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PROENZYMIC C1s ASSOCIATED WITH CATALYTIC AMOUNTS OF C1r

STUDY OF THE ACTIVATION PROCESS

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Summary

1. Proenzymic C1s isolated from human plasma by euglobulin precipitation and DEAE-cellulose chromatography is associated with trace amounts of C1r (0.5—1% on a molar basis). Incubation for 2 h at 37°C leads to the proteolytic activation of C1s. The proteolysis is characterized by the sigmoidal appearance of $C\bar{1}s$ esterase activity and of the typical heavy (57 000-dalton) and light (28 000-dalton) fragments of C1s on sodium dodecyl sulphate -polyacrylamide gel electrophoresis.

2. The C1s activation process observed is markedly temperature and concentration dependent, and the rate of activation is decreased by calcium and high ionic strength (I = 0.9). Diisopropyl phosphorofluoridate, benzamidine, polyanethol sulfonate and pentosane polysulphate inhibit the activation, which is also sensitive to C1-inactivator and anti-$C\bar{1}r$ IgG. From the kinetic experiments and from the inhibition characteristics, the activation of C1s can be attributed to the presence of C1r, which appears to undergo activation and then to activate secondarily C1s.

Introduction

The first component of complement C1 is a multimolecular complex made up of three types of subcomponents, C1q, C1r, C1s. C1r and C1s are both proteases and exist normally in blood as proenzymes which become activated by interaction of C1 with immune complexes. The activation of C1 initiates

Abbreviations: EDTA, ethylenediaminetetracetic acid; Tos-Arg-OMe, *p*-toluene sulfonyl-L-arginine methyl ester. The nomenclature of the components of complement is that recommended by World Health Organization (1968). Activation of a component is indicated by a bar. Enzymatic activities are expressed in nanokatals (nkat) as recommended in Enzyme Nomenclature (1973).

the chain reaction of the other complement components, C2—C9.

No convincing experimental observations have yet been published to explain the mechanism of activation of proenzymic C1r, but it is known that activated C1r is able to cleave and thereby activate proenzymic C1s. It is now established that proenzymic C1s can be activated by a number of extrinsic enzymes including plasmin, kallikrein or trypsin, but C1s does not undergo self-activation [1].

As C1r and C1s are found in a 1 : 1 stoicheiometry in serum C1, which represents an unusual enzyme to substrate ratio, it is of interest to study the activation of C1s by a catalytic amount of C1r which is found to copurify with C1s [1]. Results obtained suggest that this associated proenzymic C1r is able to undergo activation and then activate C1s. The characteristics of the activation process and the influence of various inhibitors are reported.

Materials and Methods

Materials. Di-isopropyl phosphorofluoridate was purchased from Sigma. di-iso[^{3}H]propyl phosphorofluoridate was obtained from the Radiochemical Centre, Amersham (U.K.). Benzamidine · HCl and sodium polyanethol sulfonate were from Eastman Kodak Co. (U.S.A.). Soybean trypsin inhibitor was purchased from Worthington Biochemical Corp. (U.S.A.). Pentosane polysulphate was a generous gift from Clin-Midy (France).

Purification of C1s. Proenzymic and activated C1s were purified from human serum as described elsewhere [1] by euglobulin precipitation followed by DEAE-cellulose chromatography and affinity chromatography on anti-$C\bar{1}r$ IgG-Sepharose 6B. For the activation studies presented here, the final step of the purification procedure was omitted, and the eluate from DEAE-cellulose was used as the final C1s preparation. This eluate was concentrated to 1—2 mg/ml and di-isopropyl phosphorofluoridate was eliminated by dialysis against 20 mM Tris · HCl, 150 mM NaCl (pH 7.6).

Preparation of anti-$C\bar{1}r$ IgG. Anti-$C\bar{1}r$ antiserum was obtained by immunization of rabbits with $C\bar{1}r$ prepared according to Takahashi et al. [2]. The IgG fraction was prepared from pooled rabbit anti-$C\bar{1}r$ antisera by sodium sulphate precipitation [3] and subsequently treated with 2 mM di-isopropyl phosphorofluoridate for 30 min at 30°C. No cross-reaction between anti-$C\bar{1}r$ IgG and C1s was detectable by double-diffusion.

Purification of $C\bar{1}$-inactivator. $C\bar{1}$-inactivator was purified from pooled citrated human plasma as described by Reboul et al. [4].

Purification of $C\bar{1}r$. For specific activation studies $C\bar{1}r$ was purified to homogeneity by the method of Gigli et al. [5].

Protein determination. Purified protein solutions were routinely estimated from absorbance measurements at 280 nm using $E^{1\%}_{1cm} = 9.5$ for C1s and $C\bar{1}s$ [5], $E^{1\%}_{1cm} = 11.5$ for $C\bar{1}r$ [5] and $E^{1\%}_{1cm} = 4.5$ for $C\bar{1}$-inactivator [6].

Measurements of $C\bar{1}s$ activity. The esterase activity of $C\bar{1}s$ was usually measured at 25°C with 1.5 mM Tos-Arg-OMe, 50 mM Tris · HCl, 150 mM NaCl, 1 mM EDTA (pH 7.6) using a spectrophotometric assay as described previously [7].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Preparation of 6% polyacrylamide gels and treatment of protein samples were as described

elsewhere [1]. For quantitative estimation of protein bands, the gels were stained with Coomassie Blue [8] and scanned at 550 nm using a Beckman Acta C III spectrophotometer equipped with a gel scanning attachment. $C\bar{1}s$ was labelled with di-iso[^{3}H]propyl phosphorofluoridate as described elsewhere [1]. Gels loaded with radioactive samples were cut into 2.0-mm slices which were counted in 10 ml scintillation fluid.

Results

General features of C1s activation

As shown in the preceding paper [1], proenzymic C1s isolated from DEAE-cellulose became activated on incubation at 37°C. This instability was overcome by adding a final step of purification, affinity chromatography on anti-$C\bar{1}r$ IgG-Sepharose 6B, and the activation phenomenon was consequently attributed to the presence of trace amounts of C1r and/or $C\bar{1}r$.

Further studies on C1s isolated from DEAE-cellulose showed that the activation curve was sigmoidal, as measured by the increase of $C\bar{1}s$ Tos-Arg-OMe esterase activity (Fig. 1). The increase of esterase activity correlated well with the appearance of the characteristic heavy (57 000) and light (28 000) fragments of C1s observed on sodium dodecyl sulphate polyacrylamide gel electrophoresis of the corresponding reduced and alkylated samples (Fig. 1). This correlation indicates clearly that the observed activation proceeded through a proteolytic split of proenzymic C1s.

The activation process followed closely the equation:

$$\mathrm{Log}_{10}\frac{A}{(A_\infty - A)} = k \cdot t + C$$

where k, A_∞ and C are respectively the second-order rate constant, the final esterase activity of $C\bar{1}s$ and a constant (Fig. 1). Similar results were reported by Morgan and Nair [9], who concluded that the activation of C1s was autocatalytic. As purified proenzymic C1s was shown to be unable to undergo any activation by itself [1], it appears likely that the sigmoidal activation reported by these authors, as well as the activation process described here may be explained by the activation of a contaminant protease which secondarily activates C1s.

C1s incubated for 2 h at 37°C was able to bind ^{3}H-labelled di-isopropyl phosphorofluoridate. Radioactivity was entirely recovered in the $C\bar{1}s$ band on sodium dodecyl sulphate-polyacrylamide gels of unreduced samples (Fig. 2a). After reduction and alkylation, 94% of the total radioactivity was found in the light fragment of $C\bar{1}s$ (Fig. 2b). $C\bar{1}r$ contamination of C1s was not visible after staining of the gels and the quantity of radioactivity associated with the expected running position of $C\bar{1}r$ light chain (Fig. 2b) was insignificant. It was concluded from the sensitivity of labelling method that the maximum possible quantity of $C\bar{1}r$ in the sample was 0.8% (mol/mol) relative to $C\bar{1}s$. Labelling of C1s in the same conditions without prior activation led to an incorporation of 0.5% of di-iso[^{3}H]propyl phosphorofluoridate compared with incorporation by activated C1s, and the total radioactivity was found associated with the unreduced $C\bar{1}s$ band and with the reduced and alkylated $C\bar{1}s$ light fragment.

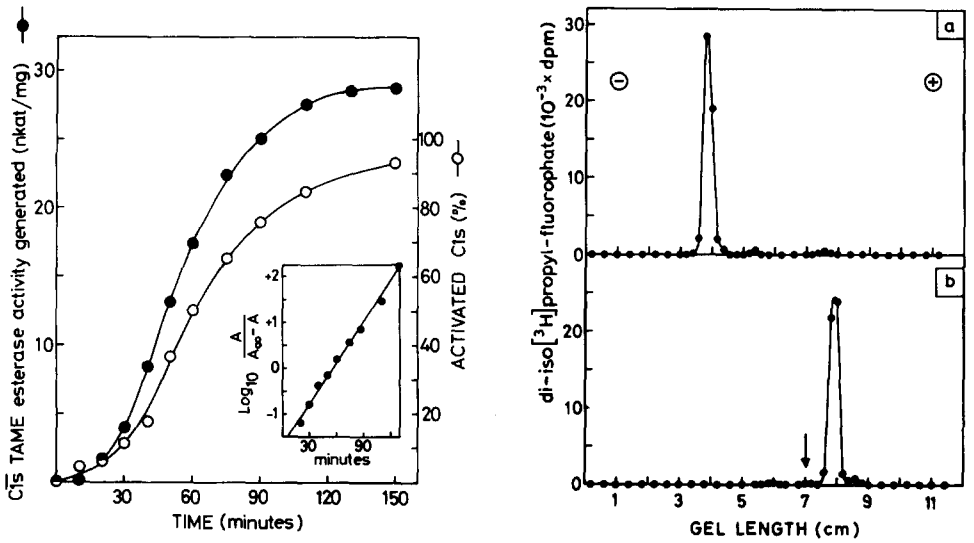

Fig. 1. Activation of C1s, correlation between structure and activity. C1s obtained from DEAE-cellulose (see Materials and Methods) was incubated at 0.88 mg/ml in 20 mM Tris · HCl, 150 mM NaCl, 1 mM EDTA (pH 7.6) for various periods at 37°C. Samples were removed and cooled in ice. Tos-Arg-OMe esterase activity was estimated on each sample. Samples were then reduced and alkylated and examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The percentage of activated C1s (○———○) was determined from gel scanning as described in Materials and Methods. Variation of Log_{10} $A/(A_\infty - A)$ versus incubation time (insert) was calculated from the observed esterase activities (A), and the maximum observed $\bar{C1}s$ esterase activity (A_∞).

Fig. 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of $\bar{C1}s$ labelled with di-iso[^{3}H]-propyl phosphorofluoridate. C1s obtained from DEAE-cellulose (see Materials and Methods) was incubated at 1.80 mg/ml in 20 mM Tris · HCl, 150 mM NaCl, 1 mM EDTA (pH 7.6) for 2 h at 37°C. Activated C1s was then treated with 1 mM di-iso[^{3}H]propyl phosphorofluoridate (0.44 Ci/mmol) for 30 min at 30°C. After dialysis against 50 mM Tris · HCl, 150 mM NaCl (pH 7.6), samples were submitted to sodium dodecyl sulphate polyacrylamide gel electrophoresis and radioactivity was measured in 2-mm gel slices as described in Materials and Methods. Both gels were loaded with about 21 μg $\bar{C1}s$. (a) Unreduced sample. (b) Reduced and alkylated sample. The arrow indicates the expected running position of C1r light fragment. DFP, di-isopropyl phosphorofluoridate.

The C1s activation process observed here was markedly temperature dependent. As shown in Table I, the activation rate was very slow at 25°C. The rate was greatly enhanced at 37°C and obeyed an exponential increase between the limits studied. It was also shown that the activation rate was strongly dependent on the concentration of C1s (Fig. 3) and that dilution of the sample induced an increase of the lag phase of the activation curve. The temperature dependence, as well as the influence of the concentration clearly indicate that the activation process was enzymatic. In the latter case, however, it is not possible to discriminate between an effect on the substrate (C1s) concentration and an effect on the activator concentration.

Effects of calcium and ionic strength on C1s activation

In order to characterize further the activation process, the influence of calcium and ionic strength were studied. As shown in Fig. 4, the rate of activation of C1s in EDTA was strongly dependent on the ionic strength. A maximal value was found at low ionic strength (I = 0.15) whereas the activation rate was

TABLE I

TEMPERATURE DEPENDENCE OF C1s ACTIVATION

C1s obtained from DEAE-cellulose (see Materials and Methods) was incubated at 0.93 mg/ml in 20 mM Tris · HCl, 120 mM NaCl, 0.5 mM EDTA (pH 7.6) for 30 or 45 min at different temperatures. Samples were cooled in ice and tested for Tos-Arg-OMe esterase activity as described in Materials and Methods.

Temperature (°C)	$\overline{C1s}$ esterase activity generated (nkat/mg)	
	30 min	45 min
25	0.6	0.75
28	1.0	1.2
31	1.8	2.8
34	4.6	8.0
37	11.3	18.2

greatly decreased at high ionic strength (I = 0.9). Addition of calcium almost completely prevented activation at higher ionic strengths although activation still occurred slowly at lower ionic strength.

As ionic strength has previously been shown to affect the thermal stability of $\overline{C1s}$, presumably by an induced conformational change in the molecule [1], it is likely that the above effect of ionic strength on C1s activation may be explained by a conformational change in proenzymic C1s, though an effect on the activator itself cannot be excluded.

Ziccardi and Cooper [10] reported that proenzymic C1r incubated at 37°C

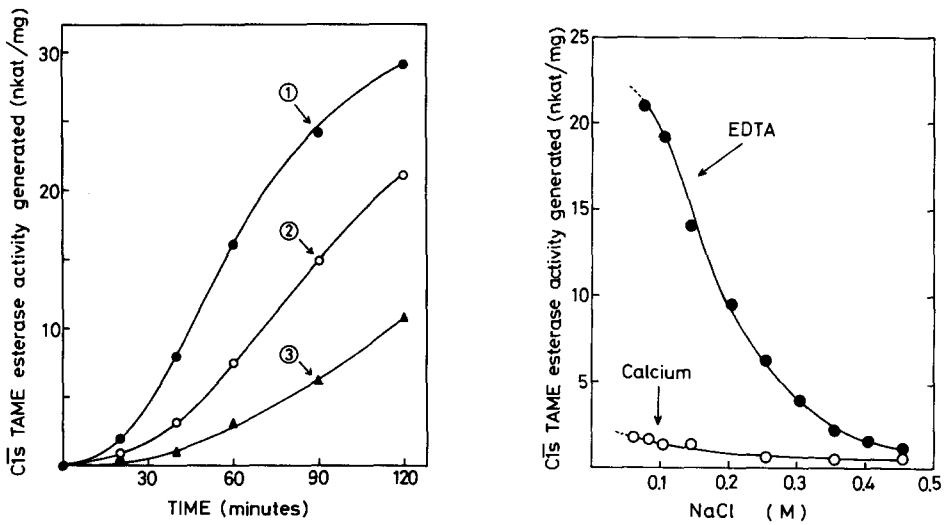

Fig. 3. Activation of C1s. Effect of concentration. C1s obtained from DEAE-cellulose (see Materials and Methods) was incubated at different concentrations in 20 mM Tris · HCl, 150 mM NaCl, 1 mM EDTA (pH 7.6) for various periods at 37°C. Samples were removed, cooled in ice and tested for Tos-Arg-OMe esterase activity as described in Materials and Methods. Curve 1: C1s, 1.70 mg/ml; curve 2: C1s, 0.85 mg/ml; curve 3: C1s, 0.42 mg/ml.

Fig. 4. Effect of calcium and ionic strength on C1s activation. C1s obtained from DEAE-cellulose (see Materials and Methods) was incubated at 0.93 mg/ml for 60 min at 37°C in 20 mM Tris · HCl, 62—455 mM NaCl, 0.5 mM $CaCl_2$ or EDTA (pH 7.6). Samples were cooled in ice and tested for Tos-Arg-OMe esterase activity as described in Materials and Methods. ●———●, EDTA; ○———○, $CaCl_2$.

was able to undergo activation, the activation process being inhibited by calcium. Consequently, a direct effect of calcium on contaminant C1r may explain the effect of calcium observed here. Again however, an effect on the substrate, C1s, cannot be excluded, as calcium was shown to affect the sedimentation coefficient of proenzymic and activated C1s [11,1] possibly by inducing a calcium-dependent dimerization.

Effect of various inhibitors on C1s activation

As the activation of C1s was shown to be correlated with the appearance of the heavy and light fragments of $C\bar{1}s$ on sodium dodecyl sulphate polyacrylamide gel electrophoresis of reduced and alkylated samples, this technique was used to monitor the effect of inhibitors on the activation process. Results presented in Table II show that complete inhibition of the activation process required relatively high concentrations of di-isopropyl phosphorofluoridate (5 mM) and benzamidine (5 mM). Polyanethol sulfonate, pentosane polysulphate and $C\bar{1}$-inactivator were also found to inhibit C1s activation, whereas soybean trypsin inhibitor had no effect. As the inhibitors used blocked the appearance of the characteristic chains of $C\bar{1}s$, it must be assumed that their effect was to inhibit either the activation or the activity of the protease responsible for C1s activation.

Ziccardi and Cooper [10] found that activation of proenzymic C1r was inhibited by di-isopropyl phosphorofluoridate, polyanethol sulfonate and $C\bar{1}$-inactivator. Our results may suggest consequently that the enzyme respon-

TABLE II

EFFECT OF INHIBITORS ON C1s ACTIVATION

C1s obtained from DEAE-cellulose (see Materials and Methods) was incubated at 0.84 mg/ml in 20 mM Tris · HCl, 150 mM NaCl, 1 mM EDTA (pH 7.6) for 90 min at 37°C, in the presence of different inhibitors. Samples were then reduced and alkylated and examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Gels were stained with Coomassie Blue and scanned as described in Materials and Methods. The percentage of activated C1s was measured from gels scans and the inhibition of C1s activation was calculated relatively to a reference sample incubated without inhibitor.

Inhibitor	Concentration	Inhibition (%)
Di-isopropyl phosphorofluoridate	1 mM	44.1
	5 mM	96.3
	10 mM	98.0
Benzamidine	0.1 mM	< 1.0
	1 mM	39.2
	5 mM	89.6
Polyanethol sulphonate	25 μg/ml	92.5
	250 μg/ml	97.2
Pentosane polysulphate	1 mg/ml	62.9
	3 mg/ml	93.9
$C\bar{1}$-inactivator	0.1 mg/ml	100
Soybean trypsin inhibitor	0.25 mg/ml	0

sible for C1s activation is a serine protease which exhibits the characteristics of $C\bar{1}r$.

The inhibition of C1s activation by $C\bar{1}$-inactivator was investigated by a different approach, as shown in Fig. 5. An excess of this inhibitor added before incubation totally blocked the generation of $C\bar{1}s$ esterase activity (curve 2), confirming the results shown in Table II. The same amount of $C\bar{1}$-inactivator added in the course of incubation (curve 3) titrated part of the $C\bar{1}s$ generated, but the activation continued. These results indicate that $C\bar{1}s$ was titrated preferentially by $C\bar{1}$-inactivator and suggest that the affinity of $C\bar{1}s$ for $C\bar{1}$-inactivator is greater than that of $C\bar{1}r$. This is in agreement with titration experiments of $C\bar{1}s$ by $C\bar{1}$-inactivator in the presence of $C\bar{1}r$ [4] which show that $C\bar{1}r$ does not compete effectively with $C\bar{1}s$ for $C\bar{1}$-inactivator binding. In the case of the addition of $C\bar{1}$-inactivator before incubation (curve 2), activated $C\bar{1}s$ is absent and the inhibitor is thus able to block the activation or activity of C1r.

Inhibition of C1s activation by anti-$C\bar{1}r$ IgG

In order to further identify the protease responsible for C1s activation, the

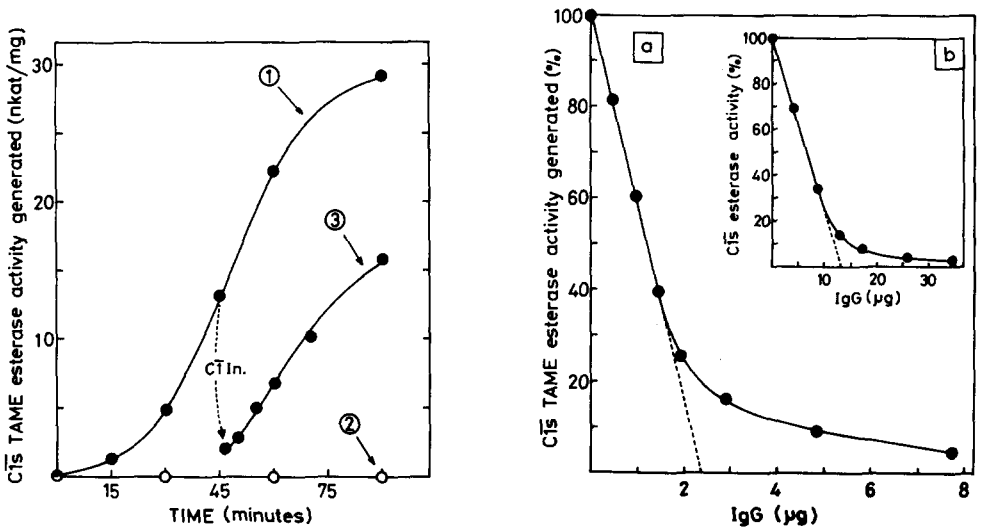

Fig. 5. Effect of $C\bar{1}$-inactivator on C1s activation. C1s obtained from DEAE-cellulose (see Materials and Methods) was incubated in triplicate at 0.73 mg/ml in 20 mM Tris · HCl, 95 mM NaCl, 2 mM EDTA (pH 7.6) for various periods at 37°C, $C\bar{1}$-inactivator was omitted (curve 1) or added at 0.20 mg/ml either before the incubation (curve 2), or after a 45 min incubation (curve 3). Samples were removed, cooled in ice and tested for Tos-Arg-OMe esterase activity as described in Materials and Methods.

Fig. 6. Inhibition of C1s activation by anti-$C\bar{1}r$ IgG. (a) C1s obtained from DEAE-cellulose (see Materials and Methods) was incubated at 0.64 mg/ml in 20 mM Tris · HCl, 150 mM NaCl, 1 mM EDTA (pH 7.6) for 90 min at 37°C in the presence of various amounts of anti-$C\bar{1}r$ IgG. Samples were cooled in ice and tested for Tos-Arg-OMe esterase activity as described in Materials and Methods. Residual activity is expressed as a percentage of the activity measured on a reference sample incubated without IgG. (b) C1s (0.63 mg/ml) was incubated with $C\bar{1}r$ (37 μg/ml) in 15 mM Tris · HCl, 15 mM sodium phosphate, 135 mM NaCl, 2 mM EDTA (pH 7.5) for 30 min at 37°C in the presence of various amounts of anti-$C\bar{1}r$ IgG. Samples were cooled in ice and tested for Tos-Arg-OMe esterase activity as described in Materials and Methods. Residual activity is expressed as a percentage of the activity measured on the reference sample incubated without IgG.

effect of Anti-$C\bar{1}r$ IgG on C1s activation was studied. As shown in Fig. 6a, increasing amounts of anti-$C\bar{1}r$ IgG added to C1s were able to inhibit its activation. The same inhibition was found for the activation of C1s by purified $C\bar{1}r$ (Fig. 6b) but larger amounts of anti-$C\bar{1}r$ IgG were needed. In both cases, the inhibition was linear from 0 to 70%, and the amount of anti-$C\bar{1}r$ IgG corresponding to 100% inhibition was determined by extrapolation. By comparison of the two systems, the amount of C1r contamination in C1s preparations could be estimated to about 1% on a molar basis, in close agreement with the estimation given above by di-iso[^{3}H]propyl phosphorofluoridate labelling.

Discussion

It appears from the experiments presented here that $C\bar{1}r$ is the enzyme responsible for the observed activation of C1s. This conclusion is supported principally by the effect of inhibitors and anti-$C\bar{1}r$ IgG on the activation process. It is likely that C1r exists in native C1s preparations as a proenzyme which is able to undergo activation on incubation and then to activate C1s.

Takahashi et al. [12] have reported that proenzymic C1r exhibited spontaneous activation and concluded from kinetic analysis that this reaction was autocatalytic. These experiments, however, were done with partially purified C1r and therefore a contribution of contaminating proteases to the activation process cannot be excluded. On the other hand, Ziccardi and Cooper [10] have reported spontaneous activation of proenzymic C1r by proteolytic cleavage. In this case, no experimental evidence was reported for an autocatalytic process, as added $C\bar{1}r$ has no effect on the rate of C1r activation.

In the case of the activation process described here, it is not possible to reach a definite conclusion on the mechanism of activation of C1r, although the kinetic experiments approximate to an autocatalytic process. On a molar basis, $C\bar{1}r$ was shown to represent about 1% relative to C1s in the preparations studied. This indicates that the activation process of C1s by $C\bar{1}r$ was catalytic and therefore different from that supposed to occur in the C1 complex, where C1r and C1s are present in an equimolar ratio [5]. The observation that traces of C1r copurify with C1s may be of a functional significance, as this association may reflect a strong residual affinity of the two subcomponents, even in the absence of calcium.

Calcium was shown to inhibit C1s activation. By comparison with the experiments reported by Ziccardi and Cooper [10], it seems possible that this effect may be principally on the activation of C1r. However, the calcium-dependent dimerization of C1s [1,11] could also partly explain the inhibitory effect of calcium by masking the site susceptible to proteolysis in the C1s dimer. Naff and Ratnoff [13] and Gigli et al. [5] have shown that calcium slows the rate of proteolysis of C1s by $C\bar{1}r$. In these cases also, it is not possible to distinguish between an effect on $C\bar{1}r$ or on C1s.

The activation process was highly dependent on the ionic strength. This could be explained by a conformational change in the C1s molecule, as was suggested elsewhere for $C\bar{1}s$ [1]. However, as there is no report on the effect of ionic strength on C1r and $C\bar{1}r$, an effect on this subcomponent cannot be excluded.

Several mechanisms can account for the inhibition of C1s activation by anti-$C\bar{1}r$ IgG. Our working hypothesis is that this inhibition was on the activity of $C\bar{1}r$ rather than on its activation. This is favoured by the observation that the activation of C1s by relatively large amounts of purified $C\bar{1}r$ is also inhibited by Anti-$C\bar{1}r$ IgG. The formation of antigen-antibody complexes between $C\bar{1}r$ and anti-$C\bar{1}r$ IgG may block the access of C1s to $C\bar{1}r$ active site and thus prevent C1s activation. Alternatively, the binding of anti-$C\bar{1}r$ IgG to $C\bar{1}r$ may induce a conformational change in the C1r molecule, which loses its catalytic properties.

In conclusion, it appears that the mechanism of activation of proenzymic C1r remains the key-question. More precise study of the activation of isolated C1r is an essential step to full elucidation of the activation mechanism of C1r in the C1 complex. Such studies require the same precautions as for C1s, particularly with regard to contamination by trace protease, in order to avoid erroneous interpretations.

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