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PURIFIED PROENZYME C1r

SOME CHARACTERISTICS OF ITS ACTIVATION AND SUBSEQUENT PROTEOLYTIC CLEAVAGE

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Summary

1. Upon incubation for 1 h at 37°C, proenzymic C1r was activated by a proteolytic cleavage comparable to that observed in vivo; after reduction and alkylation, two fragments of apparent molecular weights 57 000 and 35 000 were evident on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. The activation kinetics were slightly sigmoidal and nearly independent of C1r concentration. They were characterized by a marked thermal dependence (activation energy = 45 kcal/mol). The reaction was inhibited by calcium and *p*-nitrophenyl-*p'*-guanidinobenzoate, but poorly sensitive to di-isopropyl phosphorofluoridate. The dependence of the activation rate on pH was unusual; it decreased progressively in the acid range (pH 4.5—6.5) which coincides with the dissociation of the C1r-C1r dimer. Above pH 6.5, the rate increased slightly and showed no clear maximum. These results are consistent with an intramolecular autocatalytic activation mechanism involving the pro-site of each subunit of the C1r-C1r dimer.

2. During a 5 h incubation period at 37°C, $C\bar{1}r$ underwent two proteolytic cleavages which led to the successive removal of two fragments, α (35 000) and

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Abbreviations: pNpGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; IgG, immunoglobulin G; IgM, immunoglobulin M. 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. $C\bar{1}r$.

β (7000—11 000) from each subunit, leaving a dimeric molecule of reduced size (M_r = 110 000; $s_{20,w}$ = 6.1 *S*). The proteolytic process was nearly independent of $\overline{C1}$r concentration and characterized by a pH optimum at 8.5—9.0, and a high activation energy (36.8 kcal/mol). Calcium and *p*-nitrophenyl-*p'*-guanidinobenzoate, and also di-isopropyl phosphorofluoridate and benzamidine were inhibitors of this reaction. The product, $\overline{C1}$r II, retained the original antigenic properties of $\overline{C1}$r and a functional active site, but lost the capacity to bind $\overline{C1}$s. These results are consistent with an autocatalytic intramolecular proteolysis mediated by the active site of each subunit of the $\overline{C1}$r-$\overline{C1}$r dimer.

Introduction

C1, the first component of complement, is activated through the sequential activation of its sub-components C1r and C1s [1,2]. In both cases, the activation is known to occur through the proteolytic splitting of a peptide bond, converting the single-chain zymogen into an active proteinase comprised of two polypeptide chains linked together by at least one di-sulfide bond [3,4].

C1s was shown not to be autoactivatable [5] and its activation is known to be mediated by the active site of $\overline{C1}$r [6]. However, the origin of the proteolytic cleavage which leads to C1r activation in C1 is less obvious; since the third sub-component C1q has not been found to show any proteinase activity, the first proteolytic event of C1 activation must be attributed to C1r itself. This view is supported by the experiments of Dodds et al. [2] showing that C1r is activated autocatalytically in C1. Some discrepancy exists however, since there are conflicting reports that isolated C1r does [7,3,8] or does not [2] autoactivate.

In the same way, isolated $\overline{C1}$r was found to undergo proteolytic cleavage on incubation in the fluid phase [8,9]. In order to reevaluate the question of the intrinsic proteinase activity of C1r and $\overline{C1}$r, we have studied the effect of incubation on the structural and functional properties of these proteins. The results obtained suggest that the dimeric structures of C1r and $\overline{C1}$r allow, in these proteins, successive intramolecular autocatalytic reactions of limited proteolytic cleavage mediated successively by the pro-site (C1r) and the active site ($\overline{C1}$r).

Materials and Methods

Materials. Citrated human plasma was obtained from the Centre de Transfusion Sanguine, Grenoble. Serum was prepared as described previously [10] and stored at −80° C prior to use. pNpGB was purchased from Merck. Benzamidine and di-isopropyl phosphorofluoridate were obtained from Sigma. Soybean trypsin inhibitor was from Worthington. $Na^{125}I$ and [1,3-^{3}H]di-isopropyl phosphorofluoridate were purchased from Amersham.

Preparation of immune aggregates. Rabbit anti-ovalbumin IgG was purified by (Na_2SO_4) precipitation [11] and IgG-ovalbumin aggregates were prepared at equivalence as described previously [12].

Purification of proenzymic C1r and C1s. The activated C1 sub-components were purified by the method described previously [12]. In order to obtain pro-

enzymic C1r and C1s, this method was modified as follows. Unless otherwise stated, all manipulations were performed as close to 0°C as possible. Human serum (1 l) was thawed and centrifuged for 15 min at 20 000 × *g*, pH was adjusted to 7.0, then 50 μg/ml soybean trypsin inhibitor, 1 mM di-isopropyl phosphorofluoridate and 1 mM pNpGB were added and serum was incubated for 15 min at 0°C. Insoluble IgG-ovalbumin aggregates (1 g) were suspended in the serum and the mixture was incubated for 25 min at 0°C. The C1-(IgG-ovalbumin) aggregates were centrifuged for 5 min at 10 000 × *g* and washed, first with 200 ml of 20 mM Tris-HCl, 120 mM NaCl, 5 mM $CaCl_2$ (pH 7.0) containing 40 μg/ml soybean trypsin inhibitor, 1 mM di-isopropyl phosphorofluoridate and 1 mM pNpGB, then twice with 200 ml of the same buffer but without soybean trypsin inhibitor.

The C1-(IgG-ovalbumin) aggregates were suspended four times at 0°C in 50 ml of 40 mM Tris-HCl, 56 mM EDTA (pH 7.0) containing 5 mM di-isopropyl phosphorofluoridate and 1 mM pNpGB, and centrifuged for 5 min at 10 000 × *g*. The four supernatants containing C1r and C1s were pooled, centrifuged for 15 min at 20 000 × *g*, and dialysed overnight at 0°C against 20 mM sodium phosphate, 5 mM EDTA (pH 7.4) containing 1 mM di-isopropyl phosphorofluoridate and 0.1 mM pNpGB. The dialysed proteins were applied to a 2.5 × 15 cm DEAE-cellulose column equilibrated with 20 mM sodium phosphate, 5 mM EDTA (pH 7.4) containing 1 mM di-isopropyl phosphorofluoridate. After washing the column with the starting buffer, proenzymic C1r and C1s were eluted successively with a linear NaCl gradient (400 ml of 20 mM sodium phosphate/5 mM EDTA/1 mM di-isopropyl phosphorofluoridate (pH 7.4) and 400 ml of the same buffer containing 0.25 M NaCl). The fractions containing proenzymic C1r and C1s were pooled as indicated for $C\bar{1}r$ and $C\bar{1}s$ [12] and fresh di-isopropyl phosphorofluoridate was added to 1 mM.

C1r was concentrated to 0.5—1.0 mg/ml by ultrafiltration, then dialysed against 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4) and stored at 0°C. The purified protein (6—8 mg) showed no detectable reaction on double-diffusion with antisera against C1q, C1s, C3, C4, $C\bar{1}$-inhibitor, IgG, IgM and plasminogen. The percentage of $C\bar{1}r$ in the C1r preparations used was between 4 and 8%, as measured by the amount of the A and B chains on SDS-polyacrylamide gels of reduced samples.

C1s was further purified by affinity chromatography on anti-$C\bar{1}r$ IgG-Sepharose 6B, as previously described [12], but in the presence of 1 mM di-isopropyl phosphorofluoridate. After concentration to 1—1.5 mg/ml, the purified protein (9—12 mg) was dialysed against 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4) and stored frozen at −20°C.

C1q was released from the IgG-ovalbumin aggregates and purified on CM-cellulose as described previously [12]. The immune aggregates were washed three times in 200 ml of 20 mM Tris-HCl/120 mM NaCl/5 mM $CaCl_2$ (pH 7.0), each time incubating for 30 min at 30°C, and centrifuging for 5 min at 10 000 × *g*. Finally the aggregates were incubated for 30 min at 30°C in this buffer but containing 2 mM di-isopropyl phosphorofluoridate, and resuspended in 25 mM Tris-HCl/150 mM NaCl (pH 7.0) containing 0.02% (w/v) sodium azide. The recovery of aggregates for re-use was 85—95%.

Immunodiffusion analysis. Double-diffusion was performed according to

Ouchterlony and Nilsson [13] in 1% (w/v) agarose/10 mM EDTA/150 mM NaCl/0.1 M sodium phosphate (pH 7.0).

Labelling of proteins. Labelling of C1r and $C\bar{1}r$ by ^{125}I in the presence of lactoperoxidase and labelling of $C\bar{1}r$ by [1,3-^{3}H]di-isopropyl phosphorofluoridate were performed as described in the preceding paper [14].

SDS-polyacrylamide gel electrophoresis. Preparation of non-reduced, or reduced and alkylated samples, and electrophoresis on gels containing 6% (w/v) acrylamide was as described in the preceding paper [14]. Proteins were stained with Coomassie blue [15] and estimated on the gels by scanning at 550 nm. Gels loaded with radioactive samples were cut into 1 mm slices which were counted as described previously [14].

Measurement of $C\bar{1}r$ protease activity. $C\bar{1}r$ (25 μg/ml) was incubated with proenzyme C1s (0.5 mg/ml) in 5 mM triethanolamine-HCl/145 mM NaCl/2 mM EDTA (pH 7.4) for 30 min at 37°C. The resulting esterase activity of $C\bar{1}s$ was measured with *p*-toluene sulfonyl-L-arginine methyl ester as substrate, as described in the preceding paper [14].

Results

1. *Characteristics of the activation of proenzymic C1r*

Kinetics of activation. Proenzymic C1r behaved on SDS-polyacrylamide gels as a single-chain protein of apparent molecular weight 91 000 in the non-reduced form and 85 000 in the reduced and alkylated form. As shown in Fig. 1a, the incubation of C1r at 37°C led to the progressive cleavage of the mole-

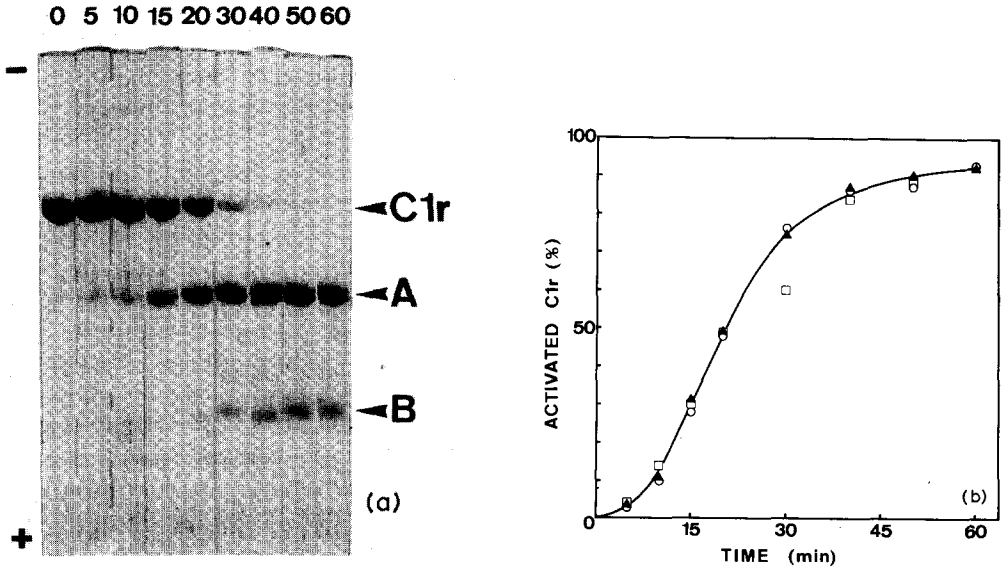


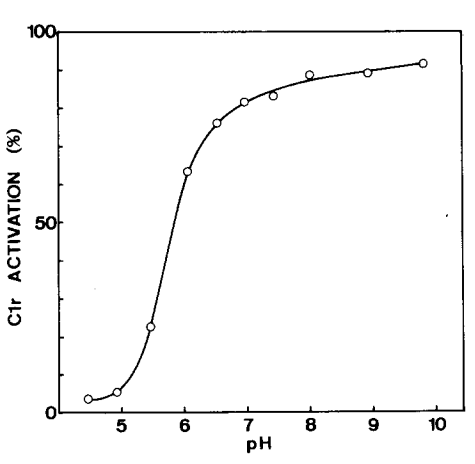
Fig. 1. Kinetics of the activation of proenzymic C1r. C1r was incubated in 5 mM triethanolamine-HCL/145 mM NaCl (pH 7.4) for different periods at 37°C. After reduction and alkylation, samples were analysed by SDS-polyacrylamide gel electrophoresis, as illustrated in Fig. 1a. The amount of activated C1r (Fig. 1b) was obtained from the scanning of the gels after staining with Coomassie blue. The C1r concentration during incubation was 0.320 mg/ml (○), 0.409 mg/ml (□) and 0.663 mg/ml (▲ and Fig. 1a).

cule which, after reduction and alkylation, separated on SDS-polyacrylamide gels into two fragments of apparent molecular weights 57 000 and 35 000, indistinguishable from the typical A and B chains of $C\bar{1}r$ [4,12]. In the absence of reducing agent, the two fragments remained linked together within a molecule of apparent molecular weight 96 000, identical to $C\bar{1}r$. This cleavage of C1r led to its activation, as revealed by the appearance of an active site able to cleave and activate C1s.

As illustrated in Fig. 1b, the kinetics of C1r cleavage were slightly sigmoidal, with a lag-phase of 5—10 min at 37°C. At this temperature, the cleavage was complete after 1 h (Figs. 1a and b). These kinetics were reproducible, as shown by the superposition of the curves obtained from three different C1r preparations, each at a different concentration (Fig. 1b).

Influence of C1r concentration. Labelling of C1r by ^{125}I in the presence of lactoperoxidase did not appreciably modify the kinetics of C1r activation, and ^{125}I-labelled C1r was, therefore, used to study the influence of protein concentration on the activation process. ^{125}I-labelled C1r incubated at 350 μg/ml for 35 min at 37°C was 84% activated, while the proportion activated when the protein was diluted 5-fold was 77%. Although it was not possible to measure the reaction at very low concentrations, it appears that dilution has only a small effect which can be explained by an increased susceptibility of C1r to thermal inactivation.

Influence of pH. As illustrated in Fig. 2, the activation of C1r was strongly inhibited at acid pH, with an inflexion point at pH about 5.7. On the other hand, the reaction was not inhibited at alkaline pH, in fact the rate increased



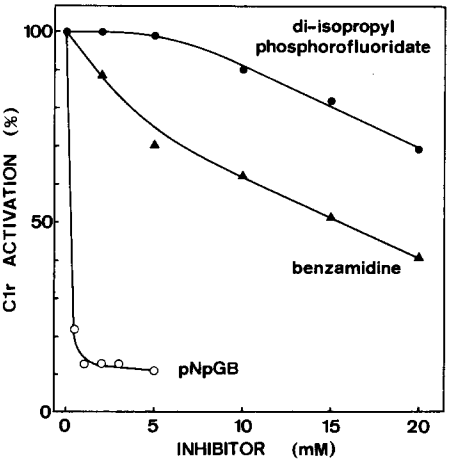


Fig. 2. Influence of pH on C1r activation. C1r (0.455 mg/ml) was incubated for 35 min at 37°C in 0.15 M NaCl/20 mM glycine/2 mM EDTA/30 mM sodium acetate (pH adjusted to 4.5—9.8 with Tris). After reduction and alkylation, samples were analysed by SDS-polyacrylamide gel electrophoresis, and the amount of activated C1r was determined from the scanning of the gels after staining with Coomassie blue.

Fig. 3. Effect of inhibitors on C1r activation. C1r (0.32 mg/ml) was incubated in 50 mM triethanolamine-HCl/145 mM NaCl/1 mM EDTA (pH 7.4) for 40 min at 37°C, in the presence of di-isopropyl phosphorofluoridate, pNpGB or benzamidine (0—20 mM). The amount of activated C1r was determined from SDS-polyacrylamide gel electrophoresis, as described in Fig. 3.

slightly between pH 7.0 and 10.0. The pH curve obtained was therefore very different from those typical of classical enzymatic reactions.

It is noteworthy that the activation reaction was almost completely inhibited at pH 5.0, where C1r was found to be in its monomeric form [14], whereas sucrose gradient ultracentrifugation showed that C1r was a dimer either at pH 6.0 or 10.0.

Effect of inhibitors. The effect of four serine proteinase inhibitors on C1r activation is illustrated in Fig. 3. The most potent inhibitor was pNpGB, which, at 1 mM concentration, caused nearly 90% inhibition. Benzamidine was active at very high concentrations, while inhibition by di-isopropyl phosphorofluoridate started at 10 mM and was very restricted. Soybean trypsin inhibitor had no effect on C1r activation.

The observed effect of these serine proteinase inhibitors points to the 'enzymatic' nature of the C1r activation reaction, and indicates that it involves an active site, or a pro-site with a reactive serine. The differences in reactivity of the three inhibitors tested may be the result of their different accessibility to the active site, depending on their hydrophobicity. More likely, the differences may be explained by the feeble reactivity of the pro-site with benzamidine and di-isopropyl phosphorofluoridate.

The inhibition of C1r activation by pNpGB was reversed by removal of the inhibitor by dialysis, which indicated that the enzyme responsible for activation reacted reversibly with pNpGB, contrary to $C\bar{1}r$ which was found to form a stable acyl-enzyme complex on reaction with pNpGB [16,17]. The most likely hypothesis therefore is that the activation of C1r is mediated by the pro-site of this sub-component.

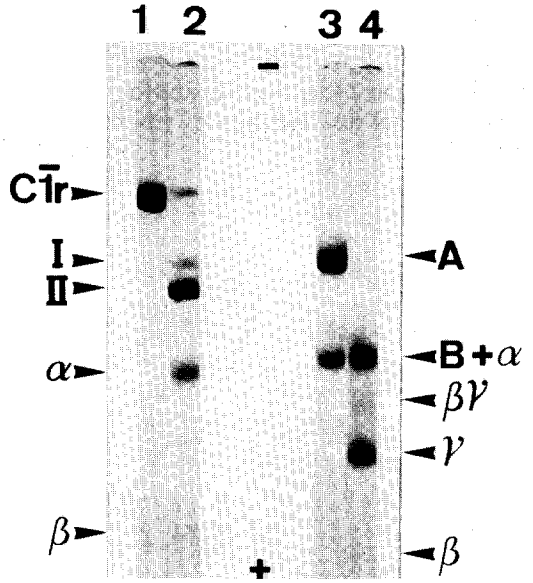


Fig. 4. Proteolytic cleavage of $C\bar{1}r$. $C\bar{1}r$ was analysed by SDS-polyacrylamide gel electrophoresis, before (gels 1 and 3) or after (gels 2 and 4) incubation for 6 h at 37°C in 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4). Proteins were stained with Coomassie blue. Gel 1, $C\bar{1}r$, non-reduced; gel 2, proteolysed $C\bar{1}r$, non-reduced; gel 3, $C\bar{1}r$, reduced and alkylated; gel 4, proteolysed $C\bar{1}r$, reduced and alkylated. The fragment β, which stains poorly, is barely visible.

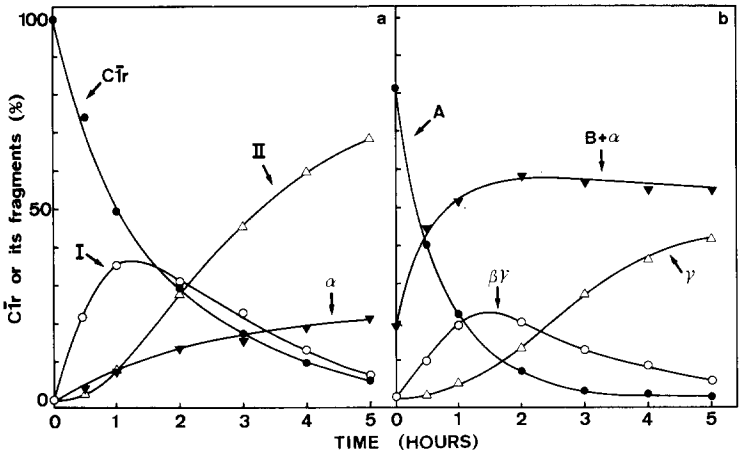


Fig. 5. Kinetics of the C1r proteolytic cleavage. C1r (0.49 mg/ml) was incubated in 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4) for different periods at 37°C. Samples were analysed by SDS-polyacrylamide gel electrophoresis and the relative amounts of C1r and its fragments were determined by scanning of the gels after staining with Coomassie blue. (a) Non-reduced fragments; (b) reduced and alkylated fragments.

In the absence of Ca^{2+}, the addition of EDTA did not modify the activation process. On the other hand, the C1r cleavage was completely inhibited by calcium, within the concentration range tested (0.5—5 mM). The specific binding of calcium to C1r (Villiers, C., unpublished results) might be the direct cause of the observed inhibition.

Influence of temperature. The effect of temperature on C1r activation was characterized by a linear Arrhenius plot within the temperature range studied (25—35°C), with an activation energy of 45 kcal/mol, corresponding to a Q_{10} of 12. These values point to the very high thermal dependence of the reaction.

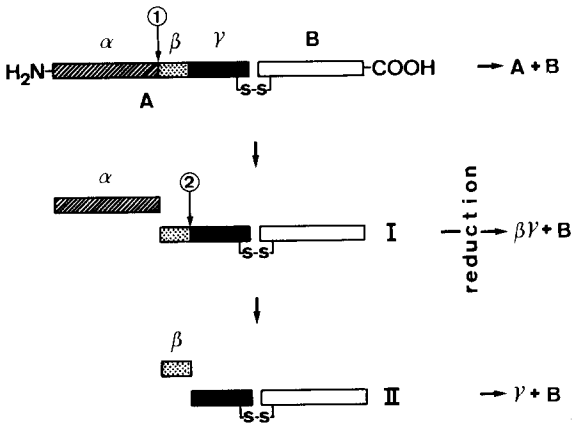


Fig. 6. Schematic drawing of the sequence of proteolytic splits in monomeric C1r. The relative location of the peptides within the A chain is hypothetical.

2. Further proteolytic cleavage of C1r

Characterization of the fragments and kinetics of the reaction. The incubation of C1r at 37°C led to the further proteolytic cleavage of the molecule, as shown by SDS-polyacrylamide gel electrophoresis (Fig. 4). After 6 h, the unreduced protein was cleaved into four fragments, the major ones being fragments II and α (Fig. 4, gel 2). After reduction and alkylation, it can be seen that the typical C1r A chain has disappeared while four fragments are now evident, one of them running at the level of the C1r B chain (Fig. 4, gel 4).

The measurement of apparent molecular weight of each fragment, and the kinetics of appearance of unreduced fragments (Fig. 5a) showed that the ent steps of C1r proteolytic cleavage, which are illustrated in Fig. 6. The kinetics of appearance of unreduced fragments (Fig. 5a) showed that the decrease of monomeric C1r (85 000) was progressive, without lag-phase, and correlated with the increase of fragment α (35 000). The first cleavage gave the transient fragment I (65 000), which, upon the second cleavage, became the fragment II (55 000) (Fig. 5a, Fig. 6).

The kinetics of appearance of the reduced and alkylated fragments (Fig. 5b) indicate that the cleavages occurred in the A chain (57 000), which completely disappeared within 4 h, during which time fragment α appeared, running with the native B chain (35 000). The first cleavage gave the transient fragment βγ (26 500) which was subsequently cleaved into two fragments γ (19 500) and β (7000—11 000), the last one being too poorly stained for accurate quantitation. In each C1r subunit, two successive splits occur (Fig. 6), which result in the release of a large αβ section (42 000—45 000) which represents nearly half the original C1r molecule. These results are consistent with those obtained by Assimeh et al. [8] and by Okamura and Fujii [9].

Effect of cleavages on the sedimentation coefficient of C1r. After 5 h incubation at 37°C, the sedimentation coefficient of C1r at neutral pH was decreased from 7.1 *S* to 6.1 *S*, while it dropped to 3.8 *S* when centrifugation was performed at pH 4.0. It appears therefore that at neutral pH, the α and β fragments of C1r dissociate from the rest of the molecule, which nevertheless retains its dimeric structure. Hence, in agreement with the gel-filtration experiments of Assimeh et al. [8], the proteolytic cleavage of C1r leads to a dimeric molecule of reduced size, comprised of two fragments II (Fig. 6), and of molecular weight approx. 110 000.

Labelling by ^{125}I. Labelling of C1r by ^{125}I in the presence of lactoperoxidase did not appear to modify the proteolytic process. When iodination was performed after cleavage of C1r the β and γ fragments were labelled, whereas they were found less accessible in the intact molecule. The labelling of fragment α was not modified, but the accessibility of the B chain was significantly decreased after the proteolytic splits, which indicates a rearrangement of the protein after removal of the αβ section.

Influence of C1r concentration. ^{125}I-labelled C1r was used to study the influence of C1r concentration on its proteolytic process. During an incubation period of 3 h at 37°C at pH 7.4, decreasing the C1r concentration from 400 to 90 μg/ml led to a relative decrease of the proteolytic reaction of about 5%.

Influence of pH. The influence of pH on the proteolytic cleavage of C1r is shown in Fig. 7. The first cleavage, as illustrated by the decrease of native C1r

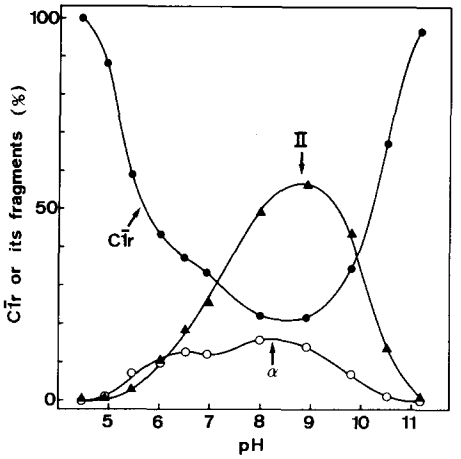


Fig. 7. Effect of pH on C1r proteolytic cleavage. C1r (0.36 mg/ml) was incubated for 3 h at 37°C, in 0.15 M NaCl/20 mM glycine/2 mM EDTA/30 mM sodium acetate (pH adjusted to 4.5—11.2 with Tris). Non-reduced samples were analysed by SDS-polyacrylamide gel electrophoresis, and the relative amounts of C1r and its fragments II and α were determined by scanning the gels after protein staining.

and by the increase of fragment α, was characterized by an asymmetrical curve, with an optimal pH of 8.5 and a shoulder at pH 6.0—6.5. The second cleavage, leading to fragment II, was characterized by a bell-shaped curve, with an optimal pH of 8.9.

Influence of temperature. C1r was incubated for 150 min at temperatures ranging between 27°C and 37°C. The Arrhenius plot was linear in this range, with an activation energy of 36.8 kcal/mol, corresponding to a Q_{10} of 7.2, which points out the high thermo-dependence of the reaction.

Effects of inhibitors. As illustrated in Table I, C1r proteolytic cleavages were totally inhibited in the presence of calcium or pNpGB. Di-isopropyl phosphorofluoridate, and, to a lesser extent, benzamidine were also inhibitors, whereas soybean trypsin inhibitor had no effect. These characteristics are consistent

TABLE I

EFFECT OF SOME INHIBITORS ON C1r PROTEOLYTIC CLEAVAGE

C1r (0.5 mg/ml) was incubated in 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4) for 3 h at 37°C, in the presence of different inhibitors. After reduction and alkylation, samples were analysed by SDS-polyacrylamide gel electrophoresis. The extent of proteolysis was determined by scanning the gels stained with Coomassie blue and the inhibition ratio was calculated relative to a reference sample incubated without inhibitor.

Inhibitor	Concentration	Inhibition (%)
Soybean trypsin inhibitor	1 mg/ml	0
Benzamidine	2 mM	44.7
	10 mM	74.8
Di-isopropyl phosphorofluoridate	2 mM	90.4
	10 mM	99.5
pNpGB	0.5 mM	100
$CaCl_2$	5 mM	100

TABLE II

EFFECT OF THE PROTEOLYTIC CLEAVAGE ON THE C1s ACTIVATING CAPACITY OF $C\bar{1}r$

$C\bar{1}r$ (0.49 mg/ml) was incubated for different periods at 37°C, in 5 mM triethanolamine-HCl/145 mM NaCl/1 mM $CaCl_2$ or EDTA (pH 7.4). The remaining proteinase activity of $C\bar{1}r$ was measured by its capacity to activate proenzyme C1s. $C\bar{1}s$ activity was monitored on *p*-toluene sulfonyl-L-arginine methyl ester (see Materials and Methods).

Incubation period (h)	$C\bar{1}r$ activity remaining	
	EDTA	$CaCl_2$
0	100	100
1	99	93.3
2	100	100
3	100	100
5	101	102

with the hypothesis of a reaction catalysed by the $C\bar{1}r$ active site: this has been found to react irreversibly with pNpGB [16,17] and with di-isopropyl phosphorofluoridate [18,19]. On the other hand, benzamidine was shown to be a reversible inhibitor of $C\bar{1}r$ [20,17] and this perhaps explains its lesser efficiency. The inhibition by Ca^{2+}, which was also described by Assimeh et al. [8], could stem from the specific binding of calcium to $C\bar{1}r$ [21], which perhaps locks the molecule in a stable conformation.

Effect of proteolytic cleavages on the protease-like and antigenic properties of $C\bar{1}r$. As shown in Table II, after an incubation period of 5 h at 37°C, corresponding to 90% proteolysis (see Fig. 5), $C\bar{1}r$ fully retained its C1s-activating capacity.

Labelling of partially cleaved $C\bar{1}r$ by [1,3-^{3}H]di-isopropyl phosphorofluoridate showed that the transient fragment I and the final fragment II retained the

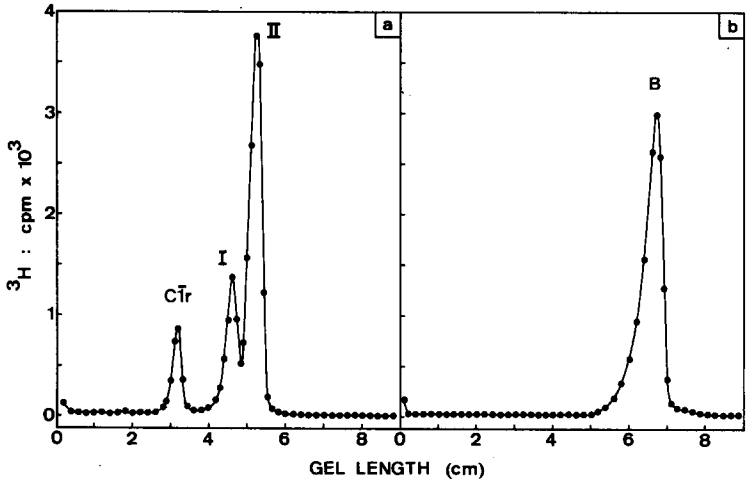


Fig. 8. Labelling by [1,3-^{3}H]di-isopropyl phosphorofluoridate of $C\bar{1}r$ after proteolytic cleavage. $C\bar{1}r$ (0.50 mg/ml) was incubated in 5 mM triethanolamine-HCl/145 mM NaCl (pH 7.4) for 4 h at 37°C, then labelled by [1,3-^{3}H]di-isopropyl phosphorofluoridate and analysed by SDS-polyacrylamide gel electrophoresis. (a) Non-reduced sample; (b) reduced and alkylated sample.

initial $C\bar{1}r$ property of binding di-isopropyl phosphorofluoridate (Fig. 8a). After reduction and alkylation, ^3H radioactivity was entirely recovered under the peak corresponding to the $C\bar{1}r$ B chain (Fig. 8b), in agreement with the findings of Assimeh et al. [8]. Therefore, it clearly appears that the proteolytic process does not modify the B chain which bears the active serine.

As judged by double-diffusion analysis, proteolytically cleaved $C\bar{1}r$ retained the property to bind to anti-$C\bar{1}r$ antibodies. Hence, in agreement with previous results [8], it is likely that the fragment II contains the major antigenic determinants of native $C\bar{1}r$.

Effect of $C\bar{1}r$ proteolytic cleavages on the $C\bar{1}r$-$C\bar{1}s$ interaction. $C\bar{1}r$ proteolytically cleaved for 5 h at 37°C totally lost its capacity to bind $C\bar{1}s$: as shown by sucrose gradient ultracentrifugation, proteolytically cleaved $C\bar{1}r$ mixed with $C\bar{1}s$ in the presence of calcium was found entirely in its dimeric (6.1 *S*) form, whereas under the same conditions, native $C\bar{1}r$ was associated with $C\bar{1}s$ in a 8.7 *S* $C\bar{1}r_2$-$C\bar{1}s_2$ complex [14]. The specific binding of calcium to $C\bar{1}r$ [21] was also abolished by proteolytic cleavages (Villiers, C., unpublished results). As the proteolytic process leads to the removal of the $\alpha\beta$ section of $C\bar{1}r$, it is likely that this part is at least partially implied in the interaction with $C\bar{1}s$ and binding of calcium.

Discussion

Activation of proenzymic C1r

The experiments reported here point out the ability of purified proenzymic C1r to undergo activation in solution.

C1r activation can be considered as independent of concentration, which rules out an intermolecular activation mechanism and is consistent with an autocatalytic intramolecular mechanism. Three reports of the 'spontaneous' activation of proenzymic C1r in the fluid phase have been published [3,7,8], whereas a fourth group concluded that C1r is autoactivatable only when bound in C1 complexed to antibody-antigen aggregates [2].

In the experiments reported by Ziccardi and Cooper [3] and Assimeh et al. [8], activation was observed for purified C1r and gave two fragments similar to those we obtained, which were indistinguishable from the A and B chains of $C\bar{1}r$. The activation process described by these authors was also inhibited by calcium but occurred more rapidly (10—15 min at 37°C) than in our case. Also, it is worthy of note that, in contrast with our results, the activation described by Ziccardi and Cooper [3] was completely prevented by 10 mM di-isopropyl phosphorofluoridate.

Dodds et al. [2] have shown the slow, variable activation of C1r in solution observed by them to be inconsistent with an intermolecular autocatalytic process and attributed it to traces of contaminating proteinases. On the other hand, these authors showed that, when incorporated in C1 bound to immune complexes, C1r was activated autocatalytically. Basically, the question is therefore to know whether the autoactivating capacity of C1r arises 'de novo' within the C1 complex, or is already a property inherent in the isolated C1r molecule, regulated by the interaction of C1r with the other C1 sub-components and by the C1-immune complexes interaction. Comparison of the activation of C1r in

C1 described by Dodds et al. [2], with the activation of isolated C1r reported here shows that both reactions have identical inhibition characteristics with regard to pNpGB (reversible inhibition) and to di-isopropyl phosphorofluoridate (no inhibition below 10 mM) and that both show comparable kinetic curves with a lag-phase.

Our hypothesis, therefore, is that the activating cleavage of C1r, whether in C1 or in the isolated sub-component, is likely to involve the same basic mechanism of intramolecular autocatalysis mediated by the pro-site of C1r. The interaction between C1q, C1r and C1s within the complex might modify the binding of calcium by each individual sub-component and thus relieve the calcium-dependent inhibition observed for isolated C1r. In the same way, the interactions between the C1 sub-components and the C1-immune complex interactions might decrease the very high level of energy required for the activation of isolated C1r, and thus facilitate the reaction.

The involvement of the pro-site of C1r in the activation reaction is compatible with current knowledge on zymogens of proteinases, which for a long time were thought to be totally inactive but, most of them, are likely to bear some catalytic activity towards substrates of the corresponding enzyme [22,23] and react with specific proteinase inhibitors [24]. Considering the dimeric structure of C1r, the internal activation may involve one of two distinct mechanisms, whether the cleavage of each monomer is mediated by its own pro-site, or by the pro-site of the adjacent monomer. The inhibition of the activation observed at acid pH can be assumed to be the consequence of the dissociation of the C1r-C1r dimer which occurs in the same pH range [14]. The activation reaction itself is therefore likely to occur within a hydrophobic area insensitive to the variations of proton concentration. Hence, the most probable of the two proposed mechanisms appears to be the second one, since it implies mutual contacts between the two subunits of the dimer, which favours the formation of hydrophobic areas.

C1r was labelled by ^{125}I in the presence of lactoperoxidase, either before or after activation by incubation. In both cases, the distribution of radioactivity in the A and B chains was comparable to that obtained for $C\bar{1}r$ [14], which would indicate that both proenzyme and activated forms of C1r have similar external structure. Given that the two monomers of $C\bar{1}r$ interact through their A chain and are likely to have their B chain, which bears the active serine, in contact with the solvent [17], one must assume the existence of a transient form of C1r, in which the active serine of each subunit comes into contact with the peptide bond to be cleaved in the adjacent subunit. This form might correspond to an unstable energised state, which would account for the high activation energy noted above. This transient form, able to interact reversibly with pNpGB and to mediate a single proteolytic cleavage, might be equivalent to the intermediary form $C1r^*$, which was previously suggested to explain the activation of C1r in C1 [2,25].

Proteolytic cleavage of $C\bar{1}r$

The proteolytic processes described for $C\bar{1}r$ by Assimeh et al. [8] and by Okamura and Fujii [9] are both closely comparable to the one we observed. As the $C\bar{1}r$ preparations used in each case were purified by very different methods,

the involvement of the same contaminating proteinase appears unlikely. Moreover, labelling of $C\bar{1}r$ by [1,3-^{3}H]di-isopropyl phosphorofluoridate after partial proteolytic cleavage did not give evidence for any other proteinase than $C\bar{1}r$ and its fragments I and II (see Fig. 8), while di-isopropyl phosphorofluoridate was a potent inhibitor of the proteolytic reaction. The proteolytic splits were also observed after prolonged incubation of proenzymic C1r, but the reaction started in this case only after C1r activation has occurred, in agreement with the results obtained by Assimeh et al. [8]. These observations, as well as the other characteristics of the proteolytic cleavage of $C\bar{1}r$, are consistent with an intramolecular autocatalytic reaction, comparable to that involved in C1r activation, but mediated in this case by the active site of $C\bar{1}r$. Within the dimeric $C\bar{1}r$ molecule, this reaction would involve successively two enzymes which also are their own substrate: firstly $C\bar{1}r$, then its fragment I (see (Fig. 6).

It is noteworthy that the removal of the $\alpha\beta$ fragment, representing nearly half the original molecule, does not decrease the proteinase-esterase activity, or the antigenicity of $C\bar{1}r$. Indeed, in agreement with other results which suggest a relative independence of the A and B chains of $C\bar{1}r$ [14], each chain appears to have definite functions to which the other one does not greatly contribute. The A chain of $C\bar{1}r$ was shown to be involved in the inter-subunit $C\bar{1}r$-$C\bar{1}r$ interaction, as well as in the interaction between $C\bar{1}r$ and $C\bar{1}s$ [14]. As the second function, together with binding of calcium to $C\bar{1}r$, was abolished by the proteolytic cleavage, it is tempting to locate the corresponding site within the $\alpha\beta$ region which is removed on this cleavage (see Fig. 6). On the other hand, as the original $C\bar{1}r$ dimeric structure is retained by the final proteolytic fragment II, the $C\bar{1}r$-$C\bar{1}r$ interaction site may be logically situated within the γ part of the A chain (see Fig. 6), although refolding of the protein during proteolysis cannot be excluded.

It appears likely that the $C\bar{1}r$ B chain contains the greater part of the proteinase-esterase site and the antigenic determinants of native $C\bar{1}r$. This is in agreement with previous experiments suggesting that these two functions at least partially overlap; indeed, the reaction of $C\bar{1}$-inhibitor with $C\bar{1}r$ abolishes the antigenicity of $C\bar{1}r$ [26,27], while the formation of immune complexes between $C\bar{1}r$ and anti-$C\bar{1}r$ IgG inhibits the proteinase activity of $C\bar{1}r$ [5]. Comparable processes of limited proteolysis were described for plasmin [28] and for rabbit C1s [29]. In both cases, proteolytic cleavage was shown to occur in the N-terminal A chain and did not significantly decrease the enzymatic activity of the protein. The release of an N-terminal peptide also occurs in the course of the activation reaction of some proteases [22]. In the case of $C\bar{1}r$, the function of these cleavages, which leave the active site intact, remains to be determined.

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