of Ca^{2+} (Cooper, 1985), this is not true for the proenzyme C1s–C1r–C1r–C1s tetramer formed in the presence of Ca^{2+} , as this does not auto-activate and shows no tendency to aggregate (Villiers *et al.*, 1984). It appears likely, therefore, that the inhibition of C1r activation observed in the presence of Ca^{2+} is the specific consequence of the binding of Ca^{2+} to the protein. Given that the C1r Ca^{2+} -binding site is located in its *N*-terminal α region (Villiers *et al.*, 1980), probably in the epidermal growth factor-like domain II (Arlaud *et al.*, 1987a), this leads to the hypothesis that a Ca^{2+} -dependent conformational change is transmitted from this region to the $(\gamma B)_2$ catalytic domains, inducing inhibition of the autoactivating ability of these domains. In this hypothesis, the $(\gamma B)_2$ domains would represent a simplified molecule containing all the structures that are necessary for activation, but lacking an essential element involved in the control of activation. Evidence for a comparable Ca^{2+} -dependent transconformation has already been obtained in the case of protein C, a vitamin K-dependent serine proteinase which also comprises an epidermal growth factor-homologous region thought to be responsible for Ca^{2+} binding, and a serine proteinase domain (Steiner *et al.*, 1980; Laurell *et al.*, 1985; Öhlin & Stenflo, 1987).

The activation of both C1r and its $(\gamma B)_2$ catalytic domains was inhibited at high ionic strength. In the case of $(\gamma B)_2$, complete inhibition of the activation of the proenzyme was obtained in the presence of 2 M-NaCl, whereas intermolecular activation was not affected or only slowed down in these conditions. These data thus clearly indicate that the observed inhibition is exerted at the level of the intramolecular mechanism and that the overall activation process is initiated by this mechanism, not by trace amounts of activated material. The inhibitory effect was entirely reversible, as the autoactivating ability of the $(\gamma B)_2$ domains was fully restored, even after incubation for 1 h at 37 °C with 2 M-NaCl, by decreasing the NaCl concentration to 0.145 M. This effect, therefore, was neither due to denaturation nor the consequence of a decreased solubility of the $(\gamma B)_2$ domains, as no aggregation or precipitation was observed at high ionic strength. High-pressure gel permeation and sucrose-density-gradient ultracentrifugation also indicated that monomerization of the $(\gamma B)_2$ domains did not occur at high ionic strength, and this was confirmed by chemical cross-linking with 1-ethyl-3-[3-(dimethyl-amino)propyl] carbodi-imide and by small-angle neutron scattering, the latter technique indicating that the $(\gamma B)_2$ domains have the same radius of gyration, either at physiological ionic strength or in the presence of 2 M-NaCl (Aude, C., Zaccari, J. & Arlaud, G., unpublished work). The most likely hypothesis, therefore, is that high ionic strength stabilizes the conformation of $(\gamma B)_2$ in a stable, non-activatable state, presumably by stabilizing hydrophobic interactions. Thus, despite the apparent non-specificity of the effect with respect to the chemical nature of the salt, this is probably due to the specific blocking of an essential step of the intramolecular activation mechanism.

Interestingly, the effect of ionic strength on the

activation of native C1r was significantly different from that observed with the isolated (γB)₂ domains: the inhibition curve was slightly sigmoidal (Fig. 5), and inhibition was significantly lower in the case of C1r, with a difference ranging from 15–35% between 0.2 M and 1.5 M-NaCl. This shows that, besides the specific effect exerted at the level of the catalytic domains, ionic strength also affects the conformation of the *N*-terminal $\alpha\beta$ region, and that both effects are interdependent. This is in agreement with the report by Busby & Ingham (1987) that the *N*-terminal α region exhibits an irreversible low-temperature transition that is abolished in the presence of Ca²⁺ and shifted at high ionic strength. This also strengthens the hypothesis, proposed above, that C1r activation is governed by a regulatory mechanism involving transmission of a conformational signal from the *N*-terminal region to the catalytic domains.

This study also confirms that activation of proenzyme C1r correlates with an irreversible increase in intrinsic fluorescence, and demonstrates that this change occurs within the (γB)₂ domains, in agreement with previous interpretation (Villiers *et al.*, 1983) and consistent with the fact that, except for a tryptophan residue at position 281, all tryptophan residues of C1r are located within the γB fragment (Arlaud & Gagnon, 1983; Arlaud *et al.*, 1987b). Whether the observed increase precedes or follows cleavage of the Arg-Ile bond that determines the formation of the active site is still an open question. However, both the irreversibility of this increase and the fact that 2 M-NaCl, which prevents the cleavage, also prevents the fluorescence increase, favour the second hypothesis, as proposed previously (Villiers *et al.*, 1983; Colomb *et al.*, 1984). The availability of the proenzyme form of C1r catalytic domains will allow a detailed study of this question, and of the molecular events associated with C1r autoactivation.

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