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Mechanism of Serpin Action: Evidence That C1 Inhibitor Functions as a Suicide Substrate†

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ABSTRACT: Serpins form a family of structurally related proteins, many of which function in plasma as inhibitors of serine proteases involved in inflammation, blood coagulation, fibrinolysis, and complement activation. To further characterize the mechanism by which serpins inhibit their target enzymes, we have studied the effect of temperature on the reaction of C1 inhibitor and the serine protease plasma kallikrein. At both 38 and 4 °C, C1 inhibitor (M_r 105 000) is cleaved by α-kallikrein (M_r 85 000 and 88 000) at position P_1 (Arg^{444}) of the reactive center, a reaction that leads to the formation of a covalent bimolecular enzyme–serpin complex (M_r 195 000) and cleaved but uncomplexed serpin (M_r 95 000). Between 38 and 4 °C, the product distribution is temperature-dependent, with more cleaved C1 inhibitor (M_r 95 000) formed at lower temperatures and correspondingly less M_r 195 000 complex. Studies employing intrinsic tryptophan fluorescence and ^1H NMR spectroscopy show that this behavior is not caused by temperature-dependent conformational changes of kallikrein or C1 inhibitor. C1 inhibitor also behaves in this manner with the light chain of kallikrein and, to a lesser extent, with plasmin and C$\bar{1}$s. These data are best explained by a branched reaction pathway, identical with the scheme describing the mechanism of action of suicide substrates. This scheme involves the formation of an enzyme–inhibitor intermediate, which can be stabilized into a covalent complex and/or dissociate into free enzyme and cleaved inhibitor, depending on the reaction conditions.

C1 inhibitor, the missing or dysfunctional protein in hereditary angioedema (Donaldson & Evans, 1963), is a member of the serpin superfamily of protease inhibitors, which also includes α_1-antitrypsin, antithrombin III, and the plasminogen activator inhibitors (Huber & Carrell, 1989; Schapira & Patston, 1991). C1 inhibitor is the only inhibitor in serum for the C$\bar{1}$r and C$\bar{1}$s subcomponents of the first component of complement (Sim et al., 1979). C1 inhibitor is also the predominant inhibitor of the blood coagulation cascade enzymes plasma kallikrein and activated factor XII, although some of the inhibitory capacity of normal plasma against kallikrein is mediated by α_2-macroglobulin (Schapira et al., 1982; van der Graaf et al., 1983a; de Agostini et al., 1984; Pixley et al., 1985), antithrombin III (Olson & Choay, 1989), and activated protein C inhibitor (Meijers et al., 1988). Using assays specific for kallikrein–C1 inhibitor and kallikrein–α_2-macroglobulin complexes, Harpel et al. (1985) showed that, in normal plasma activated by kaolin, temperature has a dramatic effect on the distribution of this serine protease between the two inhibitors. At 37 °C, 67% of kallikrein is bound to C1 inhibitor and 33% to α_2-macroglobulin; at 4 °C, 85% is bound to α_2-macroglobulin and 15% to C1 inhibitor (Harpel et al., 1985). Earlier evidence that C1 inhibitor is inefficient at low temperature was provided by studies where normal plasma was supplemented with radioiodinated prekallikrein, activated with kaolin, and the distribution of ^{125}I-kallikrein between various inhibitors was evaluated by SDS–PAGE and autoradiography (van der Graaf et al., 1983a), and by investigations on kallikrein-dependent cold-promoted activation of factor VII (van Royen et al., 1978). However, the above-referenced studies did not address the question of why C1 inhibitor is inefficient at low temperature. The relative inefficiency of C1 inhibitor in the cold was also noticed by Cameron et al. (1989).

In the present paper, we describe a study in a purified system of the effect of temperature on the reactivity of C1 inhibitor with plasma kallikrein. Although C1 inhibitor was cleaved by kallikrein at an identical site at 38 and 4 °C, it functioned more efficiently as an inhibitor at 38 °C than at 4 °C. However, even at 38 °C, there was significant proteolysis of C1 inhibitor without enzyme inhibition. Since C1 inhibitor displayed this behavior with other serine proteases, it is a

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general property of this serpin. These results have important implications for the understanding of the mechanism by which C1 inhibitor inhibits its target enzymes.

Materials and Methods

Materials. Chemical reagents and D_2O (99.8%) (Sigma, St. Louis, MO), electrophoresis reagents (Bio-Rad, Richmond, CA), column chromatography resins (Pharmacia, Piscataway, NJ), the chromogenic substrates H-D-Pro-Phe-Arg-*p*-nitroanilide (S-2302)[1] and S-2251 (Kabi Vitrum Inc., Franklin, OH), and *p*-nitrophenyl *p'*-guanidinobenzoate (NPGB) and D-phenylalanyl-L-phenylalanyl-L-arginine chloromethyl ketone (Calbiochem, San Diego, CA) were obtained from the designated supplier. Me-CO-Lys-(ϵ-Cbo)-Gly-Arg-*p*-nitroanilide (Spectrozyme C_1-E) was a gift from Dr. Richard Hart, American Diagnostica, Greenwich, CT.

Electrophoretic Studies. SDS-PAGE, nonreduced, was performed with a Bio-Rad Protean II slab gel system, using the method of Laemmli (1970). The sample buffer contained 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.002% Bromophenol Blue. The acrylamide concentration was 4% in the stacking gel and 7.5 or 7.5–10% gradient in the separating gel. Samples were boiled for 3 min prior to being loaded. Western blots were carried out by using Bio-Rad Mini Gel and Mini Trans-Blot systems. Proteins were detected with rabbit antibodies against C1 inhibitor or kallikrein and revealed with goat anti-rabbit IgG conjugated with horseradish peroxidase, 4-chloro-1-naphthol/H_2O_2 being the developing reagent.

Proteins. Human plasma kallikrein in the α form was prepared as published (Burger et al., 1986). Kallikrein light chain was purified as follows: 1 mg of kallikrein was reduced and carboxymethylated using dithiothreitol and iodoacetamide, as described by van der Graaf et al. (1982). After dialysis into 20 mM Tris-HCl, pH 7.4, 1 M NaCl, and 0.02% sodium azide, the light chain was separated by chromatography on soybean trypsin inhibitor–agarose (Nagase & Barrett, 1981). Human C$\bar{1}$s was purchased from Enzyme Research Laboratories, South Bend, IN. Human plasminogen was prepared by lysine–Sepharose chromatography (Deutsch & Mertz, 1970) and activated with human kidney urokinase (Sigma, St. Louis, MO). Purified human C1 inhibitor was a gift from Dr. E. P. Paques, Behring, Marburg, Germany. α_1-Antitrypsin–Arg^{358} and α_1-antitrypsin-Ala^{357}-Arg^{358} (Patston et al., 1990) were provided by Michael Courtney and Rainer Bischoff, Transgene, Strasbourg, France. Kallikrein and plasmin activities were standardized by active-site titration with D-Phe-Phe-Arg chloromethyl ketone (Kettner & Shaw, 1981), which had been standardized with active-site-titrated trypsin. TPCK-treated trypsin (Worthington, Freehold, NJ) was active-site-titrated with NPGB (Chase & Shaw, 1970). The concentration of C$\bar{1}$s was as indicated by the supplier; C$\bar{1}$s was assessed to be fully active by its ability to be completely complexed with an excess of C1 inhibitor. Serpin concentrations were determined from the rate of inhibition, under pseudo-first-order conditions, of C$\bar{1}$s (for C1 inhibitor) and plasma kallikrein (for α_1-antitrypsin mutants), using published values for the second-order rate constants (Patston et al., 1990).

Kallikrein, Plasmin, and C$\bar{1}$s Inhibition Titrations. The various proteases were incubated at a final concentration of 0.2 μM in buffer (20 mM sodium phosphate, pH 7.4, 150 mM sodium chloride, 1 mg/mL bovine serum albumin, and 0.02% sodium azide) with inhibitor concentrations ranging from 0.02 to 2 μM at the temperature under investigation. The incubation times used were determined from calculations using inhibition rate constants. Control experiments (see Results and Discussion) indicated that these incubation times were sufficient for completion of the inhibition reactions. The following conditions were used. Kallikrein and its light chain were incubated with C1 inhibitor at 4 °C for 5 days; kallikrein was incubated with C1 inhibitor at 8 °C for 4 days; kallikrein and its light chain were incubated with C1 inhibitor at 38 °C for 18 h; kallikrein was incubated with the α_1-antitrypsin mutants at 4 °C for 18 h and at 38 °C for 2 h. C$\bar{1}$s was incubated with C1 inhibitor at 38 °C for 2 h and at 4 °C for 18 h. Plasmin was incubated with C1 inhibitor at 38 °C for 2 h; plasmin was incubated with the α_1-antitrypsin mutants at 38 °C for 1 h; plasmin was incubated with all inhibitors at 4 °C for 18 h. In all cases, a control incubation containing no inhibitor was included. After incubation, the residual enzymatic activity was measured with the appropriate chromogenic substrate (S-2302 for α-kallikrein and kallikrein light chain, S-2251 for plasmin, and Spectrozyme C_1-E for C$\bar{1}$s). With S-2302, a 0.6 mM solution was prepared in 85 mM sodium phosphate buffer, pH 7.6, containing 127 mM NaCl. C_1-E (0.75 mM) and S-2251 (0.6 mM) were used at the designated concentrations in 50 mM Tris-HCl buffer, pH 7.4, containing 127 mM NaCl. Ten microliters of the solution to be tested was added to 0.33 mL of substrate at 37 °C, and the absorbance change at 405 nm was continuously recorded with a Gilford 260 spectrophotometer. Results are plotted as the fraction of enzyme remaining (E/E_0) vs the ratio of the initial inhibitor to initial enzyme concentration (I_0/E_0). The intercept on the abscissa is the apparent stoichiometry of the reaction. The partition ratio r was determined, the apparent stoichiometry being equal to $1 + r$ (Waley, 1985).

Isolation and Partial Sequence of C1 Inhibitor Cleavage Peptides. SDS-PAGE and Western blot studies revealed that the protein mixture obtained by the incubation of kallikrein with C1 inhibitor includes the following components: (a) the kallikrein–C1 inhibitor complex (M_r 195 000); (b) native C1 inhibitor (M_r 105 000); (c) cleaved C1 inhibitor (M_r 95 000); (d) unreacted kallikrein (M_r 88 000); (e) a peptide (M_r 5000) which is derived from the carboxy-terminal end of C1 inhibitor [data not shown; see also Weiss and Engel (1983), Nilsson et al. (1983), and van der Graaf (1983a)]. The following protocol was used to obtain C1 inhibitor cleavage peptides: 160 μg of kallikrein (1.88 nmol) was incubated with 205 μg of C1 inhibitor (1.75 nmol) on ice or at 38 °C for 18 h; at the end of incubation, protein mixtures were made 10% with β-mercaptoethanol and boiled for 3 min. The peptides were then purified by reverse-phase high-pressure liquid chromatography on a PLRP-S 300A column (250 × 4.6 mm; Polymer Laboratories, Amherst, MA) in 5% acetonitrile/0.1% trifluoroacetic acid. Elution conditions were essentially as described by Aulak et al. (1988). The purity of the peptides was verified by SDS-PAGE analysis and immunoblotting using polyclonal antisera against C1 inhibitor and kallikrein (not shown). Only one C1 inhibitor cleavage peptide was detectable. This peptide eluted at the same acetonitrile concentration as the C-terminal peptide produced by trypsin digestion of C1 inhibitor (Aulak et al., 1988). The yield of peptide was the same at both temperatures, as judged by comparison to the amount of unreacted C1 inhibitor in each sample. Peptide sequencing was performed at the Protein Chemistry Labora-

[1] Abbreviations: S-2302, H-D-Pro-Phe-Arg-*p*-nitroanilide; S-2251, H-D-Val-Leu-Lys-*p*-nitroanilide; Spectrozyme C_1-E, Me-CO-Lys-(ϵ-Cbo)-Gly-Arg-*p*-nitroanilide; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; α_1-AT, α_1-antitrypsin.

tory of the Vanderbilt University Biochemistry Department using an Applied Biosystems 470A protein sequencer and a 120A PTH analyzer.

Intrinsic Fluorescence. The intrinsic tryptophan fluorescence of kallikrein and C1 inhibitor was measured by using an ISS Greg 2000 fluorometer operating in the photon-counting mode. The proteins were in 20 mM sodium phosphate, pH 7.4, and 150 mM sodium chloride. The excitation wavelength was 295 nm (bandwidth = 12 nm). At this wavelength, the proteins had an absorbance of 0.1. Emission spectra were performed from 310 to 410 nm (bandwidth = 12 nm), in 1-nm increments. Fluorescence intensity was measured relative to a reference signal to compensate for fluctuations in the excitation source. The temperature of the sample cuvette was controlled by a circulating water bath.

Nuclear Magnetic Resonance Spectroscopy. C1 inhibitor and kallikrein were concentrated by using an Amicon XM-50 membrane concentrator to 0.1 mM in 20 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and made in D_2O. Prior to concentration, α-kallikrein was incubated with 2 mM diisopropyl fluorophosphate. SDS-PAGE analysis of kallikrein after the recording of the NMR spectra indicated that this treatment efficiently prevented conversion of α-kallikrein into the β form, as reported previously (Burger et al., 1986). ^{1}H NMR spectra were recorded on a narrow-bore Bruker AM 400 spectrometer equipped with a 5-mm ^{1}H probe. A sweep width of 6024 Hz, a data block size of 8K points zero-filled to 16K prior to Fourier-transformation, and a pulse angle of 60° were employed. The intensity of the residual water peak was reduced by using a low-power presaturation pulse of 0.5-s duration. For some spectra, a Carr-Purcell-Meiboom-Gill pulse train $[90°_x-(\tau-180°_y-\tau)_n]$ was used (Meiboom & Gill, 1958). Chemical shifts are given relative to an external standard of dimethylsilapentanesulfonate at 0 ppm.

Results and Discussion

Effect of Temperature on the Inhibition of Plasma Kallikrein by C1 Inhibitor: SDS-PAGE Studies. The reaction between C1 inhibitor and its target enzymes leads to the formation of covalent bimolecular enzyme-inhibitor complexes and of proteolytically cleaved C1 inhibitor (Harpel & Cooper, 1975; Weiss & Engel, 1983; de Agostini et al., 1985, 1988). We have examined the effect of temperature on the inhibition of plasma kallikrein by C1 inhibitor. C1 inhibitor (M_r 105 000; Figure 1, lane A) was incubated at 0 or 38 °C with plasma kallikrein (M_r 85 000 and 88 000; Figure 1, lane B), and the products of the reaction were analyzed by SDS-PAGE. Following incubation of kallikrein and C1 inhibitor at 0 °C, the predominant reaction product was cleaved C1 inhibitor (M_r 95 000), with only a small amount of kallikrein-C1 inhibitor complex (M_r 195 000) being formed and the majority of the kallikrein remainining uncomplexed (Figure 1, lane C). However, when kallikrein was incubated with C1 inhibitor at 38 °C, complex formation was prevalent (M_r 195 000; Figure 1, lane D) with correspondingly less cleaved inhibitor being formed. These results provide a qualitative demonstration that the nature of the products of the reaction between kallikrein and C1 inhibitor is dependent on the reaction temperature. Importantly, additional SDS-PAGE studies revealed that the M_r 195 000 kallikrein-C1 inhibitor complex formed at 38 °C remained stable after an 102-h incubation at 23, 4, or -20 °C (not shown). Thus, the appearance of cleaved C1 inhibitor (M_r 95 000; Figure 1, lane C) is not a result of dissociation of the stable kallikrein-C1 inhibitor complex by low temperature, SDS, or electrophoresis conditions.

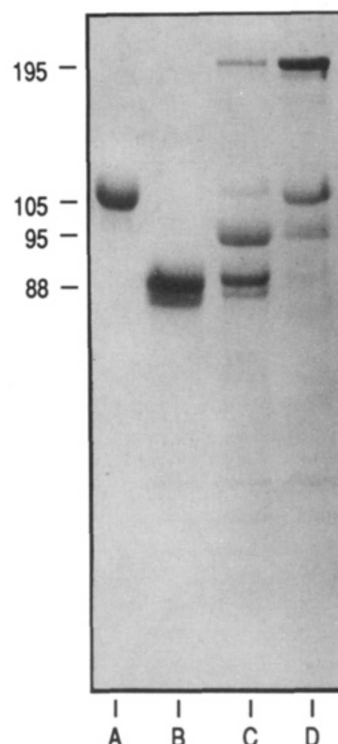


FIGURE 1: SDS-PAGE analysis (7.5%, unreduced) of C1 inhibitor (M_r 105 000, lane A) and plasma kallikrein (M_r 88 000 and 85 000, lane B). (Lanes C and D) Protein mixture resulting from the incubation of kallikrein and C1 inhibitor at 0 °C (lane C) and 38 °C (lane D). Ten micrograms of each protein in a total volume of 60 μL was incubated at 0 or 38 °C for 2 h, as indicated. After incubation, samples were mixed with sample buffer, boiled, and run on a 1.5-mm-thick, 7.5% polyacrylamide gel, which was stained with Coomassie Blue R250. Left margin numbers are $M_r \times 10^{-3}$.

Cleavage Peptide Characterization. A possible mechanism for the SDS-PAGE data reported in the preceding paragraph is that C1 inhibitor was cleaved by kallikrein at different sites at low and high temperature. Thus, experiments were undertaken to identify the cleavage sites. The peptides released from C1 inhibitor during incubation with kallikrein at 0 or 38 °C were purified by reverse-phase high-pressure liquid chromatography and sequenced (see Materials and Methods). The amino-terminal sequence of both peptides was Thr-Leu-Leu-Val-Phe-Glu-Val-Gln-Gln- demonstrating that C1 inhibitor was cleaved by kallikrein at the reactive site position P_1 (Salvesen et al., 1985; Bock et al., 1986) at both 0 and 38 °C. The observation that kallikrein cleaves C1 inhibitor at the same site at low and high temperature indicates that a common reaction pathway is shared up to the proteolytic event, irrespective of whether the outcome of the reaction is complex formation (and enzyme inhibition) or proteolysis without inhibition (i.e., turnover of substrate).

Effect of Temperature on the Conformation of C1 Inhibitor and Kallikrein. Protein conformational changes could be responsible for the effect of temperature on the reaction between kallikrein and C1 inhibitor. This possibility was explored by examining the intrinsic fluorescence and ^{1}H NMR spectra of native unreacted kallikrein and C1 inhibitor. Tryptophan intrinsic fluorescence spectra were recorded at temperatures ranging from 5 to 30 °C, with excitation at 295 nm and measuring emission between 310 and 410 nm. The spectra were integrated, normalized to the value at 5 °C, and plotted as function of temperature. The pattern observed with kallikrein is illustrated in Figure 2. It was concluded that kallikrein did not undergo conformational changes within the temperature range under investigation. No sharp transition

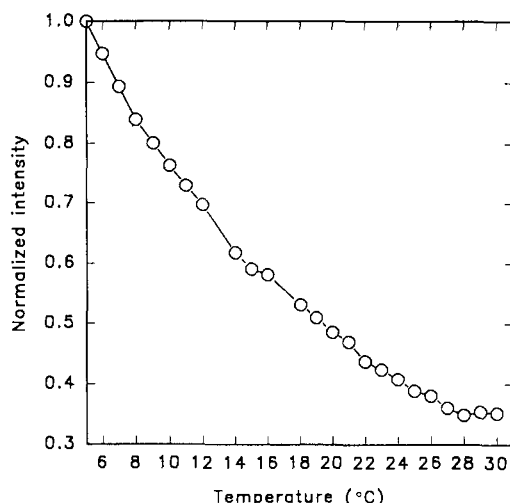


FIGURE 2: Total fluorescence intensity of kallikrein as a function of temperature (normalized to the value at 5 °C). The fluorescence spectra of tryptophan residues was measured by excitation at 295 nm and by measuring the emission between 310 and 410 nm. To show the temperature-induced quenching of fluorescence, the data for the spectra at each temperature were integrated over the complete wavelength range and plotted as a function of temperature.

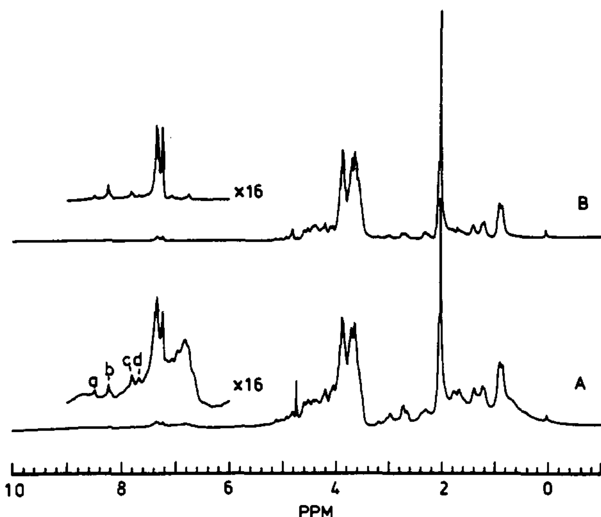


FIGURE 3: 400-MHz ^{1}H NMR spectra of the C1 inhibitor at 298 K. (A) Normal ^{1}H spectrum. (B) Carr-Purcell-Meiboom-Gill spectrum of the same sample. Insets: 16-fold amplifications of the regions between 6 and 9 ppm.

of fluorescence was observed (Figure 2), nor was there any red or blue spectral shift (not illustrated). The large monotonic temperature-dependent quenching of kallikrein fluorescence seen in Figure 2 was reversible on temperature lowering; thus, it was not caused by protein adsorption to the quartz cuvette. Because of the lack of transition, this effect was interpreted as a direct effect of temperature on tryptophan in a polar environment, but not as an indicator of a change in the conformation of kallikrein (Gally & Edelman, 1962; Cantor & Schimmel, 1980; Freifelder, 1982). Intrinsic fluorescence spectra of C1 inhibitor showed a small (10%) quenching effect between 5 and 30 °C, no spectral shift being observed (not illustrated).

The ^{1}H NMR spectrum of C1 inhibitor at 25 °C is shown in Figure 3A, with, in the inset, a 16-fold expansion of the downfield region between 6 and 9 ppm. The peaks marked a–d were attributed to hypermobile histidine C(2) protons. Figure 3B shows a Carr–Purcell–Meiboom–Gill spectrum of the same sample. The Carr–Purcell–Meiboom–Gill pulse

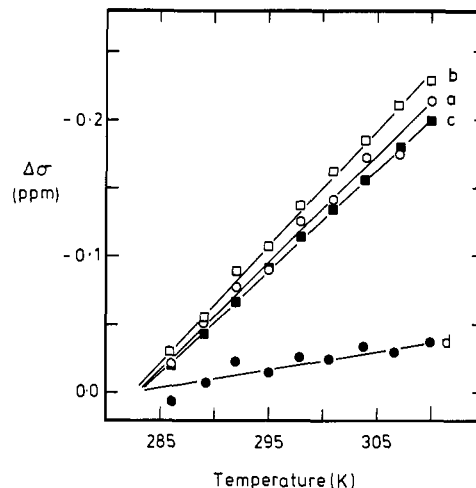


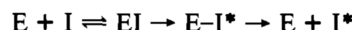
FIGURE 4: Variation in chemical shifts of the C1 inhibitor histidine [C(2)] protons shown in Figure 3 as a function of temperature.

sequence is used to observe the resonances with longer T_2 values and thus narrower resonances (Campbell et al., 1975). These spectra have a better signal to noise ratio and allow for easier interpretation. The effect of temperatures between 12 and 35 °C on the chemical shifts of the four histidine C(2) protons was determined (Figure 4). Although the chemical shift of each proton changed uniquely as a function of temperature, there was no indication of sharp structural transitions. The change in chemical shift seen is consistent with a change in the pK_a of the histidines (Gettins, 1987). Four hypermobile histidine C(2) protons were also detectable in the ^{1}H NMR spectrum of kallikrein. Changes in the chemical shifts of these protons were linear between 12 to 35 °C (not shown). In addition, two strongly upfield-shifted methyl group resonances, which were expected to be sensitive to the conformation of the protein, showed no change in chemical shift over this temperature range. A similar lack of temperature dependence was found for four sets of resonances from mobile tyrosine side chains.

The intrinsic fluorescence and NMR data presented in this section demonstrate that neither kallikrein nor C1 inhibitor undergoes large conformational changes within the temperature range under investigation, although minor conformational changes in localized areas of the proteins, such as the active-site region, may not be detectable by these methods. It is unlikely, however, that temperature-dependent isomerization of either native kallikrein or C1 inhibitor is the mechanism responsible for the differences seen between lanes C and D of Figure 1.

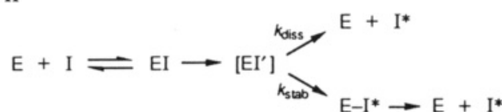
Implications for the Reaction Mechanism. It is believed that C1 inhibitor inhibits plasma kallikrein by using the mechanism employed by other serpins for inhibition of their target serine proteases. This reaction has been reported (Travis & Salvesen, 1983) to proceed according to the mechanism shown in Scheme I where E is the serine protease, I is the native serpin, EI is the reversible Michaelis complex between E and I, I* is the reactive site cleaved (between P_1 and P_1') serpin, and E–I* is the stable and irreversible complex between E and I*. E–I* complexes are believed to be covalent since they resist denaturation by SDS or urea. Because E–I* complexes are dissociated by exposure to nucleophiles, it has been proposed that they contain an ester bond between the carbonyl group of the serpin P_1 residue and the γ-hydroxyl group of the active-site serine of the target enzyme (Travis & Salvesen, 1983).

Scheme I



$$E + I \rightleftharpoons EI \rightarrow E\text{–}I^* \rightarrow E + I^*$$

Scheme II



The stability of E–I* complexes upon prolonged incubation is illustrated by the observation that the half-lives for the breakdown of the stable complexes formed between thrombin and antithrombin III (Danielsson & Björk, 1983) or chymotrypsin and α_1-antitrypsin (Vercaigne-Marko et al., 1987) are about 3 days. In the case of the complex between kallikrein and C1 inhibitor, our present data indicate that dissociation is also very slow since no detectable breakdown of the M_r 195 000 species is observed after 4 days. Therefore, our observation (Figure 1, lanes C and D) that two reaction products (kallikrein–C1 inhibitor complex, M_r 195 000; and cleaved C1 inhibitor, M_r 95 000) are detectable after a 2-h incubation of kallikrein and C1 inhibitor cannot be accommodated by Scheme I. In contrast, the formation of these two products is predicted by the mechanism of Scheme II, which describes the reaction pathway employed by suicide substrates. These compounds are also known as suicide inhibitors, or mechanism-based inhibitors, or Trojan horse inhibitors, or enzyme-activated irreversible inhibitors (Walsh, 1984; Fersht, 1985; Knight, 1986).

In Scheme II, the intermediate [EI′] is formed from EI. [EI′], which could be a tetrahedral intermediate or an acyl-enzyme, is stabilized into a bimolecular complex, E–I*, with a rate constant of k_{stab}, or it can dissociate into free enzyme, E, and reactive site cleaved serpin, I*, with a rate constant of k_{diss}. Therefore, the different product distribution seen at 0 and 38 °C (Figure 1, lanes C and D) can be explained by a differential effect of temperature on k_{diss} and k_{stab}. At 0 °C, k_{diss} is greater than k_{stab}, and one observes free enzyme (M_r 85 000 and 88 000), cleaved C1 inhibitor (M_r 95 000), and only a small amount of bimolecular complex (M_r 195 000). At higher temperatures, k_{stab} becomes larger relative to k_{diss}, and one observes the formation of more kallikrein–C1 inhibitor complex (M_r 195 000). In addition, the structure of [EI′] may be temperature-sensitive, which could influence whether k_{diss} or k_{stab} is the predominant reaction.

Effect of Temperature on the Stoichiometry of Reaction between Kallikrein and C1 Inhibitor. SDS–PAGE analyses have revealed that kallikrein and C1 inhibitor form an equimolar enzyme–inhibitor complex and, thus, that the true stoichiometry for the inhibition of kallikrein by C1 inhibitor is 1 (Figure 1; Schapira et al. 1981; van der Graaf et al., 1982). However, with inhibitors obeying the mechanism described in Scheme II, measurements of residual enzyme activity often yield apparent stoichiometry values >1 (because inhibition does not take place at every catalytic cycle). The partition ratio r, where $r = k_{diss}/k_{stab}$, can be calculated, the apparent stoichiometry being equal to $1 + r$. This ratio represents the number of catalytic turnovers per inactivation event (Walsh, 1984; Waley, 1985; Fersht, 1985; Knight, 1986). In the present study, the apparent stoichiometries for the inhibition of kallikrein by C1 inhibitor were determined by titrating fixed amounts of enzyme with increasing amounts of inhibitor and then quantitating kallikrein residual amidolytic activity with the chromogenic substrate S-2302. For some of the data points, it was shown that inhibition had reached completion, since kallikrein residual activity was not reduced upon further incubation of the enyzme–inhibitor mixture. For other points, completion of the reaction was demonstrated by the observation that, at a specific inhibitor/enzyme ratio, a 2-fold change in the absolute concentration of the reactants was not accompanied by a change in the residual activity of the enzyme. Different apparent stoichiometries were obtained, dependent on the incubation temperature: 2.5 at 38 °C (Figure 5, squares); 3.5 at 8 °C (Figure 5, triangles); 6.1 at 4 °C (Figure 5, circles). These values correspond to partition ratios of 1.5, 2.5, and 5.1, respectively. Thus, the partition ratio for the reaction of kallikrein and C1 inhibitor, which is already significant at 38 °C, increases by a factor of 3.4 when the reaction temperature is reduced to 4 °C.

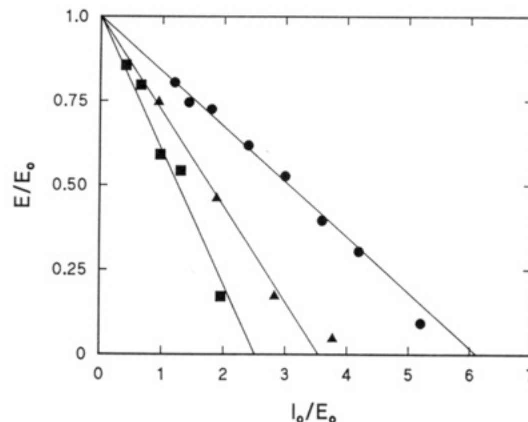


FIGURE 5: Apparent stoichiometries of the inhibition of kallikrein by C1 inhibitor. Kallikrein was incubated at 4 °C (circles), 8 °C (triangles), or 38 °C (squares) with various concentrations of C1 inhibitor. After incubation to completion, residual kallikrein amidolytic activity was determined on the chromogenic substrate S-2302.

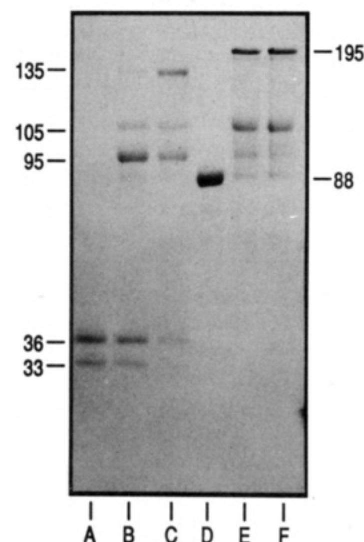


FIGURE 6: SDS–PAGE analysis (7.5–10%, 1.5-mm-thick, unreduced) of the reaction between kallikrein light chain (M_r 33 000 and 36 000, lanes A–C), C$\bar{1}$s (M_r 88 000, lanes D–F), and C1 inhibitor (M_r 105 000, lanes B, C, E, and F) at 4 °C (lanes B and E) and 38 °C (lanes C and F). Ten micrograms of each protein was incubated at the temperature indicated for 1 h in a volume of 60 μL prior to addition of sample buffer, boiling, and electrophoresis. Staining was with Coomassie blue. Margin numbers are $M_r \times 10^{-3}$.

Effect of Temperature on the Reaction of Kallikrein and C1 Inhibitor with Other Serpins and Enzymes. The reactions of C1 inhibitor and kallikrein with other enzymes and inhibitors were studied by SDS–PAGE and by determination of their partition ratios. Figure 6 shows the SDS–PAGE of incubations of kallikrein light chain (lane A) and C$\bar{1}$s (lane D) with C1 inhibitor at 4 °C (lanes B and E) and 38 °C (lanes C and F), respectively. The results seen with the light chain of kallikrein were similar to those observed with two-chain α-kallikrein (Figure 1, lanes C and D). In addition, C$\bar{1}$s

Table I: Partition Ratios for the Reaction of Various Serine Proteases with C1 Inhibitor and α_1-Antitrypsin Reactive Site Mutants

	temp (°C)	partition ratio: C1 inhibitor	α_1-AT–Arg^{358}	α_1-AT–Ala^{357}-Arg^{358}
kallikrein	4	5.25	0.1	0.1
	38	1.5	0.1	0.1
kallikrein light chain	4	10.0	ND[a]	ND
	38	3.6	ND	ND
C$\bar{1}$s	4	0.2	ND	ND
	38	0.05	ND	ND
plasmin	4	1.26	0.5	1.24
	38	0.6	0.5	1.24

[a] ND, not determined.

formed a complex with C1 inhibitor at 4 and 38 °C, although cleaved C1 inhibitor (M_r 95 000) was generated at both temperatures. To quantitate these effects, partition ratios were determined at 4 and 38 °C for reactions of C1 inhibitor with kallikrein light chain, C$\bar{1}$s, and plasmin. For comparison, partition ratios were also determined for the inhibition of kallikrein and plasmin by α_1-antitrypsin–Arg^{358} and α_1-antitrypsin–Ala^{357}-Arg^{358}. These α_1-antitrypsin reactive site mutants have inhibitory activity against kallikrein and plasmin (Patston et al., 1990). Partition ratios of all reactions involving C1 inhibitor increased at lower temperatures (Table I). The magnitude of the increase was 2.1-fold with plasmin, 2.8-fold with kallikrein light chain, 3.5-fold with α-kallikrein, and 4-fold with C$\bar{1}$s. Thus, with the various enzymes under investigation, the reactivity of C1 inhibitor was temperature-sensitive. This property is not shared by the α_1-antitrypsin mutants nor has it been reported for any other serpin. The cleavage of α_1-antitrypsin–Ala^{357}-Arg^{358} by plasmin seen in a previous study (Patston et al., 1990) is consistent with the partition ratio of 1.24 reported here. The partition ratio for C1 inhibitor with kallikrein light chain was higher than that for kallikrein both at high and at low temperature. This strongly suggests that the heavy chain of α-kallikrein [in α-kallikrein, heavy chain and light chain are covalently linked by disulfide bonds (Burger et al., 1986)] interacts with C1 inhibitor and influences the inhibition reaction. Extensive proteolysis of C1 inhibitor by kallikrein light chain was also seen by van der Graaf et al. (1983b). The value of the partition ratio was unique to each serpin–protease pair, and the magnitude of the temperature effect, in the case of C1 inhibitor, was dependent on the protease. It should be noted that, even at high temperatures, the kallikrein–C1 inhibitor and kallikrein light-chain–C1 inhibitor inhibition reactions are less efficient, on the basis of their partition ratios, than the other pairings tested. Interestingly, this situation probably exists in vivo since large amounts of cleaved C1 inhibitor are detectable in the plasma of patients with sepsis, who have activation of the blood coagulation intrinsic system and the classical pathway of complement (Nuijens et al., 1989).

Concluding Remarks. The two principal observations reported in this study are (a) that C1 inhibitor functions as a suicide substrate for plasma kallikrein and other serine proteases and (b) that the partition ratio for the reactions between this serpin and its target enzymes is markedly dependent on temperature.

The notion that serpins could be treated as suicide substrates was first suggested by studies on the reaction between thrombin and antithrombin III (Fish & Björk, 1979). It was subsequently shown that, in the absence of heparin, there is minimal formation of cleaved antithrombin III; in contrast, in the presence of high-affinity heparin, a significant increase in the apparent stoichiometry is observed, concomitant with the formation of reactive site cleaved serpin (Björk & Fish, 1982; Olson, 1985). Heparin has a qualitatively similar effect on the reaction between C$\bar{1}$s and C1 inhibitor (Weiss & Engel, 1983). In addition, since they observed that the reactive site mutant α_1-antichymotrypsin L358R has increased apparent inhibitory stoichiometry for chymotrypsin, Rubin et al. (1990) proposed that suicide inhibition could explain their data. Because our present results demonstrate that such a mechanism is also applicable to C1 inhibitor (and probably to α_1-antitrypsin), suicide inhibition seems to represent a general mechanism for the reaction between serpins and their target serine proteases.

A key characteristic of suicide inhibition is that the distribution of the reaction products is determined by the partition ratio r. When $r = 1$, the rate constant for the formation of a stable enzyme–substrate complex (k_{stab} in Scheme II) and the rate constant for substrate cleavage without concomitant enzyme inhibition (k_{diss} in Scheme II) have identical values. When $r = 0$, the reaction leads exclusively to the formation of a stable enzyme–substrate complex (since $k_{diss} = 0$), and the reaction mechanism is as described in Scheme I. When $r > 1$, substrate turnover without inhibition of the target enzyme becomes the predominant event (Walsh, 1984; Waley, 1985; Fersht, 1985; Knight, 1986). Apart from providing a biochemical explanation for the inefficiency of C1 inhibitor in the cold (van Royen et al., 1978; van der Graaf et al., 1983a; Harpel et al., 1985; Cameron et al., 1989), our observation that temperature can influence the product distribution of the reaction between C1 inhibitor and its target serine proteases identifies a novel mechanism for regulating the reactivity of this serpin. Further studies on C1 inhibitor characteristics at different temperatures should provide new insights on how the partition ratio for the reaction between this serpin and serine proteases is controlled. Also, it will be important to determine whether some as yet unidentified ligand can mimic this property in vivo. At a more general level, assessing whether regulation of serine protease–serpin reactions at the partition ratio level is a mechanism operative for the control of blood coagulation, fibrinolysis, and complement activation should be of considerable interest.

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