Use of Site-Directed Mutagenesis To Investigate the Basis for the Specificity of Hirudin

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ABSTRACT: Regions of hirudin important for its inhibitory activity with thrombin have been examined by site-directed mutagenesis. Since thrombin has a primary specificity for basic amino acids, each of the three basic residues and the histidine in hirudin were mutated to glutamine. Mutation of Lys-47 caused a small increase (9-fold) in the dissociation constant whereas the other mutations were without effect. These results indicate that hirudin is different from most other inhibitors of serine proteases in that interactions with the primary specificity pocket of its target enzyme are not crucial to its inhibitory activity. The acidic nature of the carboxyl region of hirudin was found to be important for its interaction with thrombin. Single and multiple mutations of carboxyl-terminal glutamate residues (57, 58, 61, and 62) to glutamine caused increases in the dissociation constant. This value increased with the number of mutations and reached a maximum of 61-fold when all four glutamate residues were mutated. Kinetic studies indicated that in all cases where an increase in dissociation constant was observed, it was predominantly due to a decrease in the association rate constant.

Hirudin is a polypeptide of 65 or 66 amino acids that can be isolated from the leech *Hirudo medicinalis* (Bagdy et al., 1976; Markwardt, 1970; Dodt et al., 1986). It reacts rapidly with thrombin to form a tight, noncovalent complex (K_d = 10^{-14} M; Stone & Hofsteenge, 1986) and shows an absolute specificity for thrombin (Walsmann & Markwardt, 1981). Three different forms of hirudin have been identified (Bagdy et al., 1976; Dodt et al., 1984, 1986; Harvey et al., 1986). These forms are highly homologous (about 85%), and all appear to have antithrombin activity. Northern analysis indicates the existence of three distinct mRNA species coding for hirudin while Southern analysis suggests the existence of several genes (Harvey et al., 1986). The three-dimensional structure of one of these forms of hirudin has been determined in solution by using NMR (Sukumaran et al., 1987; Clore et al., 1987). These studies indicate that hirudin consists of three domains: a central core, a protruding "finger", and an exposed loop.

The specificity of thrombin is thought to be determined by interactions at three distinct regions (Fenton, 1981): (1) the primary specificity pocket which binds the side chain of the residue on the amino-terminal side of the scissile bond; the specificity of thrombin is such that only basic side chains are efficiently bound in this pocket (Blombäck et al., 1977; Mann & Lundblad, 1982); (2) an apolar binding site which binds proflavin (Berliner & Shen, 1977) and has been exploited in the design of low molecular weight substrates and inhibitors (Claeson, 1977; Kettner & Shaw, 1981; Walker et al., 1985); (3) an anion binding region which is thought to be responsible for the specific interaction of thrombin with fibrinogen (Fenton, 1981). Each of these regions has been implicated in the interaction of thrombin with hirudin (Stone et al., 1987). However, the regions of hirudin which interact with the above three regions of thrombin and, thus, determine its specificity for thrombin are unknown. Hirudin contains several basic residues, and one of these may bind in the primary specificity pocket of thrombin. Hirudin also contains several regions with a high content of acidic residues that could interact with an anionic binding site of thrombin. The carboxyl-terminal region of hirudin is particularly rich in acidic residues, and the experiments of Chang (1983) indicate that an intact carboxyl-terminal region is important for the inhibitory activity of hirudin.

In this paper, site-directed mutagenesis has been used in conjunction with kinetic analysis to identify which, if any, of the basic residues of hirudin binds in the primary specificity pocket of thrombin and to determine the contribution made by this interaction to the overall tightness of the thrombin–hirudin complex. In addition, the importance of the acidic residues in the carboxyl-terminal region of hirudin has been assessed by using the same techniques.

Experimental Procedures

Materials. The substrate D-Phe-pipecolyl-Arg-*p*-nitroanilide was from Kabi Vitrum, Molndal, Sweden, and thermolysin was obtained from Boehringer-Mannheim, Mannheim, FRG. Human thrombin was prepared as described previously (Stone & Hofsteenge, 1986) and was 97% active as determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973). Native hirudin (2000 units/mg) was purchased from Pentapharm AG, Basel, Switzerland, and was purified by HPLC as described previously (Stone & Hofsteenge, 1986); its amino acid composition and amino-terminal sequence were consistent with the sequence shown in Figure 1. Amino acid sequence analysis using an Applied Biosystems gas-phase sequencer (Hewick et al., 1981) indicated that all proteins were greater than 99% pure.

Amidolytic Assay of Thrombin. Assays were performed as described previously (Stone & Hofsteenge, 1986) at 37 °C in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.1% poly(ethylene glycol) (M_r 6000). In a previous study, it was shown that the substrate D-Phe-pipecolyl-Arg-*p*-nitroanilide could compete with hirudin by binding at the active site and at a second lower affinity site which had a dissociation constant of 0.6–0.9 mM (Stone & Hofsteenge, 1986; Stone et al., 1987). In the present study, the concentration of substrate was maintained at about 100 μM so that its binding at the lower affinity site would not be significant.

Site-Directed Mutagenesis. The pIN-III-ompA$_2$/hir plasmid used for the expression of recombinant hirudin was a gift from Dr. M. Liersch, Biotechnology Section, Ciba-Geigy AG, Basel, Switzerland, and details of its construction will be

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V-V-Y-T-D-C-T-E-S-G-Q-N-L-C-L-C-E-G-S-N-
                    10                  20

              Q                   Q
              |                   |
V-C-G-Q-G-N-K-C-I-L-G-S-D-G-E-K-N-Q-C-V-
                    30                  40

              Q       Q           Q Q
              |       |           | |
T-G-E-G-T-P-K-P-Q-S-H-N-D-G-D-F-E-E-I-P-
                    50                  60

Q Q
| |
E-E-Y-L-Q
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FIGURE 1: Amino acid sequence of recombinant hirudin. The recombinant gene for hirudin was based on the sequence of hirudin determined by Dodt et al. (1984) and represented by the single-letter code. Where mutations were made, the letter above the sequence indicates the new amino acid. In native hirudin, Tyr-63 is sulfated.

published elsewhere. The expression vector was constructed essentially as described by Takahara et al. (1985). The gene for hirudin was synthesized as previously described (Rink et al., 1984) on the basis of the amino acid sequence (shown in Figure 1) of Dodt et al. (1984) and ligated into the pIN-III-ompA$_2$ plasmid (Ghrayeb et al., 1984). For site-directed mutagenesis, the *Xba*I/*Bam*HI restriction fragment from pIN-III-ompA$_2$/hir, which contained sequences encoding hirudin and the ompA signal peptide, was subcloned into M13 mp19. Oligonucleotide-directed mutagenesis was performed according to the primer extension method of Zoller and Smith (1983). The mutagenized M13 mp19 dsDNA was used to transform *Escherichia coli* BMH 71-18 mut S (Kramer et al., 1984). ssDNA was isolated from at least 12 plaques, and dideoxy sequencing (Sanger et al., 1977) was used to identify mutants. The *Xba*I/*Bam*HI restriction fragment from the replicative form of M13 mp19 containing the mutation was recloned into pIN-III-ompA$_2$ and the resultant pIN-III-ompA$_2$/hir plasmid used to transform *E. coli* JM101.

Purification of Mutant Hirudins. Recombinant hirudins were prepared from 1-L cultures of *E. coli* JM101 in LB medium containing 250 μg of ampicillin/L. Recombinant hirudin was directed to the periplasmic space by the ompA signal sequence (Ghrayeb et al., 1984) and was released from the periplasmic space of the bacteria by osmotic shock with cold 10 mM Tris buffer, pH 8.1 (50 mL). After centrifugation, the supernatant was filtered and applied to a Mono Q fast liquid protein chromatography column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Bis-Tris/HCl buffer, pH 6.5. The hirudin activity was eluted by using a gradient of 0–300 mM NaCl in the equilibration buffer. Fractions were assayed for thrombin inhibitory activity, and those containing the bulk of the activity were pooled. The pooled fractions were further purified by reversed-phase HPLC using a gradient of 10–40% acetonitrile in 0.1% trifluoroacetic acid. Yields of recombinant hirudin were typically 1 mg/L culture.

Protein Chemical Characterization of Hirudin Molecules. The structure and purity of the hirudins were verified by analysis of amino acid composition (Heinrikson & Meredith, 1984) and by determination of the amino-terminal sequence (Hewick et al., 1981). In the five cases where a glutamic acid residue in the carboxy-terminal region was mutated to a glutaminyl residue, the mutations were confirmed by determining the sequence of a thermolytic peptide spanning residues 40–63. This thermolytic peptide was obtained by treating 50 μg of hirudin with performic acid as described by Hirs (1967).

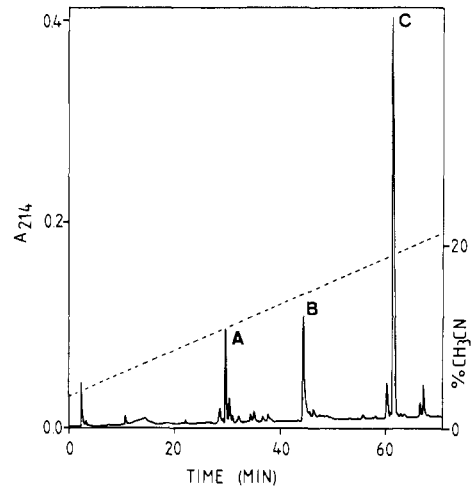

FIGURE 2: Peptide map of hirudin. The peptides resulting from cleavage of performic acid oxidized hirudin with thermolysin were separated by reversed-phase chromatography on a Vydac C_{18} column. The eluent was 0.1% (v/v) trifluoroacetic acid, and a linear gradient of acetonitrile (---) was used with a flow rate of 1.0 mL/min. The identity of peaks A, B, and C was established by sequence analysis, and they were found to correspond to residues 29–39, 1–28, and40–63, respectively. The dipeptide Leu-64-Gln-65 was not recovered. The retention times of peaks A and B did not vary between hirudins. In contrast, a progressive decrease in the retention time of peak C was observed with increases in the number of mutated glutamic acid residues.

Scheme I[a]

$$\begin{array}{ccccc} E + S & \overset{K_m}{\rightleftharpoons} & ES & \xrightarrow{K_{cat}} & E + P \\ + & & & & \\ I & & & & \\ k_1 \| k_2 & & & & \\ EI & & & & \end{array}$$

[a] $K_I = k_2/k_1$.

The sample was then dried twice, dissolved in 50 mM NH_4HCO_3 (50 μL), and digested with thermolysin (2% w/w) for 4 h at 37 °C. The digest was dried, dissolved in 0.1% (v/v) trifluoroacetic acid, and fractionated on a C_{18} reversed-phase column with a linear gradient of 0–28% (v/v) acetonitrile in 0.1% trifluoroacetic acid over 90 min. The flow rate was 1 mL/min. An example of the elution pattern obtained (that for mutant $E_4$57,58,61,62Q_4) is shown in Figure 2. The peaks were identified by amino acid sequence analysis. Peaks A, B, and C correspond to residues 29–39, 1–28, and 40–63, respectively. The sequence of peak C was determined for each of the hirudins with a carboxy-terminal substitution of glutamine for glutamate.

Theory and Data Analysis. Estimates of the dissociation constants of the thrombin–hirudin complexes and the concentrations of the hirudins were determined as described previously [data were fitted to eq 3 of Stone and Hofsteenge (1986)]. In cases where the determined dissociation constant was significantly different from that of wild-type recombinant hirudin, more detailed kinetic analyses were performed. Scheme I has previously been shown to apply to the thrombin–hirudin interaction, and progress curve data were fitted by nonlinear regression to the equation describing this mechanism [eq 4 of Stone and Hofsteenge (1986)]. These analyses yielded observed values for k_1, k_2, and K_I which must be corrected for the effect of substrate. It has previously been shown that the value of k_1 is independent of the binding of the substrate at the active site but that the observed values of k_2 and K_I should be divided by $(1 + S/K_m)$ in order to

obtain the true value (Stone & Hofsteenge, 1986; Stone et al., 1987). For these calculations, the previously determined value of 3.6 μM for the K_m of D-Phe-pipecolyl-Arg-*p*-nitroaniline with thrombin was used (Hofsteenge et al., 1986).

Results

Figure 1 shows the deduced amino acid sequence of the recombinant gene for hirudin that was used in the present study. Site-directed mutagenesis has been used to examine the importance of two classes of residues: basic residues, and acidic residues in the carboxyl-terminal region. Three lysyl residues and a histidyl residue are found in this sequence: Lys-27, Lys-36, Lys-47, and His-51. Thrombin usually cleaves adjacent to arginine residues (Blombäck et al., 1977; Mann & Lundblad, 1982), and it seemed reasonable to expect that a basic residue in hirudin would bind at the primary specificity pocket of thrombin. The charge and size properties of the lysyl residues in hirudin would approximate those of arginine, and a mutant fibrinogen in which the arginine at the cleavage site in the Aα chain was replaced by a histidine was still cleaved by thrombin (Higgins & Shafer, 1981; Southan et al., 1985). Each of the three lysines and the histidine were mutated separately to glutamine. The importance of the acidic carboxyl-terminal region was assessed by mutating Glu-61 and Glu-62, separately, and the four glutamate residues, 57, 58, 61, and 62 together, to glutaminyl residues. In the course of screening for the quadruple mutant, double and triple mutants were also obtained. It should be noted that all the mutations made were conservative in that they were not designed to create unfavorable interactions. Charged residues were changed to hydrophilic residues of about the same size. Thus, ionic interactions could be replaced by hydrogen-bonding interactions, and the observed decreases in binding energies should be regarded as the minimum contribution of the mutated residue.

The recombinant hirudins were purified by a combination of ion-exchange and reversed-phase chromatography. N-Terminal amino acid sequencing (five cycles) detected only one sequence in all samples and confirmed that the cleavage of the signal sequence occurred at the bond preceding Val-1 of hirudin in all cases. Moreover, the amino acid compositions were in good agreement with those predicted from the nucleotide sequences. Mutations involving the substitution of glutamine for glutamate in the carboxy-terminal region could not be confirmed by amino acid composition analysis. Consequently, a thermolytic peptide corresponding to residues 40–63 was isolated and sequenced for each of these mutants. In each case, the amino acid sequence obtained was in full agreement with that deduced from the DNA sequence. The fact that recombinant hirudin displayed inhibitory activity identical with that of desulfated native hirudin (see below) suggested that the disulfide bridges in the recombinant molecule were correctly formed since disruption of the disulfide bridges in native hirudin leads to loss of inhibitory activity (Bagdy et al., 1976).

The inhibitory activity of the mutant hirudins can be compared with those of native hirudin (containing Tyr-SO_3^- at position 63) and recombinant hirudin. Previously, we had not been able to obtain a direct estimate for the association rate of native hirudin with thrombin at an ionic strength below 0.2 because the rate appeared too rapid to measure with a conventional recording spectrophotometer under the conditions used (Stone & Hofsteenge, 1986). In this study, however, it was found that by lowering the concentration of thrombin in the assays from 45 to 6 pM and concomitantly lowering the concentration range of hirudin from 20–60 pM to 3–18 pM, it was possible to measure an association rate at an ionic strength of 0.125. The association rate constant (k_1; Table I) was somewhat lower than had been predicted from extrapolation from values obtained at higher ionic strengths (Stone & Hofsteenge, 1986). This prediction was made on the basis of the assumption that the dissociation rate (k_2) of the complex would be invariant with ionic strength whereas it has increased slightly (1×10^{-5} s^{-1} compared with the predicted 0.5×10^{-5} s^{-1}). The dissociation constant of the complex is, however, in excellent agreement with the value previously determined (Stone & Hofsteenge, 1986).

Table I: Kinetic Constants for the Interaction of Different Forms of Hirudin with Thrombin[a]

form of hirudin	$k_1 \times 10^{-8}$ (M^{-1} s^{-1})	$k_2 \times 10^5$ (s^{-1})	K_I (fM)
native	4.72 ± 0.01	0.98 ± 0.02	21 ± 1
desulfo–native	nd[c]	nd	207 ± 15[b]
recombinant	1.37 ± 0.03	3.17 ± 0.11	231 ± 6
K27Q	nd	nd	295 ± 37
K36Q	nd	nd	223 ± 30
K47Q	0.230 ± 0.008	4.57 ± 0.22	1999 ± 70
H51Q	nd	nd	240 ± 38
E61Q	0.957 ± 0.020	3.56 ± 0.14	372 ± 12
E62Q	0.671 ± 0.023	3.70 ± 0.21	552 ± 25
$E_2$57,58Q_2	0.218 ± 0.006	5.15 ± 0.18	2360 ± 50
$E_3$57,58,62Q_3	0.074 ± 0.001	6.38 ± 0.20	8600 ± 160
$E_4$57,58,61,62Q_4	0.045 ± 0.001	6.30 ± 0.17	14100 ± 200

[a] Assays were performed and data were analyzed as described under Experimental Procedures. The estimates for the kinetic constants are given together with their standard errors obtained from the analysis. The kinetic constants are defined in Scheme I. [b] This value was determined previously (Stone & Hofsteenge, 1986). [c] Not determined.

Hirudin isolated from leeches contains a sulfated tyrosine at position 63. Amino acid composition analysis of the recombinant hirudin treated with carboxypeptidase Y as described by Chang (1983) indicated that this residue is not sulfated in this protein. In addition, the retention time of the recombinant protein on reversed-phase HPLC corresponded to that of native hirudin that had been desulfated (Stone & Hofsteenge, 1986). The kinetic constants for the inhibition of thrombin by recombinant hirudin were determined and are given in Table I. The value obtained for the association rate constant (k_1) was 3.4-fold lower than that for native hirudin while the dissociation rate of the complex (k_2) was 3.2-fold higher. These changes in the kinetic constants were reflected in a 10-fold lower affinity of thrombin for the recombinant hirudin (K_I = 231 fM; Table I). The dissociation constant for desulfated native hirudin was previously determined to be 207 fM (Stone & Hofsteenge, 1986), and this value is in good agreement with that determined for the recombinant hirudin (Table I).

Mutation of Lys-27 (K27Q), Lys-36 (K36Q), or His-51 (H51Q) did not affect the affinity of thrombin for the hirudins containing these mutations as can be seen from the values of their dissociation constants given in Table I. Mutation of Lys-47 (K47Q) resulted in a 9-fold increase in the dissociation constant of the thrombin–hirudin complex. Analysis of kinetic data for the inhibition of thrombin by this mutant protein indicated that the decrease in the affinity was predominantly due to a 6-fold decrease in the rate of association of K47Q (Table I).

Mutation of Glu-61 (E61Q) and Glu-62 (E62Q) to glutamine caused, respectively, 1.6- and 2.3-fold increases in the dissociation constant of the complex (Table I). Multiple mutations of glutamate residues in the carboxyl-terminal region of hirudin led to greater increases in the dissociation

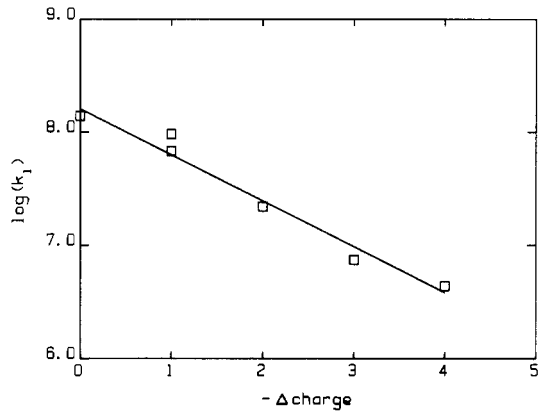

FIGURE 3: Dependence of the association rate constant (k_1) for hirudin on the charge of the molecule. The values of k_1 were determined as described under Experimental Procedures. The value for $-\Delta$ charge equals the number of glutamates mutated to glutamine, and the upper and lower points at $-\Delta$ charge = 1 represent those for E61Q and E62Q, respectively. The line drawn represents the best fit of the data obtained by weighted-linear regression for the linear dependence of log k_1 on $-\Delta$ charge.

constant. The double mutant $E_2$57,58Q_2 caused a 10-fold increase in the dissociation constant whereas the triple and quadruple mutations $E_3$57,58,62Q_3 and $E_4$57,58,61,62Q_4 led to 37- and 61-fold increases, respectively. Kinetic analysis of these mutants indicated that in all cases the decrease in affinity was predominantly due to a decrease in the association rate constant (Table I).

A certain degree of care needs to be taken when considering the effects of the multiple mutations as the possibility of changes in tertiary structure increases with each mutation. It seems probable, however, that changes in tertiary structure are not responsible for the changes in affinity observed in the present study. The effects observed are consistent with the chemical kinetic theory for the reaction between charged molecules. This theory predicts that the association rate constant will be dependent on the charge of hirudin as shown in eq 1 (Laidler, 1987):

$$\ln k_{obsd} = \ln k_\infty - A z_A z_B \quad (1)$$

where k_{obsd} is the observed value of the association rate constant (k_1), k_∞ is the association rate constant at infinite dielectric constant where the electrostatic contribution to the interaction between the two molecules will be zero, A is a constant which is dependent on the effective dielectric constant of the assay solution, the temperature, and the distance between the charged regions of the two molecules in the complex, and z_A and z_B represent the charges of the regions of thrombin and hirudin, respectively, that contribute to the electrostatic interaction. This equation predicts that a plot of log k_1 against the number of charges removed in each mutant should yield a straight line provided that all the charges removed contribute equally to the electrostatic interaction and that the mutations have not resulted in more general changes in structure. Figure 3 shows that for the carboxyl-terminal mutants of hirudin, the relationship given in eq 1 was obeyed. Thus, it seems that the effects observed are predominantly due to the removal of negative charges from the carboxyl-terminal region of hirudin.

DISCUSSION

The results obtained with hirudins in which basic residues were mutated indicate that hirudin is markedly different from most other naturally occurring inhibitors of serine proteases previously studied. The specificity of these inhibitors is determined by the interaction with the primary specificity pocket of the target protease (Travis & Salvesen, 1983; Laskowski & Kato, 1980; Carrel & Travis, 1985). With these inhibitors, changes in the residue of the inhibitor that binds in the primary specificity pocket, the P_1 residue in the nomenclature of Schecter and Berger (1967), can lead to marked alterations in the specificity of the inhibitor (Laskowski et al., 1987; Owen et al., 1983). For example, the importance of this residue in determining the specificity of α_1-proteinase inhibitor has been demonstrated by the occurrence of natural variants (Owen et al., 1983) and by site-directed mutagenesis (Jallat et al., 1986). On mutation of the reactive-site methionine of α_1-proteinase inhibitor to an arginine, the inhibitor loses its ability to inhibit neutrophil elastase and becomes an effective inhibitor of thrombin. In cases where the three-dimensional structure of serine proteases and inhibitors has been determined, it has been found that the majority of the interactions involve only a small part of the inhibitor around the reactive-site residue (Huber et al., 1974; Read et al., 1983). In contrast, hirudin does not seem to require specific interactions in the primary specificity pocket of thrombin to form a tight complex with this enzyme. Of the mutations involving the basic amino acids in hirudin, only that of Lys-47 (K47Q) caused a significant increase in the dissociation constant of the thrombin–hirudin complex, and the increase in this case was only 9-fold. This increase represents a decrease in the binding energy of 6 kJ mol^{-1} compared with the total of -75 kJ mol^{-1} for the unmutated form. Previous studies indicated that the binding of hirudin at the primary specificity pocket was not crucially important for complex formation between the two molecules; thrombin with its active site blocked with diisopropyl phosphate was still capable of forming a complex with hirudin (K_I = 26 pM; Stone et al., 1987). In this respect, hirudin is similar to the cysteine protease inhibitors called cystatins which are able to form complexes with cysteine proteases with blocked active sites (Barret, 1987). However, it should be stressed that although the binding of hirudin at the active site of thrombin is not of utmost importance for complex formation, hirudin nevertheless binds to this region of thrombin as evidenced by the fact that it is a competitive inhibitor with respect to tripeptidyl p-nitroanilide substrates (Stone & Hofsteenge, 1986). In this manner, hirudin differs from α_2-macroglobulin, which is another inhibitor for which interactions with the primary specificity pocket of its target protease are not markedly important. Proteases complexed with α_2-macroglobulin, while inactive toward high molecular weight substrates, are still capable of hydrolyzing tripeptidyl substrates, which indicates that α_2-macroglobulin is not bound at the active site (Travis & Salvesen, 1983).

Although binding of hirudin at the primary specificity pocket of thrombin appears not to make a major contribution to the formation of a tight complex, the results obtained with the Lys-47 mutant, when considered in conjunction with studies on the specificity of thrombin, suggest that Lys-47 may indeed be the P_1 residue in hirudin. The best natural and synthetic substrates of thrombin often contain a proline residue in the P_2 position (Blombäck et al., 1977). Lys-47 is preceded and followed by proline residues in the hirudin sequence. The proline in the P_2 position (Pro-46) would satisfy the specificity requirements of thrombin. Moreover, bonds between lysine and proline residues, such as that between Lys-47 and Pro-48, are usually resistant to proteolysis (Kasper, 1975). The fact that only a small change in affinity was observed on the replacement of Lys-47 by a glutamine would not be unexpected if the specificity of hirudin were determined by interactions with thrombin in regions other than the primary specificity pocket. In this case, substitution of a glutamine for a lysine

would not result in the creation of unfavorable interactions and would lead to only a small decrease in affinity. Some data exist, however, that question whether Lys-47 is the residue involved in interactions with the primary specificity pocket. Lys-47 is not conserved in all hirudin sequences; the amino acid sequence deduced from a cDNA clone isolated from leeches contains an asparaginyl residue at position 47 (Harvey et al., 1986). Moreover, the ϵ-amino group of Lys-47 is involved in electrostatic interactions with backbone carbonyl oxygen atoms (Clore et al., 1987). It is possible that the disruption of these interactions by the replacement of Lys-47 by glutamine has resulted in minor alterations in the tertiary structure of hirudin and that these alterations have led to the observed increase in the dissociation constant of the mutant protein.

Previous studies had indicated that the rate of association of hirudin and thrombin decreased markedly with increasing concentrations of salt, which suggested that ionic interactions may be involved in the formation of the complex (Stone & Hofsteenge, 1986). Subsequent studies indicated that a region of thrombin rich in positively charged residues was important for its interaction with hirudin (Stone et al., 1987). The carboxyl-terminal region of hirudin is rich in negatively charged residues (Figure 1), and it was postulated that this region was involved in the putative ionic interaction with thrombin (Stone et al., 1987). In addition, studies using chemically synthesized fragments of hirudin have demonstrated that the carboxyl-terminal region of hirudin is able to bind to thrombin and block its activity with fibrinogen (Krstenansky & Mao, 1987; Krstenansky et al., 1987). Mutations were made in this region to remove negatively charged residues. Each of these mutations caused an increase in the dissociation constant of the thrombin–hirudin complex. The effect of mutations involving Glu-57 should, however, be viewed with some caution. Structural studies indicate that Glu-57 is involved in an electrostatic interaction with Ser-9 in the central core of hirudin (Clore et al., 1987). Replacement of Glu-57 with glutamine could disrupt this interaction and may lead to alterations in the tertiary structure. The data of Figure 3 imply, however, that each of the negatively charged residues examined contribute about equally to hirudin's interaction with thrombin. These results are apparently in contrast with results obtained by Krstenansky et al. (1987) using chemically synthesized variants of the peptide 55–65. In these studies, substitution of Glu-57 for an alanine produced a much greater increase in the IC_{50} value of the peptide than substitution of any of the other three glutamates. However, the results of Krstenansky et al. (1987) and those of the present study cannot strictly be compared since the interactions important in the binding of a small fragment of a protein and the protein itself are not necessarily the same.

Other acidic residues in hirudin other than those examined may also be important for its interaction with thrombin. Clore et al. (1987) have noted that the outer surface of hirudin forms a contiguous surface of hydrophilic and charged residues, and it seems likely that some of these other charged residues may be involved in the binding to thrombin. The specificity of hirudin is unlikely to be determined by interactions in only one area such as the carboxyl terminus, and further investigations into the importance of other areas will be required.

During the revision of this paper, Dodt et al. (1988) reported the results of studies which also suggest that Lys-47 is the P_1 residue in hirudin.

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Registry No. Hirudin, 8001-27-2; thrombin, 9002-04-4.

REFERENCES

Bagdy, D., Barabas, E., Graf, L., Peterson, T. E., & Magnusson, S. (1976) *Methods Enzymol. 45*, 669–678.

Barret, A. J. (1987) *Trends Biochem. Sci. (Pers. Ed.) 12*, 193–196.

Berliner, L. J., & Shen, Y. Y. L. (1977) *Biochemistry 16*, 4622–4626.

Blombäck, J. G., Hessel, B., Hogg, D., & Claeson, G. (1977) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., Fenton, J. W., II, & Mann, K. G., Eds.) pp 275–290, Ann Arbor Science, Ann Arbor, MI.

Carrell, R. W., & Travis, J. (1985) *Trends Biochem. Sci. (Pers. Ed.) 10*, 20–24.

Chang, J.-Y. (1983) *FEBS Lett. 164*, 307–313.

Claeson, G., Aurell, L., Karlsson, G., & Friberger, P. (1977) in *Neue Methoden der Gerinnungsanalyse mit chromogenen Substraten* (Witt, I., Ed.) pp 37–48, Walter de Gruyter, New York.

Clore, G. M., Sukumaran, D. K., Nilges, M., Zarbock, J., & Gronenborn, A. M. (1987) *EMBO J. 6*, 529–537.

Dodt, J., Müller, H., Seemüller, U., & Chang, J.-Y. (1984) *FEBS Lett. 165*, 180–183.

Dodt, J., Machleidt, N., Seemüller, U., Maschler, R., & Fritz, H. (1986) *Hoppe-Seyler's Z. Physiol. Chem. 367*, 803–811.

Dodt, J., Köhler, S., & Baici, A. (1988) *FEBS Lett. 229*, 87–90.

Fenton, J. W., II (1981) *Ann. N.Y. Acad. Sci. 370*, 468–495.

Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y., & Inouye, M. (1984) *EMBO J. 3*, 2437–2442.

Harvey, R. P., Degryse, E., Stefani, L., Schamber, F., Cazenave, J.-P., Courtney, M., Tolstoshev, P., & Lecocq, J.-P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1084–1088.

Heinrikson, R. L., & Meredith, S. C. (1984) *Anal. Biochem. 136*, 65–74.

Hewick, R. M., Hunkapillar, M. W., Hood, L. E., & Dreyer, W. J. (1983) *J. Biol. Chem. 256*, 7990–7997.

Higgins, D. L., & Shafer, J. A. (1981) *J. Biol. Chem. 256*, 12013–12017.

Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 197–199.

Hofsteenge, J., Taguchi, H., & Stone, S. R. (1986) *Biochem. J. 237*, 243–251.

Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., & Steigemann, W. (1974) *J. Mol. Biol. 89*, 73–101.

Jallat, S., Carvallo, D., Tessier, L. H., Roecklin, D., Roitsch, C., Ogushi, F., Crystal, R. G., & Courtney, M. (1986) *Protein Eng. 1*, 29–35.

Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A., & Elmore, D. T. (1973) *Biochem. J. 131*, 101–117.

Kasper, C. B. (1975) in *Protein Sequence Determination* (Needleman, S. B., Ed.) pp 114–161, Springer Verlag, Berlin.

Kettner, C., & Shaw, E. (1981) *Methods Enzymol. 80*, 826–842.

Kramer, B., Kramer, W., & Fritz, H.-J. (1984) *Cell (Cambridge, Mass.) 38*, 879–887.

Krstenansky, J. L., & Mao, S. J. T. (1987) *FEBS Lett. 211*, 10–16.

Krstenansky, J. L., Owen, T. J., Yates, M. T., & Mao, S. J. T. (1987) *J. Med. Chem. 30*, 1688-1691.
Laidler, K. J. (1987) *Chemical Kinetics*, pp 191-202, Harper and Row, New York.
Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593-626.
Laskowski, M., Jr., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., Schatzley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A., & Wieczorek, M. (1987) *Biochemistry 26*, 202-221.
Mann, K. G., & Lundblad, R. L. (1982) in *Haemostasis and Thrombosis* (Colman, R. W., Hirsh, J., Maeder, V. J., & Salzman, E. W., Eds.) pp 112-126, J. B. Lippincott, Philadelphia, PA.
Markwardt, F. (1970) *Methods Enzymol. 19*, 924-932.
Owen, M. C., Brennan, S. O., Lewis, J. H., & Carrell, R. W. (1983) *N. Engl. J. Med. 309*, 694-698.
Read, R. J., Fujinaga, M., Sielecki, A. R., & James, M. N. G. (1983) *Biochemistry 22*, 4420-4433.
Rink, H., Liersch, M., Sieber, P., & Meyer, F. (1984) *Nucleic Acids Res. 12*, 6369-6387.
Sanger, F., Nicklen, S., & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157-162.
Southan, C., Lane, D. A., Bode, W., & Henschen, A. (1985) *Eur. J. Biochem. 147*, 593-600.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622-4628.
Stone, S. R., Braun, P. J., & Hofsteenge, J. (1987) *Biochemistry 26*, 4617-4624.
Sukumaran, D. K., Clore, G. M., Preuss, A., Zarbock, J., & Gronenborn, A. M. (1987) *Biochemistry 26*, 333-338.
Takahara, M., Hibler, D. W., Barr, P. J., Gerlt, J. A., & Inouye, M. (1985) *J. Biol. Chem. 260*, 2670-2674.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655-709.
Walker, B., Wikstrom, P., & Shaw, E. (1985) *Biochem. J. 230*, 645-650.
Walsmann, P., & Markwardt, F. (1981) *Pharmazie 36*, 633-660.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468-500.

Effectors of the Activation of Human [Glu^1]plasminogen by Human Tissue Plasminogen Activator†

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ABSTRACT: The activation of human [Glu^1]plasminogen ([Glu^1]Pg) by human recombinant (rec) two-chain tissue plasminogen activator (t-PA) is inhibited by Cl^-, at physiological concentrations, and stimulated by ϵ-aminocaproic acid (EACA), as well as fibrin(ogen). Chloride functions as a result of its binding to [Glu^1]Pg, with a K_i of approximately 9.0 mM, thereby rendering [Glu^1]Pg a less effective substrate for two-chain rec-t-PA. EACA stimulates the activation in Cl^--containing solutions, with a K_a of approximately 4.0 mM, primarily by reversal of the Cl^--inhibitory effect. Fibrinogen appears to exert its stimulatory properties mainly through effects on the enzyme, two-chain rec-t-PA, with a K_a of approximately 3.7 μM in activation systems containing physiological levels of Cl^-. Analysis of the results of this paper reveals that normal plasma components, Cl^- and fibrinogen, exert major regulatory roles on the ability of [Glu^1]Pg to be activated by two-chain rec-t-PA, in in vitro systems. The presence of Cl^- inhibits the stimulation of [Glu^1]Pg activation that would normally occur in the presence of fibrinogen, a result of possible importance to the observation that some degree of systemic fibrinogenolysis accompanies therapeutic use of tissue plasminogen activator.

Human plasminogen ([Glu^1]Pg), the precursor of the fibrinogenolytic and fibrinolytic enzyme plasmin, is a single-chain plasma glycoprotein containing 791 amino acids in known sequence (Wiman, 1973, 1977; Sottrup-Jensen et al., 1977; Malinowski et al., 1984; Forsgren et al., 1987). Activation of [Glu^1]Pg is concomitant with cleavage of the Arg^{561}–Val^{562} peptide bond in the zymogen and is catalyzed by a variety of proteins, such as urokinase (u-PA), streptokinase (SK), and tissue plasminogen activator (t-PA) [for a review, see Castellino (1981)].

As synthesized, t-PA is a single-chain protein containing 527 amino acid residues (Pennica et al., 1983). This protein is also found in a two-chain form, as a result of cleavage of the Arg^{275}–Ile^{276} peptide bond by plasmin (Pennica et al., 1983) and other such enzymes (Ichinose et al., 1984). t-PA is a serine protease, containing the catalytic triad at sequence positions His^{322} (Pennica et al., 1983), Asp^{371} (Pennica et al., 1983), and Ser^{478} (Pennica et al., 1983; Pohl et al, 1984). The single-chain form of t-PA is less active than the two-chain form toward small substrates (Wallen et al., 1982), but apparently, both forms of this enzyme possess similar plasminogen activator activities (Rijken et al., 1982). Whereas t-PA is a poor activator of plasminogen in the absence of certain effector molecules, its activity is greatly stimulated in the presence of

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