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EFFECT OF $C\bar{1}$-INHIBITOR, BENZAMIDINE, AND HEAT TREATMENT ON THE HYDROLYTIC ACTIVITY OF A SUBCOMPONENT OF THE FIRST COMPONENT OF HUMAN COMPLEMENT, $C\bar{1}s$

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Summary

Inhibition of one subcomponent of the first component of human complement, $C\bar{1}s$ *, by $C\bar{1}$-inhibitor, benzamidine, and heating at 52°C was investigated using various synthetic and natural substrates for $C\bar{1}s$.

The esterolytic activity of $C\bar{1}s$ toward acetyl-arginine methyl ester (AcArgOMe) was found to be more stable than that toward acetyl-glycyl-lysine methyl ester (AcGlyLysOMe) and acetyl-tyrosine ethyl ester (AcTyrOEt) against inhibition of $C\bar{1}INH$ and heating at 52°C. Also, the AcArgOMe hydrolytic activity of $C\bar{1}s$ was less susceptible to benzamidine than the AcGlyLysOMe and AcTyrOEt hydrolytic activity of $C\bar{1}s$. The K_i value calculated with AcArgOMe as the substrate for $C\bar{1}s$ was found to be 7-fold larger than that calculated with AcGlyLysOMe and AcTyrOEt as substrates, suggesting that the catalytic site for AcArgOMe is different from that for AcGlyLysOMe and AcTyrOEt.

The proteolytic activity of $C\bar{1}s$ towards C4 was found to be more stable than that towards C2 against inhibition by $C\bar{1}INH$ and heat-treatment.

These results suggested that there are two $C\bar{1}s$ species; one is responsible for the hydrolysis of C2, AcGlyLysOMe, and AcTyrOEt, and another is for the hydrolysis of C4 and AcArgOMe.

The former is more sensitive than the latter to inhibition by $C\bar{1}INH$, benzamidine, and heat-treatment.

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* The symbols for components used in this paper conform to the recommendations of the World Health Organization Committee on Complement Nomenclature, Immunochemistry (1970) 7, 137—142. Activated components were indicated by placing a bar over the numeral which refers to the active component or subcomponent.

Abbreviations: AcArgOMe, acetylarginine methyl ester; AcGlyLysOMe, acetylglycyllysine methyl ester, CbzTyrONp, carbobenzoxytyrosine paranitrophenyl ester.

Introduction

The first component of complement, $C\bar{1}$, is a Ca^{2+}-dependent complex of three subcomponents, C1q, $C\bar{1}r$, and $C\bar{1}s$ [1], and activates the classical pathway of complement by limited proteolysis of C2 and C4 [2]. The proteolysis of C2 and C4 is caused by the action of $C\bar{1}s$ subcomponent [3].

Many reports have been made on the enzymatic nature of $C\bar{1}s$ which is the only plasma protease which exhibits substrate specificities similar to both trypsin and chymotrypsin [4]. Thus, $C\bar{1}s$ is capable of hydrolyzing N-substituted esters of arginine, lysine, and tyrosine (such as acetyl-arginine methyl ester (AcArgOMe), acetylglycyllysine methyl ester (AcGlyLysOMe), tosylarginine methyl ester (TosArgOMe), acetyltyrosine ethyl or methyl ester (AcTyrOEt, AcTyrOMe), and *N*-carbobenzoxytyrosine *p*-nitrophenyl ester (CbzTyrONp), in addition to the natural substrates, C2 and C4.

It was of interest to discover whether the same catalytic site in $C\bar{1}s$ is responsible for the hydrolysis of C2, C4, and various synthetic substrates.

Gigli and Austen [5] observed that treatment of $C\bar{1}$ at 50°C and $C\bar{1}$INH was accompanied by loss of activity against C2, while leaving its activity against C4 almost intact. Kondo et al. [6] also reported that hemolytic inactivation of $C\bar{1}$ by $C\bar{1}$INH and heat was accompanied by the loss of activity against C2, TosArgOMe, and AcTyrOMe without appreciably affecting its activity against C4 and AcArgOMe. In addition, Strunk and Colten [7] suggested that there are two separate enzyme sites on $C\bar{1}$ for C2 and C4, inasmuch as no competitive inhibition between C2 and C4 was demonstrated.

Since $C\bar{1}s$ subcomponent represents the hydrolytic activity of $C\bar{1}$ against C2 and C4, these results suggested that there are separate catalytic sites on a $C\bar{1}s$ molecule or that there are two species of $C\bar{1}s$ which differ in their substrate specificity and reactivity against $C\bar{1}$INH.

We have examined the kinetics of the effect of inhibitors and heating on the hydrolytic activity of highly purified $C\bar{1}s$ subcomponent.

Materials and Methods

Materials. Citrate/phosphate/dextrose plasma was obtained from the Blood center of the Japanese Red Cross (Sapporo, Japan). Synthetic substrates, AcGlyLysOMe, AcTyrOEt, and AcArgOMe were obtained from the Protein Research Foundation, (Osaka, Japan), E. Merck (Darmstadt, F.R.G.) and Vega-Fox Biochemicals (U.S.A.), respectively. Benzamidine was from Nakarai Chemicals (Kyoto, Japan).

Preparation of human complement components. $C\bar{1}s$ was purified from human plasma by polyethyleneglycol fractionation and chromatography on IgG-Sepharose and DE-52 as previously described [8]. The $C\bar{1}s$ preparations thus purified were homogeneous in polyacrylamide gel electrophoresis with and without SDS. The functional purity of the $C\bar{1}s$ preparations was also established by determining the esterase activity after separation upon polyacrylamide gel electrophoresis. As shown in Fig. 1, the esterase activity of the $C\bar{1}s$ preparation was detected only in the extract of sectioned gels in positions corresponding to the stained band of $C\bar{1}s$ in the α-globulin region. This result

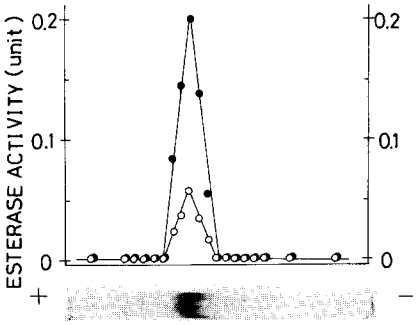

Fig. 1. Polyacrylamide gel electrophoresis of $C\bar{1}s$. $C\bar{1}s$ was electrophoresed on 5% polyacrylamide gels in duplicate by the method of Davis [9]. One gel was stained with Coomassie blue R-250 and another gel was sectioned into 1.5 mm segments. Each segment was suspended in 1 ml 0.9% NaCl for 24 h at 4°C. The esterase activity toward AcGlyLysOMe (●) and AcArgOMe (○) was determined with aliquots of each extract.

excluded the possibility that $C\bar{1}r$ subcomponent and plasmin contaminate the $C\bar{1}s$ preparations, since these proteases migrated as β- and γ-globulins, respectively, on polyacrylamide gels. Protein content of $C\bar{1}s$ was estimated from its absorbance at 280 nm, using $E^{1\%}_{1cm}$ 9.4 [10].

$C\bar{1}INH$ was purified from human plasma by the method of Reboul et al. [11] with a slight modification (unpublished data). One unit of $C\bar{1}INH$ is numerically equal to one unit of the esterase activity of $C\bar{1}s$ inhibited. C2 was purified from human plasma by affinity chromatography with C4b-Sepharose as previously described [12]. C4 was purified from human plasm by polyethyleneglycol fractionation and chromatographies with QAE-Sephadex A-50, heparin-Sepharose, and DE-52 (unpublished data). The purified C2 and C4 both gave a single band on polyacrylamide gel electrophoresis with and without SDS. Protein content of C2 and C4 was estimated from their absorbance at 280 nm, assuming $E^{1\%}_{1cm} = 10.0$.

Assay for esterase activity. $C\bar{1}s$ in 0.9 ml 0.1 M Tris-HCl (pH 8.5) was mixed with 0.1 ml ester. The stock solutions of esters were prepared by dissolving AcArgOMe or AcGlyLysOMe in water to final concentrations of 0.05 M or AcTyrOEt in methyl cellosolve to 0.1 M. After incubation for an appropriate time at 37°C, the amounts of ester hydrolyzed were determined by the hydroxamate method of Roberts [13]. One unit of the esterase activity of $C\bar{1}s$ was defined as the amount of enzyme which hydrolyzes 1 μmol AcGlyLysOMe per min at 37°C. For kinetic analyses, the esterase activity was monitored continuously using a pH-stat (Hiranuma Recording Autotitrator, Model PS 11, Japan). The reaction mixture was maintained at 37°C and pH 7.5, using 20 mM NaOH.

Assay of proteolytic activity. $C\bar{1}s$ is known to cleave C2 (M_r = 100 000) into C2a (M_r = 70 000) and C2b (M_r = 30 000) [12,14] and C4 (M_r = 200 000) into C4a (M_r = 8000) and C4b (M_r = 190 000) [15]. Based on this evidence, we determined the proteolytic activity of $C\bar{1}s$ towards C2 and C4 by measuring densitometrically the amount of products, C2a and C4a, after separation of the incubation mixture on SDS-polyacrylamide gel electrophoresis. 4.5 μg C2 and

0.02 μg $C\bar{1}s$ were incubated for 15 min at 37°C and electrophoresed in 0.1% SDS/5.6% polyacrylamide gels. The gels were stained with Coomassie blue R-250 and scanned using a dual-wavelength TLC Scanner CS-900, (Shimadzu, Japan). The areas below the peak corresponding to C2a were determined by planimetry. 25 μg C4 and 0.08 μg $C\bar{1}s$ were incubated for 15 min at 37°C and electrophoresed on 0.1% SDS/7.5% polyacrylamide gels. The areas below the peak corresponding to C4a were determined as described above.

SDS-polyacrylamide gel electrophoresis was performed according to the method of Fairbanks et al. [16] and the gels were stained with Coomassie blue R-250 as described by Burges [17].

Results

Effect of $C\bar{1}INH$ and heating at 52°C on the esterase activity of $C\bar{1}s$

Kondo et al. [6] observed that C1INH and heating at 50°C had essentially no inhibitory effect on the AcArgOMe esterase activity of the $C\bar{1}$ complex. Since AcArgOMe is known as the most susceptible substrate for $C\bar{1}r$ subcomponent [18], there remained a possibility that the AcArgOMe esterase activity detected with $C\bar{1}INH$- or heat-treated $C\bar{1}$ complex might be due to the esterase activity of $C\bar{1}r$ subcomponent.

We investigated the effect of $C\bar{1}INH$ and heat treatment on the esterase activity of $C\bar{1}s$ subcomponent using three synthetic substrates.

As shown in Fig. 2a, the esterase activity of $C\bar{1}s$ towards AcGlyLysOMe and AcTyrOEt was linearly inhibited as a function of concentration of $C\bar{1}INH$, whereas the esterase activity towards AcArgOMe was inactivated biphasically by increasing $C\bar{1}INH$ concentration. At the lower concentrations of $C\bar{1}INH$, the AcArgOMe esterase activity of $C\bar{1}s$ was only slightly inhibited.

The results of heat treatment are shown in Fig. 2b. The AcGlyLysOMe and AcTyrOEt esterase activity of $C\bar{1}s$ decreased in parallel and dropped to 10% of

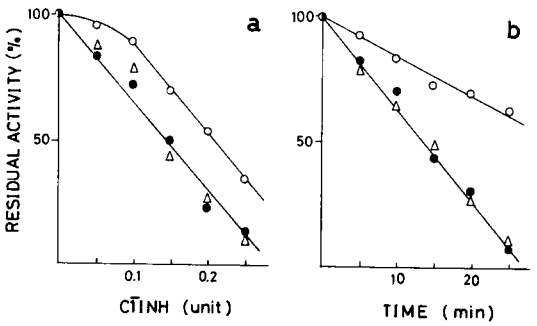

Fig. 2. Inhibition of the esterase activity of $C\bar{1}s$ by (a) $C\bar{1}INH$ and (b) heating at 52°C. (a) Mixtures containing 0.3 unit $C\bar{1}s$ and indicated amounts of $C\bar{1}INH$ in 0.9 ml 0.1 M Tris-HCl (pH 8.5) were incubated for 10 min at 37°C. The remaining $C\bar{1}s$ activity was determined with (○) AcArgOMe, (●) AcGlyLysOMe, and (△) AcTyrOEt. (b) 6 ml $C\bar{1}s$ (0.33 unit/ml) in 0.1 M Tris-HCl (pH 8.5) were heated at 52°C. At zero time and at appropriate time intervals, aliquots of 0.9 ml were removed, cooled in an ice-bath, and tested for its esterase activity with (○) AcArgOMe, (●) AcGlyLysOMe, and (△) AcTyrOEt at 37°C.

the original activity after heating at 52°C for 25 min. In contrast, the AcArgOMe esterase activity of $C\bar{1}s$ was heat-stable and decreased only to 65% of the original activity after heating under the same conditions.

Effect of $C\bar{1}INH$ and heat-treatment on the kinetic constants of $C\bar{1}s$

In order to characterize in detail the inhibition of $C\bar{1}s$ by $C\bar{1}INH$ and heat treatment, we evaluated the K_m and k_{cat} values of these esters, using the $C\bar{1}s$ preparations which had been treated with $C\bar{1}INH$ or heated at 52°C for 25 min.

Analyses of kinetic data according to the method of Lineweaver Burk yielded a linear plot (Fig. 3). The plots for free $C\bar{1}s$ preparations differed in slope and did not share a common intercept on the $1/V$ axis with any of three esters, suggesting that $C\bar{1}INH$ and heat treatment inactivated the esterase activity of $C\bar{1}s$ non-competitively. The K_m values of $C\bar{1}s$ for AcGlyLysOMe, AcTryOEt, and AcArgOMe were estimated to be 2.0, 19, and 2.0 mM, respectively. These values did not change upon treatment of $C\bar{1}s$ with heat or $C\bar{1}INH$.

The decrease in V but not in K_m upon treatment of $C\bar{1}s$ with $C\bar{1}INH$ and heating also indicated that these treatments did not affect the affinity between $C\bar{1}s$ and the esters but decreased the number of active sites of $C\bar{1}s$. In the case of heat-treatment, it was estimated that the active site of $C\bar{1}s$ for AcGlyLysOMe and AcTyrOEt was decreased approx. to 60—70% of the original activity, while the active site for AcArgOMe remained almost unchanged.

As given in Table I, $C\bar{1}INH$ decreased k_{cat} for AcGlyLysOMe and AcTyrOEt to 33—37% for the original activity, but that for AcArgOMe to 55%. On the other hand, the heat-treatment decreased the k_{cat} for AcArgOMe only to 90%, but those for AcGlyLysOMe and AcTyrOEt to 66 and 63%, respectively.

Inhibition of $C\bar{1}s$ esterase activity by benzamidine

Benzamidine is a potent competitive inhibitor for trypsin-type serine protease [19]. The inhibition of $C\bar{1}s$ by benzamidine was analyzed with a pH-stat at two inhibitor concentrations and the initial rate of reaction was calculated. The results were plotted according to the method of Dixon [20].

As shown in Fig. 4, the inhibition by benzamidine was competitive in nature as evidenced by the intersection of the lines above I-axis. The inhibition

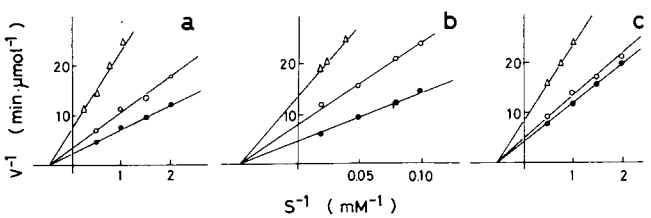

Fig. 3. Lineweaver-Burk plots of the hydrolysis of (a) AcGlyLysOMe, (b) AcTyrOEt, and (c) AcArgOMe by $C\bar{1}s$, heat-treated $C\bar{1}s$, and $C\bar{1}INH$-treated $C\bar{1}s$. The hydrolysis by native $C\bar{1}s$ (●), $C\bar{1}s$ treated at 52°C for 25 min (○), and the mixture of $C\bar{1}s$ and $C\bar{1}INH$ (molar ratio of 1.0 : 0.67) (△) was followed using a pH-stat at pH 7.5.

TABLE I

KINETIC CONSTANT, k_{cat}, FOR THE HYDROLYSIS OF SYNTHETIC ESTERS BY $C\bar{1}s$, HEAT-TREATED $C\bar{1}s$, AND $C\bar{1}INH$-TREATED $C\bar{1}s$

For the calculation of kcat, the molecular weight of $C\bar{1}s$ was assumed to be 105 000 [8]. $C\bar{1}INH$ and $C\bar{1}s$ were mixed in the molar ratio of 0.67 : 1.0. The molecular weight of $C\bar{1}INH$ was estimated to be 120 000 by SDS-polyacrylamide gel electrophoresis.

Enzyme	AcGlyLysOMe (s^{-1})	AcTyrOEt (s^{-1})	AcArgOMe (s^{-1})
$C\bar{1}s$	393	216	250
heat-treated $C\bar{1}s$	262	137	228
$C\bar{1}INH$-treated $C\bar{1}s$ **	131	80	137

constant (K_i) values were calculated from the Dixon's plots. As the K_i value is dependent on the affinity between enzyme ($C\bar{1}s$) and inhibitor (benzamidine), it has essentially no relation to the kinds of substrate used. Indeed, the K_i values were almost the same when AcGlyLysOMe and AcTyrOEt were used as the substrate; 0.70 mM for AcGlyLysOMe and 0.60 mM for AcTyrOEt. Bing [19] also reported that the K_i of benzamidine for the hydrolysis of CbzTyrONp by $C\bar{1}s$ was estimated to be 0.60 mM. However, when the K_i value was evaluated with AcArgOMe as the substrate, the K_i of 4.4 mM was calculated. This value was 7-fold larger than the K_i value evaluated with AcGlyLysOMe or AcTyrOEt.

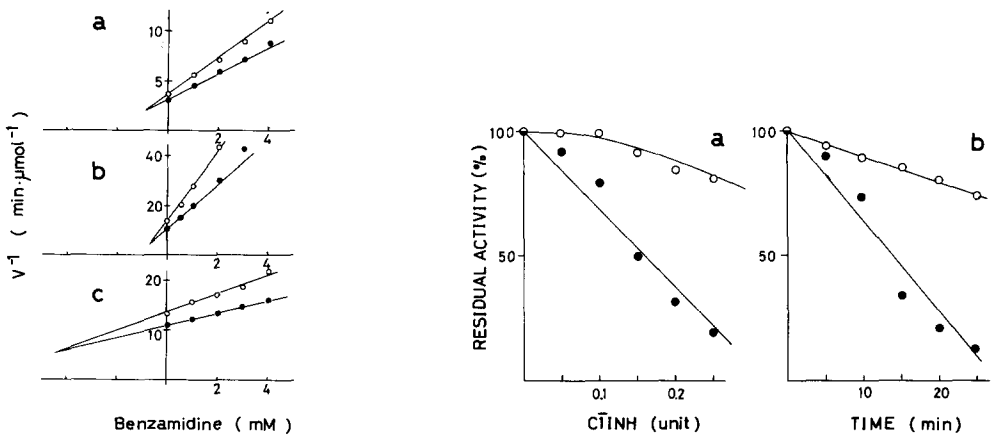

Fig. 4. Effect of the concentration of benzamidine on the hydrolysis of (a) AcGlyLysOMe, (b) AcTyrOEt, and (c) AcArgOMe by $C\bar{1}s$. The hydrolysis was followed using a pH-stat at two substrate concentrations. (a) ○, 5 mM; ●, 8 mM; (b) ○, 6 mM; ●, 10 mM; (c) ○, 5 mM; ●, 10 mM. The reciprocal of the initial velocity of the hydrolysis was plotted against the concentration of the inhibitor.

Fig. 5. Inhibition of the proteolytic activity of $C\bar{1}s$ by $C\bar{1}INH$ and heating at 52°C. Inhibition of $C\bar{1}s$ by $C\bar{1}INH$ and heat-treatment was performed under the same condition as described in the legend of Fig. 1. The remaining proteolytic activity of the $C\bar{1}s$ preparations was determined with C2 (●) and C4 (○) as substrates.

Effect of C$\bar{1}$INH and heating at 52°C on the proteolytic activity of C$\bar{1}$s

Next, we examined whether the proteolytic activity of C$\bar{1}$s toward C2 and C4 might be inhibited to different degree by C$\bar{1}$INH and heat treatment.

As shown in Fig. 5 the proteolysis of C2 by C$\bar{1}$s was decreased almost linearly by increasing the concentration of C$\bar{1}$INH, whereas the proteolysis of C4 by C$\bar{1}$s was only slightly inhibited by C$\bar{1}$INH.

The proteolysis of C4 by C$\bar{1}$s was heat-stable and about 90% of the original activity was retained after heating at 52°C for 25 min, while the proteolysis of C2 was dropped to 10% of the original activity after heating at 52°C for 25 min.

These results suggested that the catalytic site for C4 in C$\bar{1}$s was different from that for C2.

Discussion

In 1972 Kondo et al. [6] reported the apparent dissociation of C$\bar{1}$ activity on C4 and AcArgOMe from that on C2 and AcTyrOMe upon treatment with C$\bar{1}$INH and heat.

They discussed briefly that the dissociation of C$\bar{1}$ activity might be related to allosteric alteration of C$\bar{1}$ complex upon treatment with C$\bar{1}$INH and heat, and no attempt was made to explain this observation in detail.

The present studies extend their observations by showing that two separate catalytic sites seem to be responsible for the estero-proteolytic activity of C$\bar{1}$s subcomponent.

The inhibition plots of the hydrolysis of AcGlyLysOMe and AcTyrOEt against C$\bar{1}$INH and heating were similar to that of C2, while the inhibition plot of the hydrolysis of AcArgOMe were similar to that of C4. It is well recognized that synthetic substrates utilize the catalytic site for C2 and C4 of C$\bar{1}$s [21,22].

It appears likely that two separate catalytic sites are responsible for the estero-proteolytic activity of C$\bar{1}$s; one is the site for C4 and AcArgOMe and another is for C2, AcGlyLysOMe, and AcTyrOEt. The former is more stable than the latter against inhibition by C$\bar{1}$INH, benzamidine, and heat-treatment.

Kinetic analyses on the inhibition of C$\bar{1}$s by the inhibitors and heat-treatment suggested that the loss of the hydrolytic activity of C$\bar{1}$s was not due to allosteric alteration of C$\bar{1}$s but to the loss of the catalytic site of C$\bar{1}$s.

The established degree of purity of the C$\bar{1}$s preparations excluded the possibility that the heat- and C$\bar{1}$INH-resistant estero-proteolytic activity was due to some proteases contaminating the C$\bar{1}$s preparations.

At least three models can be proposed to explain the dissociation of C$\bar{1}$s activity.

(1) A C$\bar{1}$s molecule has two separate catalytic sites which differ in the substrate specificity, reactivity to inhibitors and heat stability.

(2) A C$\bar{1}$s molecule has a single active site serine and two separate substrate binding sites which differ in heat-stability.

(3) C$\bar{1}$s preparations consist of two types of C$\bar{1}$s which differ in the nature of catalytic site from each other.

Evidencce that 1 mol diisopropyl phosphorofluoridate is incorporated into 1 mol C$\bar{1}$s [23] indicates that there is one active site serine in a C1s molecule.

In addition, it was found that $C\bar{1}s$ and $C\bar{1}INH$ form an enzymatically inactive complex of 1 : 1 molar ratio [24]. Thus, model 1 seems to be very unlikely.

Model 2 may well explain the data on the heat-treatment and inhibition by benzamidine. It is difficult to explain why $C\bar{1}INH$ inhibits the hydrolysis of C2, AcGlyLysOMe, and AcTyrOEt much more effectively than that of C4 and AcArgOMe, unless we assume that $C\bar{1}INH$ does not directly block the active site serine of $C\bar{1}s$. $C\bar{1}INH$ may not completely block the active site of $C\bar{1}s$, but could modify it so that $C\bar{1}s$ continues to hydrolyze some substrates at a slower rate. However, this concept does not hold for the general mechanism on interaction of inhibitors and serine-type proteases; the active site serine of a protease interacts with the reactive site of an inhibitor to form a functionally inactive enzyme-inhibitor complex [25]. The only exception to this concept so fat reported is α_2-macroglobulin, which irreversibly binds to proteases without the involvement of any covalent linkage, and inhibits sterically the access of protein substrates to the entrapped enzyme [26]. Thus, α_2-macroglobulin does not inhibit the hydrolysis of low molecular weight substrates by the entrapped enzymes.

The binding of $C\bar{1}INH$ to $C\bar{1}s$ seems to involve the active site serine, since diisopropyl phosphonofluoridate interferes the interaction between $C\bar{1}INH$ and $C\bar{1}s$ [3,27].

Model 3 appears to be the most acceptable of these three models. There are two $C\bar{1}s$ species; one is more sensitive than the other against inhibition by $C\bar{1}INH$, benzamidine, and heat-treatment. The former represents the hydrolytic activity towards C2, AcGlyLysOMe, and AcTyrOEt, while the latter represents that towards C4 and AcArgOMe.

Although Nagaki and Stroud [28] detected immunochemically the microheterogeneity of $C\bar{1}s$, we did not have any data to suggest the microheterogeneity of the $C\bar{1}s$ preparations concerning N-terminal amino acid and electrophoretic behaviour in polyacrylamide gel. It seems that the two $C\bar{1}s$ species may differ only slightly in the primary structure around the substrate binding site, so they are judged homogeneous by usual gel electrophoresis and N-terminal analysis.

Recently, the $C\bar{1}$ complex was shown to be comprised of 1 mol C1q, and 2 mol each $C\bar{1}r$ and $C\bar{1}s$ [23]. So, it may be that 2 mol of $C\bar{1}s$ in C1 complex are 1 mol each C2-specific and C4-specific C1s species.

Additional evidence for the presence of two functionally different $C\bar{1}s$ species is now under investigation.

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