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C1 Inhibitor: Analysis of the Role of Amino Acid Residues Within the Reactive Center Loop in Target Protease Recognition

Rana Zahedi,2* Ryan C. MacFarlane,* Jeffrey J. Wisnieski, † and Alvin E. Davis III3*

Previous analysis of a naturally occurring C1 inhibitor P2 mutant (Ala439 → Val) indicated a role for P2 in specificity determination. To define this role and that of other reactive center loop residues, a number of different amino acids were introduced at P2, as well as at P6 (Ala439) and P8/9 (Gln452Gln453). Ala439→Val is a naturally occurring mutant observed in a patient with hereditary angioedema. Previous data suggested that Gln452Gln453 might be a contact site for C1s. Reactivity of the inhibitors toward target (C1s, C1r, kallikrein, β factor XIIa, and plasmin) and nontarget proteases (α-thrombin and trypsin) were studied. Substitution of P2 with bulky or charged residues resulted in decreased reactivity with all target proteases. Substitution with residues with hydrophobic or polar side chains resulted in decreased reactivity with some proteases, but in unaltered or increased reactivity with others. Second order rate constants for the reaction with C1s were determined for the mutants with activities most similar to the wild-type protein. The three P2 mutants showed reductions in rate from 3.35 × 10⁴ M⁻¹ s⁻¹ for the wild type to 1.61, 1.29, and 0.63 × 10⁴ for the Ser, Thr, and Val mutants, respectively. In contrast, the Ala439→Val and the Gln452Gln453→Ala mutants showed little difference in association rates with C1s, in comparison with the wild-type inhibitor. The data confirm the importance of P2 in specificity determination. However, the P6 position appears to be of little, if any, importance. Furthermore, it appears unlikely that Gln452Gln453 comprise a portion of a protease contact site within the inhibitor. The Journal of Immunology, 2001, 167: 1500–1506.

The C1 inhibitor (C1INH)⁴ is the only inhibitor of the complement proteases C1s and C1r, and is the primary inhibitor of the proteases of the contact phase of coagulation: plasma kallikrein, factor XIa, and factor XIIa (1–6). C1INH belongs to the superfamily of serine protease inhibitors, or serpins, many members of which are involved in the regulation of proteolytic cascades, including those participating in inflammation, coagulation, and fibrinolysis. Serpins share homologous structures and a common mode of action. They are composed of 400–450 aa residues organized into three β sheets and eight or nine α helices (7). Serpins act as suicide substrates in which the amino acid sequence of the reactive center loop mimics that of an ideal natural substrate of target proteases (8). Reaction with target protease results in formation of an initial Michaelis complex. After this initial interaction, an intermediate complex forms that either develops into a stable complex or dissociates to release the inactive cleaved serpin and the active enzyme (8). The potency of an inhibitor depends on the relative rates of these two reactions. The rate of dissociation of the stable complex is extremely slow (9).

The primary recognition element for serpin-protease association is a 15 aa residue exposed segment known as the reactive center loop. This loop varies in sequence and length; it is mobile and can adopt different conformations. Cleavage of the reactive center loop by nontarget proteases results in complete insertion of the loop into the five-stranded β sheet A, thereby converting it to a six-stranded β sheet (10). In the complex with protease, the reactive center loop is similarly inserted; the protease is thereby displaced from the site of initial interaction at one pole of the inhibitor molecule to the other pole (11–14). The amino acid sequence of the reactive center loop from approximately P5 through P6’ varies among the serpins and, to a great extent, determines the specificity of the inhibitor (15–21). The P1 residue is the major determinant of serpin specificity, as has been illustrated by analysis of both naturally occurring serpins and serpins with new unique engineered mutations (15, 20). Because a serpin with a single P1 residue may inhibit a variety of target proteases and because different serpins with the same P1 residue inhibit different proteases, other residues must be involved in specificity determination. Analyses of mutant serpins, which indicate that the P1 residue is not the sole determinant of specificity, support this assumption (16–20, 22–24). Other residues within the reactive center region also play a significant role. However, the roles of different residues appear to vary with different serpin-protease combinations.

We described a variant C1INH in which the P2 residue (Ala439) was substituted with Val (25, 26). This mutant had a decreased rate of reactivity toward C1s and C1r, and had acquired weak inhibitory activity toward trypsin, but retained normal activity against the target proteases factor XIIa and plasma kallikrein. A patient with hereditary angioedema (HAE) has been described in which the P6 residue (Ala457) was substituted with a Val (27). It was not clear whether this resulted in absolute deficiency of C1INH or in

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5 Abbreviations used in this paper: C1INH, C1 inhibitor; HAE, hereditary angioedema.
a dysfunctional inhibitor. An identical mutation at the P6 position of antithrombin, also without functional analysis, previously had been reported (28). Analysis of autoantibodies to C1INH from patients with acquired angioedema led to the identification of the P8’/P9’ (Gln453→Gln55) region as a potential contact site with C1s during complex formation. To further characterize the roles of these reactive center region residues in complex formation and in specificity determination, we have created mutant proteins with substitutions at each of these positions. In the case of P2, a variety of substitutions varying in side chain size, charge, and hydrophobicity have been constructed. We have analyzed the reactivity of these mutant proteins with target proteases, and have determined their sensitivity to heat denaturation as a measure of conformation. In addition, we have analyzed the reactivity of the mutant inhibitors with plasmin and thrombin.

Materials and Methods
Site-directed mutagenesis and expression in COS-1 cells
Mutations at the C1INH P2 position were introduced using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers corresponding to nucleotides 16,773 through 16,802 of the C1INH genomic sequence that varied in the nucleotides at positions 16,785 through 16,787 were designed. These substitutions led to replacement of Ala443 with Asn, Gly, Leu, and Thr. The same approach was used to replace the P6 Ala residue with Val and both the P8’ and P9’ residues (Gln452 and Gln453) with Ala. Mutated and wild-type proteins then were expressed transiently in COS-1 cells. For some experiments, newly synthesized proteins were metabolic radiolabeled with [35S]Met (Amersham Life Sciences, Arlington Heights, IL). Medium was harvested 72 h after transfection and dialyzed into PBS.

Thermal stability
COS-1 cell culture supernatants (100 μl) containing recombinant inhibitors were incubated with and without trypsin (5 μg) at 40, 50, 60, 70, and 80°C for 120 min, following which they were subjected to centrifugation at 14,000 × g for 30 min. Quantitation of residual Ag in the supernatant was performed using an ELISA as described by Aulak et al. (29).

Complex formation between C1INH proteins and proteases
Radiolabeled recombinant wild-type or mutant C1INHs were incubated for 1 h at 37°C with C1s (8.4 μg), C1r (6.4 μg) (Advanced Research Technologies, San Diego, CA), kallikrein (15 μg), B factor XllAs (3.5 μg) (Enzyme Research Laboratories, South Bend, IN), plasmin (0.2, 0.5, 1, 2, 5, and 10 μg/μl) and a-thrombin (0.2, 0.5, 1, 2, 5, and 10 μg/μl) (plasmin and thrombin provided by Dr. V. Donaldson, Children’s Hospital Research Foundation, Cincinnati, OH), or trypsin (0.5 μg; Sigma, St. Louis, MO). Each protease was incubated in molar excess relative to recombinant C1INH. After incubation, PMSF (final concentration, 1 mM) was added, and Triton X-100 (0.5%), deoxycholic acid (0.25%), SDS (0.5%), and EDTA (5 mM) were added to each sample. The IgG fraction of rabbit anti-human C1INH (3 μg) was added, and Triton X-100 (0.5%), deoxycholic acid (0.25%), SDS (0.5%), and EDTA (5 mM) were added to each sample. The IgG fraction of rabbit anti-human C1INH (3 μl) then was added, and samples were incubated at 4°C overnight. A suspension of fixed Staphylococcus aureus (6 μl, IgG sorb; The Enzyme Center, Boston, MA), sonicated and washed three times (with 1% Triton X-100, 1% SDS, 0.5% deoxycholate, and 5 mg/ml BSA in 0.02 M sodium phosphate and 0.15 M NaCl, pH 7.4), was added to each sample and incubated at 4°C for 1 h. The pellets (14,000 × g for 3 min) were washed once with the above washing solution and three times with a washing solution lacking BSA. Each precipitate then was dissolved in SDS sample buffer without reducing agent (15 μl, 4% SDS, 20% glycerol, 0.1 M Tris-HCl, pH 6.5, and 0.01% bromophenol blue), vortexed, boiled for 3 min, and subjected to brief centrifugation and electrophoresis on SDS- 7.5% polyacrylamide gel. Gels were fixed, dried, and exposed to x-ray film (Kodak XAR-5; Eastman Kodak, Rochester, NY) at −70°C.

Inhibitor assay and calculation of second order rate constants
COS-1 cell supernatants (40 ml) containing recombinant C1INH proteins were collected and concentrated to 2–5 ml. The C1INH concentrations of the samples were measured by ELISA (29). The concentrations of the recombinant mutant C1INH proteins after concentration were: wild type (5.5 μg/ml), Ala443→Val mutant (5 μg/ml), Ala452→Asn mutant (6.5 μg/ml), Ala452→Thr mutant (5.5 μg/ml), Ala459→Val mutant (2.5 μg/ml), and Gln453→Gln55→Ala mutant (1.6 μg/ml). The amount of active inhibitor in each C1INH preparation was determined by incubation of varying quantities of mutant inhibitor with a fixed known quantity of fully active C1s for a prolonged period, following which residual C1s esterolytic activity was determined by hydrolysis of the chromogenic substrate S-2314 (Chromogenix, Molndal, Sweden). The active concentration of C1s was determined by incubation of varying molar ratios of C1s and plasma-derived C1INH of known concentration (determined using an extinction coefficient of 3.7) (30) followed by determination of the amount of complex formation by SDS-PAGE.

For the kinetic assays, equimolar concentrations of C1s and recombinant wild-type or mutant C1INH were incubated at 37°C in PBS. At different times (0–30 min), aliquots of the reaction mix were removed and the reaction stopped by dilution in the chromogenic substrate S2314 (0.5 mM in PBS). Residual enzyme activity was quantitated by monitoring hydrolysis of the substrate (increase in absorption over time at a wavelength of 405 nm). The second order rate constants were determined using the half-time of the reaction (t1/2), which is calculated by plotting the inverse of the enzymatic rate vs time; this is related to the (Kc) through the equation t1/2 = (Kc × E0)−1, where E0 is the total enzyme concentration.

Results
Thermal denaturation profiles of the P2 mutant C1INHs
Thermal stability provides a simple, but reliable measure of the conformation of serpins (31). Normal intact serpins multimerize at temperatures of 50–60°C. This results in a decrease in epitopes detected with polyclonal anti-C1INH antisera. Therefore, with multimerization the apparent concentration of C1INH decreases. Serpins that have been cleaved near the reactive center are more stable and remain monomeric at temperatures as high as 80–90°C. Thermal denaturation curves of all the P2, the P6 Ala→Val, and the P8’/P9’ Gln→Ala mutant C1INHs were indistinguishable from that of the wild-type protein (Fig. 1). After cleavage by trypsin, all of the recombinant proteins were stable at elevated temperatures (50–90°C). These data suggest that the mutations did not induce any major conformational changes, and that each was able to undergo the conformational rearrangement with reactive center loop insertion that takes place in normal inhibitor serpins following reactive center cleavage or complex formation (10, 11). Therefore, it is likely that each of the mutants would be capable of complex formation with an appropriate protease.

![FIGURE 1. Thermal denaturation profiles of recombinant C1INH proteins. Supernatant from transfected COS-1 cells were incubated in the presence or absence of trypsin (5 μg) at different temperatures for 2 h. Samples then were subjected to centrifugation for 30 min, and residual Ag was quantitated by ELISA.](http://www.jimmunol.org/)

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Reactivity of the P2 mutant proteins with target proteases

C1s. The results of each of the recombinant inhibitors with the different proteases in excess were assessed by SDS-PAGE following incubation for 1 h at 37°C. Recombinant wild-type C1INH formed a stable complex with C1s, accompanied by the appearance of a relatively small amount of cleaved C1INH, similar to previous observations (Fig. 2A) (25, 26). Compared with the wild type, recombinant C1INH with an Asn at the P2 position showed less complex formation with C1s in the 60-min incubation period (Fig. 1A). An appreciable quantity of the native form of the inhibitor was present even after 1 h of incubation. Furthermore, no cleavage of the Ala443→Asn mutant was observed. It should be pointed out that even with the same inhibitor-protease pair, in experiments performed at different times, the amount of cleaved vs complexed inhibitor, as assessed by SDS-PAGE, may vary somewhat. This likely is a result of subtle differences in experimental conditions. Therefore, over-interpretation of moderate changes in the amounts of cleaved inhibitor should be avoided. However, as seen in Fig. 2A, the complete absence of cleavage of this mutant clearly differs from the amount observed with the recombinant wild-type protein. The results with the Ala443→Asn mutant are similar to those observed previously with the P2 Asp mutant (26). The results described here, together with previously published data (26), are summarized in Table I. Substitution of P2 with Gly, Leu, or Thr described previously with the P2 Asp mutant (26). The results with the Ala443→Val mutant show much less cleavage than with the wild type (25, 26). The wild-type inhibitor reacted more slowly with C1r than with C1s (25, 33, 34); however, in a 1-h incubation period this difference is not apparent (Fig. 3, B and D) (25). As with C1s, the reaction of the P2 Asp mutant with C1r was very slow, as indicated by the presence of residual native C1INH after a 1-h incubation at 37°C. The P2 Thr mutant did not show any alteration in reactivity with C1s following a 1-h incubation, even though the association rate constant for this reaction was slower than the reaction of the previously described P2 Ser substitution mutant (26). Neither the P6 Ala→Val nor the P8′/P9′ Gln→Ala substitutions showed any significant alteration in complex formation with C1s in comparison with the wild-type protein.

Second order association rate constants were determined, as described in Materials and Methods, for some of those mutants that appeared similar to the wild type in the above experiments (Ala443→Val, Ala443→Ser, Ala443→Thr, Ala439→Val, and Gln452/Gln453→Ala) (Table II). The rate for the wild-type protein is at the high end of the range of values reported in the literature for normal plasma-derived or recombinant C1INH (19, 32–34). Previous data, based on time course analysis of complex formation, as observed on SDS-PAGE in which recombinant C1INH was incubated with a vast molar excess of C1s, indicated that the Ala443→Val mutant was much less active than the wild-type inhibitor (25, 26). The wild-type inhibitor was fully complexed within 0.5 min, whereas the mutant required ~5 min before complex formation was complete. As expected from these data, the association rate for the Ala443→Val mutant with C1s was substantially lower than that of the wild-type protein (p = 0.04). In addition, the association rate constants of the Ser and Thr mutants were reduced in comparison with the wild-type inhibitor, although to a lesser extent than that of the Ala443→Val mutant (50–60% reduction in rate as opposed to an 80% reduction for Ala443→Val).

C1r. Recombinant wild-type C1INH reacts more slowly with C1r than with C1s (25, 33, 34); however, in a 1-h incubation period this difference is not apparent (Fig. 3, A and B) (25). As with C1s, the reaction of the P2 Asn mutant with C1r was very slow, as indicated by the presence of residual native C1INH after a 1-h incubation at 37°C. The P2 Thr mutant did not show any alteration in reactivity with C1s following a 1-h incubation, even though the association rate constant for this reaction was slower than the reaction of the

Table I. Complex formation between recombinant C1INH mutant proteins and proteases following incubation at 37°C for 60 min as assessed by SDS-PAGE

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>C1s</th>
<th>C1r</th>
<th>Kall</th>
<th>FXIIa</th>
<th>Try</th>
<th>Pl</th>
<th>Th</th>
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<td>Ala443→Val</td>
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<tr>
<td>Ala443→Asn</td>
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<td>Ala443→Asp</td>
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<tr>
<td>Ala443→Gly</td>
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<tr>
<td>Ala443→Leu</td>
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<tr>
<td>Ala443→Ser</td>
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<td>Ala443→Thr</td>
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<tr>
<td>Ala439→Val</td>
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<tr>
<td>Gln452/Gln453→Ala</td>
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</table>

Table II. Association rate constants for the interaction of C1INH mutants with C1s

<table>
<thead>
<tr>
<th>C1INH</th>
<th>kₐ(×10⁶ M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>3.35 ± 0.73</td>
</tr>
<tr>
<td>Ala443→Ser</td>
<td>1.61 ± 0.44</td>
</tr>
<tr>
<td>Ala443→Thr</td>
<td>1.29 ± 0.32</td>
</tr>
<tr>
<td>Ala443→Val</td>
<td>0.63 ± 0.40</td>
</tr>
<tr>
<td>Ala439→Val</td>
<td>2.14 ± 0.98</td>
</tr>
<tr>
<td>Gln452/Gln453→Ala</td>
<td>2.99 ± 1.07</td>
</tr>
</tbody>
</table>

FIGURE 2. Complex formation between recombinant inhibitors and C1s. COS-1 cell supernatants (100 µl) containing the different recombinant inhibitors intrinsically labeled with [³⁵S]Met were incubated with C1s for 1 h at 37°C. Samples then were immunoprecipitated with polyclonal antiserum to human C1INH and subjected to SDS-PAGE. Autoradiography was performed as described in Materials and Methods.
wild-type inhibitor with C1s. The slow reaction was more apparent with C1r; less complex was formed and small amounts of uncomplexed inhibitor remained after a 1-h incubation (Fig. 3B). The P2 Gly and Leu mutants also showed very small amounts of native uncomplexed inhibitor at the end of the incubation period (albeit less than with the Ala443Asp mutant), which may suggest a small effect on the rate of complex formation with C1r (Fig. 3, A and B). As with C1s, the P6 and P8/P9 mutanets each complexed with C1r to the same extent as did the wild-type inhibitor (Fig. 3C).

Kallikrein and factor XIIa. In the reaction with β factor XIIa, recombinant wild-type C1INH showed nearly complete complex formation with minimal amounts of cleaved inhibitor or of remaining uncleaved inhibitor. However, a significant quantity of cleaved inhibitor is released during the reaction with plasma kallikrein (Fig. 3, A–C). This is in agreement with previous observations (8, 26, 35, 36). For plasma-derived C1INH, Patston et al. (8) determined a stoichiometry of 1.05:1 and 2.5:1 for the reactions with C1s and kallikrein, respectively. Only recombinant C1INH with an Asn at the P2 position showed altered reactivity with kallikrein (Fig. 4A). The Ala443→Asp mutant with these proteases (26) (Table I). Each of the other mutants, including the P6 and the P8/P9' mutants, showed a pattern of reactivity with both plasma kallikrein and factor XIIa that was virtually identical with the reactivity of the wild-type inhibitor (Fig. 4, A–C).

Plasmin. Normal recombinant wild-type C1INH reacts slowly with plasmin; complex formation was incomplete after the 1-h incubation (Figs. 5, A and C, and 6). No cleaved wild-type inhibitor was observed in this reaction mixture. The P2 Val and Leu mutants also complexed with plasmin (Figs. 5, A and B, and 6). However, there was a large quantity of cleaved C1INH present in each of these reactions. Very little complex formation was observed between plasmin and the P2 Asn, Gly, and Thr mutants (Fig. 5, A and B). The Ala449→Val mutant complexed with plasmin to an extent similar to that with the wild-type C1INH (Fig. 5C). In contrast, the P8/P9' mutant appeared to form less complex with plasmin than did the wild type during the 60-min incubation period.

Thrombin. No significant stable complex formation was observed between wild-type C1INH and thrombin, but a moderate
amount of cleaved inhibitor was generated during the 1-h incubation (Figs. 5A and 7). The P2 Val and P2 Leu mutants each formed a small amount of stable complex with thrombin but each was cleaved more efficiently than was the wild type (Figs. 5, A and B, and 7). The Asn, Gly, and Thr mutants did not complex with, and were resistant to cleavage by, thrombin. The P6 mutant formed little or no complex with thrombin, but was cleaved to a significant extent, whereas the P8/9 mutant formed minimal or no complex, and was not cleaved.

**Trypsin.** We previously demonstrated that the Ala^443→Val mutant protein was relatively resistant to cleavage by trypsin in comparison with the wild-type protein and that it had acquired the ability to complex with trypsin at a very slow rate (26). The reactivities of each of the other mutants appeared much more similar to that of the wild-type protein in that they were readily cleaved and showed little or no complex formation with trypsin (data not shown).

**Discussion**

Substitution at the P2 position clearly has effects on reactivity with each of the proteases tested, including both target (C1r, C1s, plasma kallikrein, factor XIIa, plasmin) and nontarget proteases (thrombin, trypsin). This is in distinction to the observations with P2 substitutions in α1-antichymotrypsin, in which reactivities with factor Xa and activated protein C were markedly altered whereas that with trypsin was relatively unaffected (18). Several characteristics of the reactivities of these mutants are notable. As might be expected, bulky and/or charged amino acids were poorly tolerated at this position, and likely interfered with access of protease to the reactive center. The mutants with P2 Ala→Asp and Asn substitutions reacted extremely poorly with all of the proteases tested.
(Table I). This observation is similar to previous findings with antithrombin and protein C inhibitor (37, 38). Also, the reactivities of the inhibitors with C1r and C1s tended to parallel one another, as did the reactivities with factor XIIa and kallikrein. Normal wild-type C1INH reacts more slowly with C1r than with C1s (32, 34). This difference in rate explains the observation that on SDS-PAGE, incomplete complex formation is more apparent with C1r than with C1s. Given the similar substrate specificities of C1r and C1s and of factor XIIa and kallikrein, these results might have been expected.

 Virtually any change in side chain from the wild-type Ala resulted in decreased inhibitory activity against C1s (and C1r), whereas reactivity with kallikrein and factor XIIa was less sensitive to these substitutions. The sensitivity of reactivity with C1s to changes at P2 was apparent on SDS-PAGE for the Ala$^{443}$→Asn and Asp mutants, and for the Ser, Thr, and Val mutants on kinetic analysis. Unfortunately, there were insufficient quantities of the Ala$^{443}$→Gly and Leu variants for kinetic studies with C1s and of any of the recombinant inhibitors for kinetic analyses with proteases other than C1s. The slower rate of reactivity of each of these variants with C1r was apparent on SDS-PAGE. This high degree of specificity in the reactivity with C1s (and C1r) is consistent with data from the recently reported crystal structure of the catalytic domain of C1s (39). This demonstrated that access to the C1s binding subsites, particularly S2, is partially obstructed in comparison with many other proteases, such as trypsin. This restricted access, as suggested by Gaboriaud et al. (39), very likely explains the high degree of substrate specificity of C1s and the fact that its only inhibitor in plasma is C1INH.

The observations related to the reactivity of the P2 substitution mutants with thrombin are also of interest in relation to specificity determination. The increased reactivity of the P2 Val and Leu mutants with thrombin, as demonstrated by both increased complex formation and increased cleavage in comparison with wild type, correlates with thrombin specificity toward fibrinogen. Fibrinopeptide B, which has an Ala at the P2 position, is released more slowly than is fibrinopeptide A, which has a Val at the P2 position (40). In general, it was observed that more hydrophobic residues in the P2-P4 positions resulted in substrates that were better recognized by thrombin (41). The somewhat increased complex formation with, and cleavage of, the P2 Val and Leu mutants are consistent with these earlier observations. However, based on the previous data (41), it might have been expected that the P2 Gly mutant also would have been recognized, but it neither was complexed with nor cleaved by thrombin.

The Ala$^{439}$→Val (P6) substitution mutant, identified originally in a family with HAE (27), revealed only a moderate decrease in activity against C1s in comparison with the wild-type protein ($K_i = 2.14 \times 10^{-5} M^{-1} s^{-1}$). Its reactivity with all of the proteases, including C1s (after a 1-h incubation), was indistinguishable from normal on SDS-PAGE. Current information suggests that angioedema is mediated by bradykinin (25, 42, 43). Therefore, prevention of attacks of angioedema very likely is dependent on the inhibition of plasma kallikrein (which catalyzes the cleavage of high m.w. kininogen to release bradykinin) by C1INH. Determination of rate constants for this mutant (when available in larger quantities) with kallikrein will be required to determine whether a relatively mild dysfunction of this inhibitor is responsible for the development of angioedema in this family. In addition, this family reportedly presented with laboratory values consistent with type 1 HAE (decreased C1INH antigenic and functional levels) as opposed to type 2 (decreased C1INH functional levels with relatively normal antigenic levels). This could be explained if the mutation resulted in either a decrease in secretion of newly synthesized protein or in enhanced intracellular degradation. The amount of recombinant mutant protein synthesized by COS cells did not dramatically differ from the amount of wild-type inhibitor synthesized, which argues against this explanation. Unfortunately, these hypotheses cannot be tested directly because this family is not currently available. In the original analysis of this family, this substitution was the only mutation identified within the coding region or at intron-exon junctions (27). In addition, the argument that this mutation is sufficient to result in HAE is supported by the observation that an identical mutation in antithrombin was described in a family with recurrent venous thromboses (28).

The Gln$^{652}$Gln$^{653}$→Ala substitution was analyzed to test the hypothesis that these residues make up a contact site for C1s. This was based on the observations that autoantibodies to C1INH react with a synthetic peptide corresponding to aa 448–459 and that the epitope included residues Gln$^{652}$, Gln$^{653}$, Pro$^{654}$, and Phe$^{655}$ (44). These authors then went on to report experiments that indicated that this peptide bound directly to C1s and prevented its complex formation with C1INH (45). Substitution of Gln$^{652}$, Gln$^{653}$, or Phe$^{655}$ in this peptide abrogated C1s binding (46). This led to the conclusion that this region at the distal end of the reactive center loop constitutes a C1s contact site. The results of the experiments reported here show that substitution of both Gln$^{652}$ and Gln$^{653}$ with Ala residues has only a relatively marginal effect on the inhibition of C1s (or, of the other proteases, with the exception of plasmin) by C1INH. It seems unlikely that a contact site on the side chains of two adjacent Gln residues would be adequately replaced by Ala residues. Therefore, it seems likely that these residues, at least, are not part of a major contact site for C1s in its recognition of C1INH. Recently, it was demonstrated that C1INH can be cross-linked to fibrin by tissue transglutaminase via Gln$^{657}$, and that this immobilized C1INH retained its inhibitory activity toward kallikrein and C1s (47). The Gln$^{652}$Gln$^{653}$ mutant may prove to be valuable to analyze the potential biologic role of this cross-linking reaction.

In summary, these studies have further characterized the roles of amino acids within the reactive center loop of C1INH in specificity determination. In particular, the results emphasize the importance of the amino acid at the P2 position in recognition by the highly specific proteases C1r and C1s. Conversely, plasma kallikrein and factor XIIa are much more tolerant of variation at this position.

References


