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Kinetics of Interaction of C1 Inhibitor with Complement $C1\bar{s}$[†]

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ABSTRACT: The kinetics of inhibition of the complement serine protease, $C1\bar{s}$, by its only known inhibitor, C1 inhibitor, have been measured by a variety of methods. One method continuously monitors the loss of esterolytic activity with a synthetic substrate coupled to a chromogen while another monitors the formation of a stable (covalent) complex by high-pressure size-exclusion chromatography under dissociating conditions. Additional methods employ fluorescence probes to follow the formation of bimolecular complexes but are not expected to distinguish between covalent product and noncovalent (reversible) intermediates. There was good agreement between rate constants obtained by the various methods over a broad range of inhibitor concentrations, suggesting that noncovalent intermediates do not accumulate to a significant extent. The reaction appears to be pure second order with a bimolecular rate constant of 6.0×10^4 M^{-1} s^{-1} at 30 °C, independent of Ca^{2+}, and an activation energy of 11.0 kcal/mol. The rate increases up to 35-fold in the presence of heparin which was shown to bind to all three components (enzyme, inhibitor, and complex) with similar affinity (K_d = 2.0–3.3 μM). The fluorescent probe 1,1′-bis(anilino)-4,4′-bi(naphthalene)-8,8′-disulfonate [bis(ANS)] bound to the complex with K_d = 0.26 μM under conditions where the individual components had little affinity for the dye, consistent with the generation of one or more hydrophobic binding sites on the protein surface during complex formation.

Human C1 inhibitor (C1-Inh)[1] performs a pivotal function in the regulation of the complement system in that it is the only circulating inhibitor known to react with the activated complement proteases, $C1\bar{r}$ and $C1\bar{s}$ (Sim et al., 1979; Ziccardi, 1981; Cooper, 1985). The resulting 1:1 complexes are stable to heat and SDS but can be dissociated with hydroxylamine, suggesting the formation of a covalent ester linkage between the components (Harpel & Cooper, 1975). Although the kinetics of these reactions have been investigated in two different laboratories, there is a 40-fold difference between the bimolecular rate constants reported for the reaction with $C1\bar{s}$ (Sim et al., 1980; Nilsson & Wiman, 1983). There is also uncertainty as to the mechanism of the reaction, in particular with respect to the importance of a reversibly associated intermediate complex whose subsequent conversion to a stable (covalent) product may be rate limiting under some conditions [see also Salvesen et al. (1985)]. Part of the confusion could be due to the different methods employed. Sim et al. (1980) measured the rate of formation of a heat-stable complex by SDS–PAGE and found their data to be consistent with simple second-order kinetics up to about 70% completion of the reaction. Nilsson and Wiman (1983) measured the rate of disappearance of enzyme activity by a spectrophotometric method and emphasized a two-step mechanism. In an effort to clarify the kinetics of this important reaction, we have measured its rate by several methods covering a broad range of reactant concentrations. It is shown that the rate of the initial bimolecular reaction, as measured by loss of enzymatic activity and by changes in fluorescence intensity and polarization of extrinsic fluorescent probes, is almost indistin-

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[1] Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; bis(ANS), 1,1′-bis(anilino)-4,4′-bi(naphthalene)-8,8′-disulfonate; C1-Inh, C1 inhibitor or C1 inactivator; DTDP, 4,4′-dithiodipyridine; FITC, fluorescein isothiocyanate; HBS, 0.02 M Hepes/0.15 M NaCl, pH 7.4; HPSEC, high-pressure size-exclusion chromatography; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, 0.02 M Tris/0.15 M NaCl, pH 7.4; Z-Lys-sBzl, thiobenzyl ester of benzyloxycarbonyllysine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; kDa, kilodalton(s); PBS, phosphate-buffered saline.

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guishable from the rate of formation of a nondissociable (covalent) complex, as measured by high-pressure size-exclusion chromatography in the presence of SDS. In addition, we have examined the interaction of heparin with the components of this system and have shown by several methods that heparin binds to both the enzyme and the inhibitor as well as to the complex under conditions where the glycosaminoglycan accelerates the inhibition reaction up to 35-fold.

Materials and Methods

The substrates for the esterolytic assay, Z-Lys-sBzl and Z-Gly-Arg-sBzl, were purchased from Vega Biochemicals and from Enzyme Systems Products (Livermore, CA), respectively. The chromagen 4,4'-dithiodipyridine (DTDP) was purchased from Sigma as were heparin (grade 1 porcine intestinal mucosa) and fluoresceinamine isomer II. Fluorescein isothiocyanate (FITC) and bis(ANS) were purchased from Molecular Probes. Acetonitrile and cyanogen bromide were purchased from Eastman. All other chemicals were from Sigma.

C1-Inh was purified to homogeneity by a modification of the procedure of Nilsson and Wiman (1982) as described by Lennick et al. (1985). C1$\bar{s}$ was purified from Cohn fraction I paste as described by Bing et al. (1980) using IgG–Sepharose prepared according to Kolb et al. (1979). Both proteins were >95% pure as judged by SDS–polyacrylamide slab gel electrophoresis and HPSEC (Lennick et al., 1985). Their concentrations were determined by assuming $A_{280}(1\%) = 10.0$ and M_r 83000 for C1$\bar{s}$ (Sim et al., 1977) with corresponding values of 4.5 and 104000 for C1-Inh (Haupt et al., 1970). Harrison (1983) recently proposed a lower value of 3.6 for the $A_{280}(1\%)$ of C1-Inh. Both values are substantially less than a value of 6.0 calculated by the method of Edelhoch (1967) assuming 7 Trp and 17 Tyr (Haupt et al., 1970). An even higher value would be derived from the number of Trp and Tyr residues listed by Harrison. Because of this uncertainty, we have chosen to use the widely accepted value of 4.5.

Heparin–Sepharose and C1$\bar{s}$–Sepharose were prepared by coupling to CNBr-activated Sepharose according to the method of March et al. (1974).

C1$\bar{s}$ was labeled with FITC by incubating the protein at 1–2 mg/mL in 0.05 M $NaHCO_3$, pH 8.5, with a 14-fold molar excess of FITC for 1 or 2 h at 37 °C. Excess dye was removed by exclusion chromatography on Sephadex G-25 (Pharmacia). Degrees of labeling, determined spectrophotometrically (Ingham & Brew, 1981), ranged between 1.2 and 2.1 mol of dye per mole of protein in several preparations, and retention of esterolytic activity was 70–90%. The labeled material was homogeneous by HPSEC and SDS–PAGE. Direct visualization of UV-illuminated gels of reduced samples after SDS–PAGE indicated that the fluorescent label was preferentially incorporated into the light chain of C1$\bar{s}$. The percentage of labeled enzyme capable of forming a stable complex with excess inhibitor ranged from 65% to 90% for different preparations as assessed by HPSEC with fluorescence detection. To study the accelerating effect of heparin on the reaction of FITC–C1$\bar{s}$ with the inhibitor, an alternative labeling approach was used. C1$\bar{s}$ was loaded onto a column of heparin–Sepharose in 0.05 M $NaHCO_3$, pH 8.5. After the column was washed, a solution containing a 350-fold molar excess of FITC was loaded onto the column and allowed to incubate overnight at room temperature. After the column was washed, the labeled protein was eluted with 0.05 M Tris/1.5 M NaCl, pH 7.4, and extensively dialyzed against 0.05 M Tris/0.15 M NaCl, pH 7.4. The degree of labeling was 0.39 mol/mol.

Heparin was labeled with fluoresceinamine, carefully following the procedure of Ogamo et al. (1982) [see also Bently et al. (1985)] which couples the fluorophore to the carboxyl groups of the glycosaminoglycan by means of a carbodiimide reaction. After extensive dialysis against HBS (0.02 M Hepes/0.15 M NaCl, pH 7.4), the material was applied to a column of C1$\bar{s}$–Sepharose and washed with the same buffer until no detectable fluorescence eluted. Application of 1 M NaCl in 0.02 M Hepes, pH 7.4, produced a C1$\bar{s}$ binding fraction having a degree of labeling of 1.4 mol of fluorescein (determined from the absorbance at 495 nm) per mole of heparin [determined by the uronic acid assay (Bitter & Muir, 1962), and arbitrarily assuming an average molecular weight of 13000 for heparin]. Because of the low capacity of the column, substantial amounts of labeled heparin could be recovered by repeated passage of the unbound fraction through the column. The fractions thus obtained had progressively lower degrees of labeling. The fraction used for the experiments described here had a degree of labeling of 0.66.

Fluorescence intensity measurements were made on a Perkin-Elmer MPF4 fluorometer and polarization measurements on an SLM-8000 fluorometer, both with thermostated cell holders. Polarization values were calculated from intensities which had been corrected for blanks which were identical with the sample in every way except for the absence of fluorophore. These corrections never exceeded 10% of the sample signal.

High-pressure size-exclusion chromatography (HPSEC) was performed at room temperature using an 0.8 × 30 cm size-exclusion column (TSK G4000SW) purchased from Varian Instruments. All buffers were sterile filtered and extensively degassed prior to use. Samples were injected with a Waters U6K injector, and protein elution was monitored either by absorbance at 280 nm with a Waters Model 450 variable wavelength detector or by fluorescence with a Shimadzu RF-530 fluorescence detector.

Affinity chromatography on heparin–Sepharose was performed with a Pharmacia FPLC system. Columns of approximately 1-mL bed volume were used. Elution profiles were determined by continuous monitoring of the absorbance at 280 nm. The percentage distribution between pass-through and bound peaks was determined by weighing triplicates of the profiles which were cut out from photocopies of the chart tracings.

Kinetics of the interaction between C1$\bar{s}$ and C1-Inh were measured by loss of C1 esterase activity in a kinetic assay using synthetic peptide thioester substrates, Z-Lys-sBzl and Z-Gly-Arg-sBzl, in combination with the chromogenic thiol reagent, DTDP, as described by McRae et al. (1981). Unless otherwise stated, the buffer used in all assays was 20 mM Tris, 0.15 M NaCl, 5 mM Na-EDTA, and 9.2% Me_2SO, pH 7.4. All assays were performed at 30 °C in either a Cary 118 double-beam recording spectrophotometer or a Perkin-Elmer Lambda-5 spectrophotometer equipped with a Model 3600 data station. Stock solutions of chromogen (4.5 mM DTDP in assay buffer) and substrate (in deionized water, 9.2% Me_2SO) were prepared in advance. Buffer, chromogen (0.3 mM final concentration for Z-Lys-sBzl and 0.68 mM final concentration with Z-Gly-Arg-sBzl), substrate (0.12 mM final concentration for Z-Lys-sBzl and 0.20 mM final concentration for Z-Gly-Arg-sBzl), and inhibitor were mixed in a 1-cm quartz cuvette and allowed to equilibrate to temperature. Blanks, containing buffer, chromogen, heparin, if added, and substrate, were prepared for each assay. Reactions were initiated by adding C1$\bar{s}$ (6–10 nM final concentration) to the sample cuvette, and the reaction was monitored by the change in absorbance at 324 nm (Castillo et al., 1979). The velocity

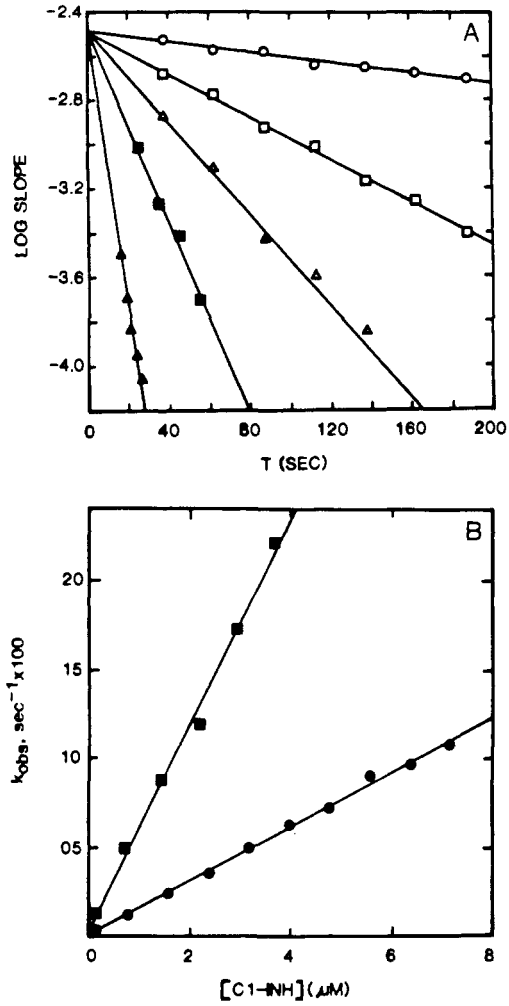

FIGURE 1: (A) Pseudo-first-order kinetics of inhibition of C1s by C1-Inh. Loss of enzyme activity was continuously monitored with the synthetic substrate Z-Lys-sBzl coupled to the chromogen dithiodipyridine (McRae et al., 1981). The solvent was 20 mM Tris containing 0.15 M NaCl, 5 mM EDTA, and 9.2% dimethyl sulfoxide at pH 7.4 and 30 °C. The concentration of C1s was 6.7 nM, and concentrations of C1-Inh were 0.16 (○), 0.79 (□), 1.6 (△), 2.4 (■), and 7.2 μM (▲). Pseudo-first-order rate constants, k_{obsd}, were calculated from the slopes determined by linear regression. (B) Dependence of k_{obsd} on C1-Inh concentration as determined with Z-Lys-sBzl (■) or Z-Gly-Arg-sBzl (●). Second-order rate constants of 6.2×10^4 and 6.0×10^4 M^{-1} s^{-1} were calculated from the respective slopes by means of eq 2.

of the enzyme-catalyzed reaction was determined from slopes of tangents to the curved plots of A_{324}nm vs. time. The concentration of enzyme remaining uncomplexed at any time was directly proportional to velocity. Since C1-Inh was always present in greater than 10-fold excess over C1s, pseudo-first-order rate constants could be derived from the initial slopes of semilog plots of enzyme activity vs. time (see Figure 1). The second-order rate constant, k_2, was then determined by fitting eq 1 [from Petersen & Clemmensen (1981)] to a

$$k_{obsd} = k_2[K_m/(K_m + S)]I \quad (1)$$

series of inactivation experiments done at various inhibitor concentrations, where k_{obsd} = the apparent first-order rate constant, k_2 = the second-order rate constant, K_m = the Michaelis constant of the enzyme for the synthetic substrate, S = substrate concentration, and I = C1-Inh concentration. Values of K_m used in the calculations, determined by Lineweaver–Burk analysis of substrate hydrolysis at 30 °C, were 2.0 mM for Z-Lys-sBzl and 70.0 μM for Z-Gly-Arg-sBzl; the latter agrees well with the value of 69 μM reported by McRae et al. (1981).

Table I: Summary of Second-Order Rate Constants for Inhibition of C1s by C1 Inhibitor

conditions	method	temp (°C)	k_2 ($\times 10^{-4}$ M^{-1} s^{-1})
TBS/EDTA/ Me_2SO	Z-Lys-sBzl	30	6.2
TBS/EDTA/ Me_2SO	Z-Gly-Arg-sBzl	30	5.9
Tris/EDTA/ Me_2SO	quenching of FITC–C1s	25	4.7
Tris/EDTA	quenching of FITC–C1s	25	4.6
Tris/Ca (5 mM)	quenching of FITC–C1s	25	5.3
PBS/EDTA	quenching of FITC–C1s	25	4.8, 5.0
TBS	polarization of FITC–C1s	32	10.5
	HPSEC of FITC–C1s	32	11.3
PBS	bis(ANS)fluorescence	30	3.5, 4.8

Second-order rate constants were also determined by fitting data obtained under second-order conditions (slight excess of inhibitor) to a modified second-order rate equation:

$$\ln (R - f)/(1 - f) = \ln R + k_2(I_0 - E_0)t \quad (2)$$

where R is the ratio of I_0 to E_0, the initial concentrations of inhibitor and enzyme, respectively, and f is the fraction of enzyme reacted, obtained by linear interpolation of the observed spectrophotometric response (absorbance, fluorescence, or polarization) between initial and final values.

RESULTS

Inhibition Kinetics by the Substrate Hydrolysis Assay. Figure 1A illustrates the rate of inactivation of C1s by C1-Inh at 30 °C in terms of the loss of the enzyme's activity toward the peptide substrate Z-Lys-sBzl. The conditions were such that less than 10% of the substrate was consumed during any given assay. Each line represents a different concentration of inhibitor, the lowest being more than 10 times that of C1s. The linearity of the semilog plots and the fact that each set of data extrapolates to the same initial velocity indicates that pseudo-first-order conditions were achieved. A similar pattern was obtained with the dipeptide substrate Z-Gly-Arg-sBzl (not shown).

Pseudo-first-order rate constants obtained with both substrates increased linearly with C1-Inh concentration as shown in Figure 1B. This implies that the reaction remains second order over the concentration range examined. According to eq 1, taken from Petersen and Clemmensen (1981), the slopes of these lines are proportional to k_2, the second-order rate constant for the inhibition reaction. The values thus calculated were 6.2×10^4 and 6.0×10^4 M^{-1} s^{-1} for the mono- and dipeptide substrates, respectively, at 30 °C. These and all other rate constants determined in this work are summarized in Table I.

A separate experiment was performed to determine if C1s is able to "turn over" C1-Inh as has been observed with other systems (Fish & Bjork, 1979; Laine et al., 1984). The enzyme (0.84 μM) was reacted with a slight excess of C1 inhibitor (0.96 μM), just enough to abolish the esterase activity, and then incubated at 37 °C in TBS containing 0.02% sodium azide. No activity toward Z-Lys-sBzl could be detected even after 5 days during which time a control sample of C1s alone retained over 80% of its activity. Nor was there a significant increase in the small amount of modified 95-kDa inhibitor which can be observed by SDS–PAGE of nonreduced samples (Weiss & Engel, 1983; van der Graaf et al., 1983). We thus conclude that the protease–inhibitor complex is stable under

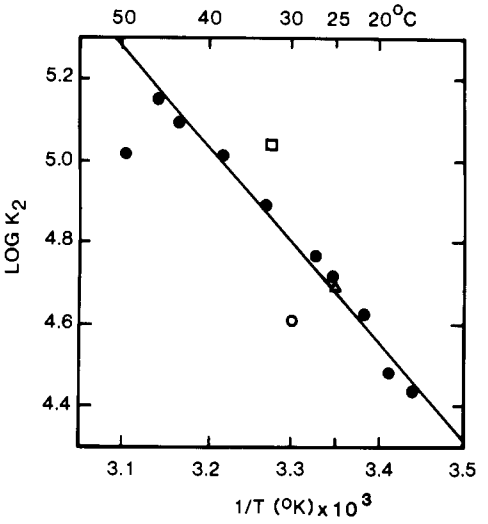

FIGURE 2: Arrhenius plot of the temperature dependence of the second-order rate constant for inhibition of C1$\bar{s}$ by C1-Inh under the conditions described in Figure 1A. At each temperature, three values of k_{obsd} were determined at C1-Inh concentrations of 0.4, 0.9, and 1.4 μM, and the corresponding value of k_2 was determined as in Figure 1B. The straight line obtained by linear regression (omitting the value near 49 °C) corresponds to an activation energy of 11 kcal/mol. The open symbols represent values determined by other methods: (○) average of two values determined with bis(ANS) (Figure 3); (△) average of values determined by FITC–C1$\bar{s}$ fluorescence (Table I); (□) value obtained by HPSEC and change in fluorescence polarization of FITC–C1$\bar{s}$ (Figure 6).

the conditions of the kinetic experiments reported here.

Effects of Temperature. The temperature dependence of the second-order rate constant for inhibition is illustrated by the Arrhenius plot in Figure 2, where the closed symbols represent values determined by the substrate hydrolysis method using Z-Lys-sBzl. All of the data fall on a single straight line except for the point at 49 °C where C1$\bar{s}$ begins to denature (Lennick et al., 1985). The slope of the fitted line is consistent with an activation energy E_a = 11.0 kcal/mol. A similar analysis of the rate of hydrolysis of Z-Lys-sBzl yielded a value of E_a = 7.8 kcal/mol (not shown). The open symbols represent values obtained by various other methods as presented below.

Studies with Noncovalent Fluorescent Probes. We previously reported that formation of a complex between C1$\bar{s}$ and C1-Inh resulted in a substantial increase in the ability to enhance the fluorescence of the hydrophobic probe 1,8-anilinonaphthalenesulfonate (ANS), relative to that of the isolated components (Lennick et al., 1985). Efforts to exploit this effect for kinetic measurements were hampered by the weak signals observed at the low protein concentrations required to slow the reaction to a measurable rate. The related probe bis(ANS) proved to be more sensitive and was equal in its ability to discriminate between the reactants and the product. Fluorescence titrations of C1$\bar{s}$ (1.2 μM), C1-Inh (0.7 μM), and complex (0.7 μM) are shown in Figure 3A where it is apparent that enhancement of bis(ANS) fluorescence in the range of 0–20 μM is much greater by the complex than by either of the components alone. A double-reciprocal plot of the data for the complex (Figure 3B) yields a value of 0.26 μM for the dissociation constant of the dye. Addition of C1-Inh to a solution containing C1$\bar{s}$ and the dye caused a time-dependent increase in bis(ANS) fluorescence, the rate of which depended upon the protein concentration. Examples are shown in Figure 3C, where the curves drawn through the normalized data points correspond to second-order rate constants of 3.5 × 10^4 and 4.8 × 10^4 M^{-1} s^{-1} at 30 °C (Table I). The average of these values appears as an open circle in Figure 2 and is only 30–40% lower than that obtained by substrate hydrolysis.

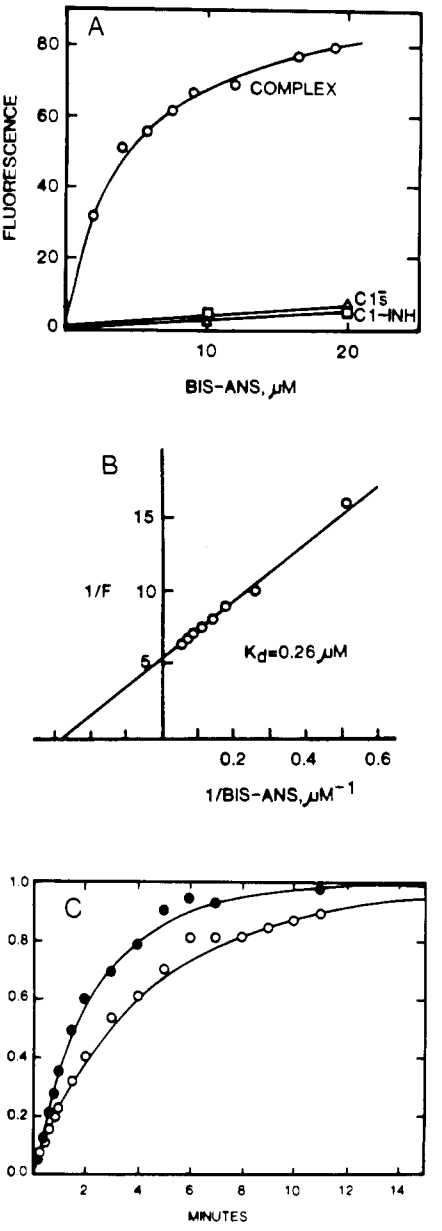

FIGURE 3: (A) Fluorescence titration of C1$\bar{s}$, C1-Inh, and the C1$\bar{s}$–C1-Inh complex with bis(ANS) at 25 °C in 0.04 M phosphate/0.15 M NaCl, pH 7.4. The blank [bis(ANS) alone] had negligible fluorescence on this scale. (B) Double-reciprocal plot of the data for the complex in Figure 3A. (C) Normalized increase in bis(ANS) fluorescence with time after addition of C1-Inh to a solution containing C1$\bar{s}$ (0.04 μM) and the dye (5 μM) at 30 °C. The C1-Inh concentrations were 0.12 (○) and 0.16 μM (●). Conditions same as in panel A. The lines represent theoretical curves corresponding to k_2 = 5.9 × 10^4 (○) and 6.0 × 10^4 M^{-1} s^{-1} (●).

Studies with Fluorescent-Labeled C1$\bar{s}$. A third approach to analyzing the kinetics of complex formation involved the use of C1$\bar{s}$ which had been covalently labeled with fluorescein (see Materials and Methods). Reaction of FITC–C1$\bar{s}$ with C1 inhibitor in PBS produced a time-dependent decrease in the fluorescence intensity of the probe to about two-thirds of the original value. Normalized plots of the intensity change vs. time are illustrated in Figure 4 for two concentrations of inhibitor at 25 °C. The curves drawn through some of the experimental points correspond to second-order rate constants of 4.8 × 10^4 and 5.0 × 10^4 M^{-1} s^{-1}, obtained by fitting the data to eq 2. This method was used to examine the effects of solution conditions on the second-order rate constant. The

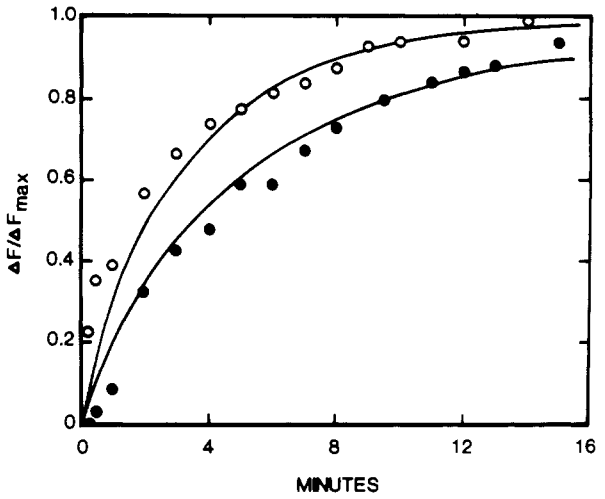

FIGURE 4: Rate of change of fluorescence intensity of FITC–C1s following addition of C1-Inh to a solution containing the labeled enzyme in 0.04 M phosphate/0.15 M NaCl, pH 7.4 at 25 °C. The concentration of C1s was 0.04 μM, and C1-Inh concentration was 0.08 (●) and 0.12 μM (○). The fluorescence actually decreases by about 35% during the course of the reaction. The data shown have been normalized, and the lines represent theoretical curves corresponding to $k_2 = 4.8 \times 10^4$ and 5.0×10^4 M^{-1} s^{-1}.

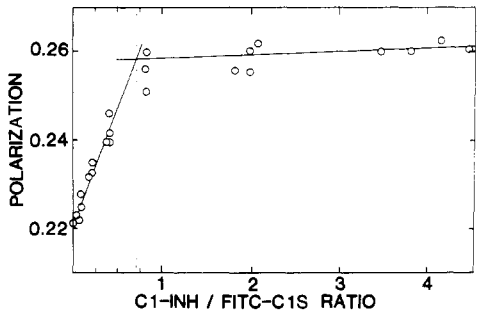

FIGURE 5: Change in fluorescence polarization of FITC–C1s (0.1 μM) upon addition of increasing amounts of C1-Inh at 25 °C in HBS (0.02 M Hepes/0.15 NaCl, pH 7.4). Sufficient time was allowed after each addition to obtain a stable value of P. The results of three separate experiments are shown.

results summarized in Table I indicate that k_2 is insensitive to changes in the buffer composition, to the presence or absence of 5 mM calcium, and to the presence of 9.2% Me_2SO used in the substrate hydrolysis assay. The average of the values in Table I appears as the open triangle in Figure 2.

The interaction of FITC–C1s with C1-Inh was also manifested by an increase in the fluorescence polarization, P. of the fluorescein label. As shown in Figure 5, titration with the inhibitor caused a progressive increase in P to a plateau value about 0.04 unit above that of FITC–C1s alone. In these experiments, sufficient time was allowed after each addition to allow the reaction to go to completion, as evidenced by the attainment of a stable P value. Extrapolation of the initial slope to the plateau level suggests a stoichiometry of about 0.7 mol of C1-Inh per mole of FITC–C1s. This indicates that some of the labeled enzyme does not react with inhibitor, consistent with the fact that this particular preparation was only about 70% as active as the unlabeled enzyme with respect to hydrolysis of Z-Lys-sBzl.

The rate of increase in polarization of FITC–C1s following addition of a slight excess of C1-Inh at 32 °C is shown by the closed circles in Figure 6. Also shown are the results of a parallel experiment in which small samples were periodically removed, mixed with SDS and PMSF to quench the reaction, and analyzed by HPSEC in the presence of 0.1% SDS to

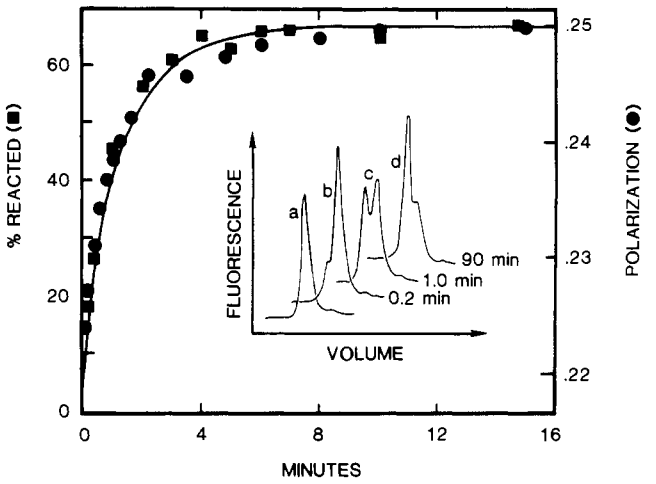

FIGURE 6: Rate of complex formation between FITC–C1s and C1-Inh at 32 °C as measured by the increase in fluorescence polarization (●) and by the appearance of a stable complex on HPSEC in SDS-containing buffer (■). The inset shows examples of fluorescence-detected elution profiles of samples containing 0.10 μM FITC–C1s and 0.16 μM C1-Inh in HBS. Samples of 0.1-mL volume were withdrawn at 0 (a), 0.2 (b), 1.0 (c), and 90 (d) min, quickly mixed with PMSF (1.0 mM final concentration) and SDS (1% final concentration), and placed on ice until they were loaded on the TSK column. The smooth curve corresponds to $k_2 = 11.0 \times 10^4$ M^{-1} s^{-1}.

determine the proportion of FITC–C1s which had been converted to a stable (covalent) complex. Examples of the fluorescence-detected elution profiles are shown in the inset of Figure 6. FITC–C1s by itself elutes as a single symmetrical peak (trace a). Within 0.2 min after addition of C1-Inh, a stable complex can be detected on the leading edge of the profile (trace b). After 1 min, approximately 50% of the fluorescent material is in the form of complex (trace c). The rate of this conversion, as shown by the open circles, is indistinguishable from the rate of change in polarization. After 10–15 min, a plateau is reached corresponding to conversion of about two-thirds of the FITC–C1s to a stable complex with the inhibitor. Continued incubation up to 90 min (trace d) did not result in further conversion or further increases in P. This result is consistent with the stoichiometric titration of Figure 5, again suggesting that about one-third of the labeled enzyme or, more accurately, one-third of the fluorescence, in this particular preparation does not react with inhibitor. Therefore, in fitting the normalized data to eq 2, we assumed the effective concentration of FITC–C1s to be two-thirds the actual concentration. This resulted in k_2 values of 10.5×10^4 and 11.3×10^4 M^{-1} s^{-1} (Table I). The line drawn through the points in Figure 6 corresponds to $k_2 = 11 \times 10^4$ M^{-1} s^{-1}. The square in Figure 2 indicates that this is slightly high, a discrepancy which would be diminished if the effective concentration of FITC–C1s were closer to the actual than assumed. The important point is that the rates determined by the two methods, one of which measures the formation of covalent complex, are indistinguishable.

The rate of inhibition of this same preparation was also analyzed by the substrate hydrolysis method under pseudo-first-order conditions (6.7 nM FITC–C1s, 160 nM C1-Inh) where uncertainty in the concentration of C1s is of little consequence. The resulting k_{obsd} at 32 °C differed by less than 10% from that of the unlabeled control. Assuming that the labeling caused no change in K_m, a value of $k_2 = 11.9 \times 10^4$ M^{-1} s^{-1} was calculated from eq 1 in good agreement with the results from the polarization and HPSEC analyses.

Acceleration by Heparin. The effect of heparin on the rate of inhibition of C1s by C1-Inh was also examined. Figure 7

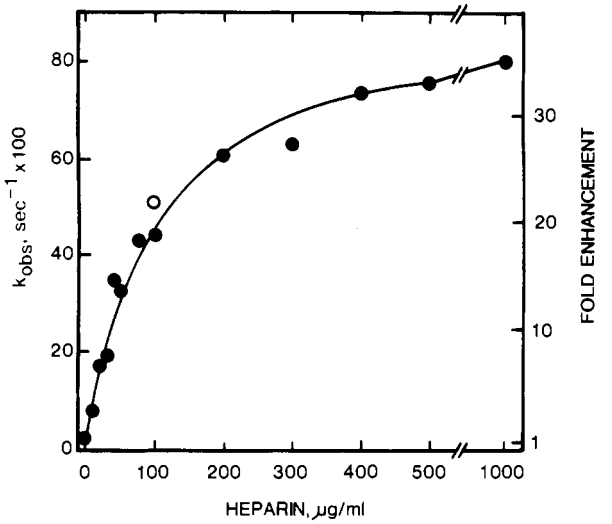

FIGURE 7: Acceleration by heparin of the inhibition of C1s̄ (6.7 nM) by C1-Inh (0.1 μM) under conditions of Figure 1. The pseudo-first-order rate constant, k_{obsd}, was determined as in Figure 1A at 30 °C. The right-hand ordinate gives the ratio of the value of k_{obsd} at any given heparin concentration to that in the absence of heparin. The open circle corresponds to data obtained by the change in fluorescence intensity of FITC–C1s̄ at 25 °C (see Figure 8).

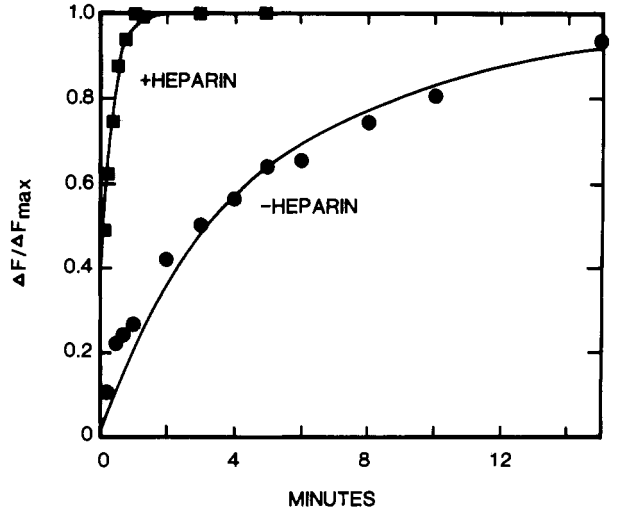

FIGURE 8: Normalized change in fluorescence intensity of FITC–C1s̄ following addition of C1-Inh in the presence (■) and absence (●) of 100 μg/mL heparin at 25 °C in TBS (0.05 M Tris/0.15 M NaCl, pH 7.4). Reactant concentrations were 0.04 μM FITC–C1s̄ and 0.08 μM C1-Inh.

shows that the pseudo-first-order rate constant determined by the substrate hydrolysis method increased progressively with increasing heparin, approaching a maximum 35-fold stimulation above 400 μg/mL with half-maximal stimulation occurring near 100 μg/mL.

The effect of 100 μg/mL heparin on the rate of reaction of FITC–C1s̄ with C1-Inh, as monitored by the decrease in fluorescence intensity at 25 °C, is shown in Figure 8. In this case, the second-order rate constant increased from 5.3×10^4 to 121×10^4 M^{-1} s^{-1}, a 23-fold enhancement. This is close to the effect obtained by the substrate hydrolysis method at the same heparin concentration (see open circle in Figure 7). The FITC–C1s̄ used in this experiment was prepared by a special procedure in which C1s̄ was bound to heparin–Sepharose during the labeling procedure in order to protect the heparin binding site(s) (see Materials and Methods). Material labeled by the conventional procedure showed much less acceleration by heparin as if the same lysine residues important for the binding of heparin had reacted with FITC.

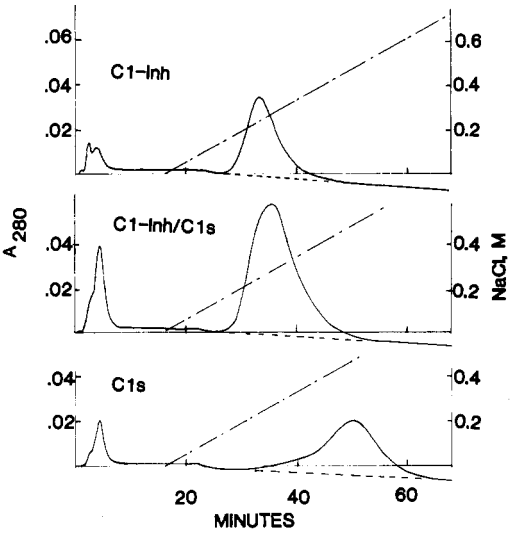

FIGURE 9: Affinity chromatography of C1-Inh, C1s̄, and their complex on heparin–Sepharose in 0.02 M Tris at room temperature. The two proteins were dialyzed against 0.02 M Tris, and 100 μL of 1 mg/mL samples was applied to a small column (bed volume approximately 1 mL) by injection through a Pharmacia V-7 valve. Bound protein was eluted with a linear gradient of NaCl using the Pharmacia FPLC system. The experiment in the center panel involved injection of 190 μL of an equivolume mixture of the two proteins which had been preincubated at room temperature for 1 h. The percent of nonbinding material in the three experiments was between 13% and 14%. The dashed line illustrates a drift in the base line due to a refractive index effect on the detector.

The interaction of these proteins with heparin was examined by affinity chromatography on a small column of heparin–Sepharose. The results are summarized in Figure 9. When C1-Inh (top panel) was applied in a low-salt buffer, 86% of the protein bound to the column and was eluted by a NaCl gradient. The peak of the elution occurred at 0.24 M NaCl. A similar pattern was seen with C1s̄ (bottom panel), except that a higher concentration of NaCl was required for elution (0.5 M). When the two proteins were mixed and incubated at 25 °C for 30 min before chromatography (center panel), 86% of the applied protein again bound to the column. The NaCl concentration required for elution was intermediate between that of the free enzyme and free inhibitor but closer to that of the latter.

A more quantitative analysis of the binding of heparin to these proteins was obtained from measurements of the fluorescence polarization of fluorescein-labeled heparin. As shown in Figure 10, P increased progressively from 0.10 to 0.23 or greater with increasing concentration of the added proteins. The dose response curve was very similar for C1s̄, C1-Inh, and the complex. In all cases, the increase in P was fully reversed by addition of excess unlabeled heparin (not shown). Scatchard-type plots (inset) gave dissociation constants of 2.0 μM for C1s̄ and 3.3 μM for C1-Inh and the complex. Thus, heparin binds reversibly and with similar strength to all three proteins in the fluid phase under conditions identical with those where the accelerating effects on the inhibition reaction were measured.

Discussion

In this study, we have used several different methods to measure the rate of inhibition of C1s̄ by C1 inhibitor. The most sensitive and convenient of these made use of synthetic peptide thiol ester substrates in combination with a chromogenic reagent, allowing the activity of low concentrations of enzyme to be continuously monitored. By varying the concentration of inhibitor under pseudo-first-order conditions, it

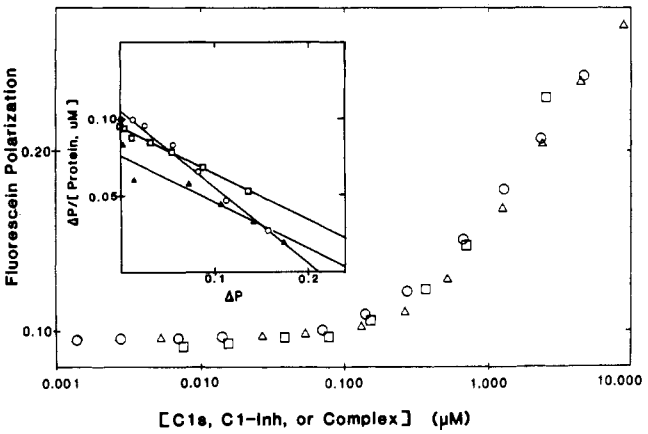

FIGURE 10: Fluorescence polarization of fluorescein-labeled heparin as a function of the concentration of added C1s̄ (○), C1-Inh (△), and their complex (□) at 25 °C in TBS. The concentration of labeled heparin in these experiments was 0.06 μM, and the concentration of unlabeled heparin required for 50% inhibition was approximately 3 μM (not shown). The inset shows Scatchard-type plots of the same data, the slopes of which correspond to K_d values of 2.0 μM (○) and 3.3 μM (□ and △).

was possible to calculate the second-order rate constant by means of eq 1. The values of K_m used for this purpose were 2.0 mM for Z-Lys-sBzl and 70 μM for Z-Gly-Arg-sBzl, determined by double-reciprocal plots of initial velocities vs. substrate concentration (not shown). The latter value compares favorably with a value of 69 μM obtained by McRae et al. (1981) for the same reaction under similar conditions. This agreement is reassuring since the calculated value of k_2 is quite sensitive to the value of K_m, especially when the substrate concentration is large relative to K_m as was the case with the dipeptide substrate. To our knowledge, a value of K_m has not been reported for Z-Lys-sBzl, perhaps because it is not a particularly good substrate for C1s̄. It does have an advantage, however, when used to measure the kinetics of inhibition. Because it is employed far below its K_m, it does not compete very effectively with C1-Inh for the active site of C1s̄, and values of k_2 are relatively insensitive to errors in K_m. The fact that very similar values were obtained with the two different substrates strengthens our confidence in the validity of the result.

Nilsson and Wiman (1983) used a tripeptide *p*-nitroanilide, S-2314, to measure the residual activity of C1s̄ at various times after addition of C1-Inh. In their procedure, enzyme and inhibitor were mixed at zero time, and the substrate was subsequently added to mixtures at different times in an effort to determine residual C1s̄ from the initial slope of absorbance vs. time. At the protein concentrations employed, the reaction appeared to be 75% complete after only 1 min at 37 °C. In our experience, since the proteins were not diluted significantly by addition of substrate, the inhibition reaction would be expected to continue at a rate comparable to that of substrate hydrolysis, complicating efforts to accurately measure the initial slope. The tendency would be to underestimate the remaining C1s̄, perhaps accounting for the order of magnitude higher estimates of k_2 compared to our values under similar conditions. These same authors also reported that small amounts of enzyme persisted in the presence of excess inhibitor long after the inhibition should have been complete. This was interpreted as evidence for an intermediate step corresponding to the reversible formation of a noncovalent complex which is only slowly converted to a stable nondissociable form, according to eq 3. From the apparent rate of decrease in C1s̄ at long times, they computed a value of $k_1 = 10^4$ s^{-1} for the

$$E + I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{-}I \xrightarrow{k_1} E\text{-}I \qquad (3)$$

second (unimolecular) step, 4 orders of magnitude lower than the value found after much effort by Olsen and Shore (1982) for the corresponding step in the reaction of thrombin with antithrombin III. It predicts an unrealistic lower limit of 30 min for the half-life of formation of a stable nondissociable complex, regardless of protein concentration. This is inconsistent with numerous observations that 10–15 min at 37 °C is sufficient to convert all or most of the C1s̄ or C1-Inh, depending on which was in excess, to a higher molecular weight form which is stable during electrophoresis in the presence of SDS (Harpel & Cooper, 1975; Reboul et al., 1977; Weiss & Engel, 1983; Lennick et al., 1985). Alternative explanations for the apparent residual C1s̄ activity include possible spontaneous hydrolysis of the substrate or the presence of altered forms of C1s̄ which react only slowly with the inhibitor (Takahashi et al., 1980).

Sim et al. (1980) directly measured the rate of formation of stable complexes between C1-Inh and ^{125}I-C1s̄ by slicing polyacrylamide gels and determining the percent of radioactivity migrating in the position of the complex. These workers were careful to quench the reaction quickly by dilution into a hot mixture of urea and SDS. Their elegant data followed second-order kinetics over 70% of the reaction with $k_2 = 1.2 \times 10^4$ M^{-1} s^{-1} at 37 °C, about 40-fold slower than Nilsson and Wiman (1983). However, their calculation of an equilibrium dissociation constant of 0.1 μM for the complex, based on the amount of unreacted ^{125}I-C1s̄ after 1 h at 37 °C, has no meaning in the context of eq 3 since all of the limiting component, assuming it to be fully active, would eventually be converted to the nondissociable form. Although there are examples of plasma protease–inhibitor complexes which slowly dissociate to produce active enzyme (Fish & Bjork, 1979; Laine et al., 1984), the covalently modified inhibitor which results from such dissociation is no longer active and thus cannot be viewed as an equilibrium dissocation product. Furthermore, we found no evidence for release of active C1s̄ from the complex even after 5 days at 37 °C under conditions where the kinetics were measured. However, we did not test in the presence of heparin, which has been shown to enhance the production of modified inhibitors (Weiss & Engel, 1983; Olson, 1985).

The large discrepancy between the rates reported by Nilsson and Wiman (1985) and Sim et al. (1984) and the possibility that the discrepancy could be due to the different methodologies employed prompted us to further investigate the kinetics by several methods. The substrate hydrolysis method measures loss of active enzyme and would presumably not distinguish between noncovalent intermediates and stable complexes. At the other extreme is the HPSEC method which, like the electrophoretic method of Sim et al., detects only the stable complex. The various fluorescence methods cannot be unambiguously classified in this respect. However, we obtained very similar rate constants by all of the methods despite the wide range of concentrations employed (6.7–100 nM for C1s̄ and 0.04–7.2 μM for C1-Inh). This argues strongly against the accumulation of significant quantities of reversible intermediate under the conditions employed. For all practical purposes, the reaction is pure second order, the second step being simply too fast to be of any consequence from a kinetic point of view. A similar conclusion was reached by van der Graaf et al. (1983) for the reaction of C1-Inh with kallikrein.

The values of k_2 obtained by us are intermediate between those reported by the other two groups but closest to those of

Sim et al. (1980). Our activation energy of 11 kcal/mol also agrees very well with their value of 11.7 kcal/mol. Assuming a value of 1.3 μM for the physiological concentration of C1-Inh (Ziccardi, 1981), we calculate a half-life of about 5 s for free C1s̄ in blood, about 10-fold shorter than that estimated by Travis and Salvesen (1983), but still slow relative to other plasma protease inhibitors. This could be due to geometrical and size constraints which slow the diffusion of the components, both of which are highly elongated and asymmetric (Tschopp et al., 1980; Odermatt et al., 1981). Another factor limiting the rate could be electrostatic repulsion between the components, since both have net negative charge at neutral pH (Cooper, 1985). The rate of inhibition was unaffected by Ca^{2+} in spite of the fact that C1s̄ self-associates to form a dimer in the presence of that ion (Valet & Cooper, 1974; Arlaud et al., 1977). van der Graaf et al. (1983) reported that inhibition of kallikrein by C1-Inh was unaffected by high molecular weight kininogen which forms a complex with the heavy chain of that enzyme. These observations are consistent with the proposed multidomain structure of C1s̄ (Colomb et al., 1984; Villiers et al., 1985; Cooper, 1985) and other serine proteases (Neurath, 1984; van der Graaf, 1983) in which one domain contains the catalytic site while the other independently participates in protein–protein interactions. Even in whole C1̄, the available data suggest that the active sites of C1s̄ remain freely accessible to the inhibitor providing the system is fluid phase. However, when bound to antibody-coated erythrocytes, C1̄ is inhibited much less effectively (Medicus & Chapuis, 1980; Caughman et al., 1982; Tenner & Frank, 1986), presumably owing to additional charge repulsion and steric effects.

Although the anticomplement activity of heparin has been known for many years, the mechanism of its action has not been elucidated. Caughman et al. (1982) showed that the concentration of heparin required to inhibit C1̄ hemolytic activity was greatly increased in the absence of C1-Inh, confirming an important role for the inhibitor in this phenomenon. Such would be expected from the strong accelerating effects of heparin on the reaction of C1-Inh with the free enzymes (Sim et al., 1980; this work). Detailed interpretation of the catalytic mechanism requires knowledge of the interaction of heparin with the various components of the system. Studies by McKay et al. (1981) suggested that free C1-Inh had little affinity for heparin–Sepharose but that its complexes with C1r̄ and C1s̄ bound readily. We found that all three species, i.e., C1s̄, C1-Inh, and their complex, bind tightly to heparin–Sepharose under conditions where the kinetic experiments were performed; salt concentrations substantially higher than physiological were required to elute the proteins from the column. The apparent stronger affinity for heparin–Sepharose of C1s̄ relative to C1-Inh in terms of their position of elution in a NaCl gradient did not show up in the titration experiments. This illustrates the hazard of inferring fluid phase behavior from solid-phase experiments where surface density, orientation, and steric factors may confuse the issue. Titration of fluorescent-labeled heparin provided some quantitative insight into the strength of the fluid-phase interaction and suggested that all three components have similar dissociation constants near 2–3 μM. Assuming an average molecular weight of 10000–20000, 100 μg/mL heparin corresponds to a molar concentration 5–10-fold greater than our estimate of the K_d. This suggests that all three components would be saturated under conditions of maximal rate enhancement and argues against a catalytic mechanism in which single molecules of heparin initiate multiple rounds of complex formation. Our results are consistent with a catalytic mechanism whereby heparin, by interacting with both the enzyme and the inhibitor, increases the probability of an encounter between them. The possibility that heparin-induced conformational changes are also involved cannot be excluded. However, if heparin causes a conformational change in C1s̄, it probably does not involve the active site since concentrations of heparin up to 100 μg/mL had no effect on the rate of hydrolysis of Z-Lys-sBzl (data not shown). Furthermore, heparin does not affect the intrinsic fluorescence of C1s̄ or C1-Inh. Additional studies are required before a more detailed mechanism can be proposed.

The much greater binding of bis(ANS) by the C1s̄–C1-Inh complex, relative to the individual components suggests conformational changes associated with the creation of one or more hydrophobic sites on the surface of the complex. This is consistent with earlier studies showing an increased exposure to the solvent of hydrophobic (aromatic) amino acid side chains during complex formation (Lennick et al., 1985). Additional evidence for conformational differences between the complex and the free components comes from recent work of de Agostini et al. (1985 and private communication). These workers, using monoclonal antibodies, showed that reaction of C1-Inh with kallikrein, factor XIIa, or C1s̄ leads to the emergence of an epitope, located on the inhibitor, which is not detectable on the parent enzyme or inhibitor molecules. Such conformational changes could be involved in the recognition of protease–inhibitor complexes by receptors involved in their clearance from circulation in the blood (Gonias et al., 1983).

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Registry No. C1s, 80295-70-1; C1 inhibitor, 80295-38-1.

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Aplysia Oxymyoglobin with an Unusual Stability Property: Kinetic Analysis of the pH Dependence

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ABSTRACT: Native oxymyoglobin (MbO_2) was isolated directly from the radular muscle of *Aplysia kurodai* and was examined for its stability properties over the wide range of pH 4-13 in 0.1 M buffer at 25 °C. When compared with sperm whale MbO_2 as a reference, *Aplysia* MbO_2 is found to be extremely unstable with an unusual pH dependence for its autoxidation rate. Kinetic analysis has revealed that *Aplysia* MbO_2, lacking the distal histidine, does not show such a proton-catalyzed process that can play a dominant role in the autoxidation reaction of sperm whale MbO_2, involving the distal histidine as its catalytic residue. Rather, *Aplysia* MbO_2 contains two kinds of dissociable groups with pK_a = 4.2 and 6.1, respectively, both probably being carboxyl groups and both also being responsible for an increase in its autoxidation rate in the acidic pH range. Therefore, the extreme susceptibility of *Aplysia* MbO_2 to autoxidation comes mainly from the rate constant for a nucleophilic displacement of O_2^- from MbO_2 by an entering water molecule, with the iron ending up as the ferric form. Its value is found to be 100 times higher than the corresponding value for sperm whale MbO_2. In relation to structural evidence, these findings suggest that the heme pocket of *Aplysia* MbO_2 is open enough to allow easier attack of the solvent water molecule on the FeO_2 center.

Unlike mammalian myoglobins, *Aplysia* myoglobins contain only a single histidine residue, lacking the usual distal one (Tentori et al., 1973; Suzuki et al., 1981; Takagi et al., 1984), and the circular dichroism magnitude being about two-thirds that of sperm whale myoglobin (Shikama et al., 1982). Furthermore, the hydropathy profiles obtained from the amino acid sequence of *Aplysia* myoglobins are quite different from that of sperm whale myoglobin, especially on the distal side of the heme iron (Takagi et al., 1984).

We have recently succeeded in isolating native oxymyoglobin directly from the radular muscle of *Aplysia kurodai*, a common species around the Japanese coast, and have examined its spectral and stability properties. The absorption spectrum of *Aplysia* MbO_2[1] is very similar to those of mammalian oxymyoglobins. Its stability, however, is quite different from those of the mammalian oxymyoglobins, and *Aplysia* MbO_2 is found to be extremely susceptible to autoxidation (Shikama & Katagiri, 1984).

In this paper, we describe a mechanistic analysis of the unusual pH dependence for the stability properties of *Aplysia* MbO_2. This kinetic analysis seems to be of great interest if

[1] Abbreviations: MbO_2, oxymyoglobin; metMb, metmyoglobin; HbO_2, oxyhemoglobin; metHb, methemoglobin; MES, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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