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14. COS-1 cells maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) were washed three times with PBS and incubated for 30 min in RPMI medium without methionine and cysteine and containing 5% FBS. After preincubation, 3 ml of RPMI medium without methionine and cysteine and supplemented with [^{35}S]methionine (0.25 mCi/ml) and [^{35}S]cysteine (0.25 mCi/ml) was added to each 150-mm tissue culture dish and incubated for 2 hours at 37°C.
15. Antibodies used were: Antibody to TCR-α, A2B4, murine IgG2a monoclonal antibody [L. E. Samelson, R. N. Germain, R. H. Schwartz, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6972 (1983)]; antibody to CD3-ζ, rabbit antibody to mouse affinity-purified antibody [A. M. Weissman, L. E. Samelson, R. D. Klausner, *Nature* **324**, 480 (1986)]. Antibodies to CD3-δ directed against a COOH-terminal peptide of the murine δ chain [L. E. Samelson, A. M. Weissman, F. A. Robey, I. Berkower, R. D. Klausner, *J. Immunol.* **137**, 3254 (1986)] were affinity-purified and covalently bound to CNBr-activated Sepharose 4B beads.
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20. Cotransfected cells were harvested and solubilized, and lysates were subjected to immunoprecipitation with either antibody to CD3-δ or antibody to Tac. Samples were run under reducing conditions with 13% SDS–polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred electrophoretically to nitrocellulose [S. M. King, T. Otter, G. B. Whitman, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4717 (1985)] and immunoblotted with antibodies to CD3-δ as described previously [M. D. Patel, L. E. Samelson, R. D. Klausner, *J. Biol. Chem.* **262**, 5831 (1987)]. Band intensities on autoradiograms were determined by scanning densitometry with a Hoeffer densitometer and computer analyzed with the Application II data analysis program (Dynamic Solutions, Pasadena, CA).
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The Structure of a Complex of Recombinant Hirudin and Human α-Thrombin

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The crystallographic structure of a recombinant hirudin-thrombin complex has been solved at 2.3 angstrom (Å) resolution. Hirudin consists of an NH_2-terminal globular domain and a long (39 Å) COOH-terminal extended domain. Residues Ile^1 to Tyr^3 of hirudin form a parallel β-strand with Ser^{214} to Glu^{217} of thrombin with the nitrogen atom of Ile^1 making a hydrogen bond with Ser^{195} Oγ atom of the catalytic site, but the specificity pocket of thrombin is not involved in the interaction. The COOH-terminal segment makes numerous electrostatic interactions with an anion-binding exosite of thrombin, whereas the last five residues are in a helical loop that forms many hydrophobic contacts. In all, 27 of the 65 residues of hirudin have contacts less than 4.0 Å with thrombin (10 ion pairs and 23 hydrogen bonds). Such abundant interactions may account for the high affinity and specificity of hirudin.

THROMBIN (E.C. 3.4.21.5) IS A GLYcoprotein that functions as a serine proteinase when it is generated in the final events of blood coagulation (*1*). α-Thrombin converts fibrinogen into clottable fibrin by exhibiting specificity largely attributed to an anion binding exosite distinct from the catalytic site (*2*). The molecule consists of two peptide chains of 36 and 259 residues linked by a disulfide bond (*3*). The crystallographic structure of human α-thrombin inactivated with D-Phe-Pro-Arg-chloromethyl ketone (PPACK) at 1.9 Å resolution (*4*) shows structural similarity to trypsin-like proteases but with insertions (loops at Leu^{60} and Thr^{149}) (*5*) that protrude around the active site and narrow the substrate binding cleft.

The principal inhibitor of thrombin in blood is antithrombin III; however, the most potent natural inhibitor is hirudin from the European medicinal leech *Hirudo medicinalis* (*6*), which consists of a compact NH_2-terminal head and a long polypeptide COOH-terminal tail (Fig. 1). The structures of two recombinant hirudins have been determined in solution by nuclear magnetic resonance (NMR) (*7*). The NH_2-terminal head along with the first 10 residues of the COOH-terminal tail were resolved but the remaining 16 residues and residues 31 to 36 were disordered. We report the crystallographic structure determination of a recombinant hirudin α-thrombin complex at 2.3 Å resolution (*8*) in which the COOH-terminal tail of hirudin is ordered.

The structure of hirudin in the complex is composed of two domains (Figs. 2 and 3). The folding of the NH_2-terminal domain appears to be intimately related to the presence of a three-disulfide core. Residues h-Cys^6-Cys^{14} and h-Cys^{16}-Cys^{28} orient nearly perpendicular at a distance of 4.7 Å between the midpoints of the bridges. Conversely, h-Cys^{16}-Cys^{28} and h-Cys^{22}-Cys^{39} are nearly parallel with a comparable distance (5.3 Å) (*9*). The Cys interactions cause the loop segments B, C, and D (Fig. 1), which form the double-loop structure of hirudin, to fold into three different three-dimensional loops, each stabilized by antiparallel β structure. In segment B, residues h-Cys^{14} to Cys^{16} and h-Asn^{20} to Cys^{22} form a short antiparallel β stretch connected by a type II′ β turn. In segment D, h-Lys^{27} to Gly^{31} and h-Gly^{36} to

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Val[40] form longer β strands. However, the turn between these strands has little density and is disordered in the complex (*10*). The result of the disulfide and antiparallel β interactions is that the NH_2-terminal domain of hirudin is dominated by four spatially distinct loop structures (Fig. 3) similar to those observed by NMR (*7*).

The NH_2-terminal domain of hirudin binds at the active site region, and the 39 Å long COOH-terminal tail occupies a narrow canyon. A novel interaction occurs between hirudin and α-thrombin in the active site that involves the three NH_2-terminal residues of hirudin (Fig. 3), which form a parallel β strand with Ser[214] to Glu[217] of thrombin. This segment is an antiparallel interaction in all natural serine protease inhibitor complexes (*11*) and also in PPACK-thrombin. The NH_2-terminal nitrogen of h-Ile[1] is within hydrogen-bonding distance of the Oγ of Ser[195] (3.2 Å). The side chain atoms of h-Ile[1] and h-Tyr[3] participate in numerous nonpolar contacts with hydrophobic residues of thrombin: h-Ile[1] is in the S2 subsite of thrombin in contact with His[57], Tyr[60a], Trp[60d], and Leu[99], whereas the ring of h-Tyr[3] occupies a hydrophobic cleft (made by Leu[99], Ile[174], and Trp[215]) in a manner similar to D-Phe of PPACK-thrombin. This hydrophobic pocket of thrombin may be the apolar binding site reported for indole and delineated by Bode *et al.* (*4*) in the structure of PPACK-thrombin. The side chain of h-Thr[2] only penetrates the edge of the specificity pocket of thrombin. However, binding through this site is not obligatory, since hirudin binds to catalytically inactivated forms of thrombin (*6, 12*).

Most of the NH_2-terminal domain of hirudin is not in contact with the thrombin surface (Fig. 2) but many interactions exist at the interface of the two. Residues h-Leu[13] and h-Pro[46] are both 3.5 Å from Pro[60c] of the Tyr[60a] to Thr[60i] insertion, which narrows the active site cleft (*4*). Residues h-Asp[5], h-Glu[17], h-Ser[19], and h-Lys[47] form salt bridges or hydrogen bonds with thrombin. Lastly, h-Asn[20] and h-Lys[24] also interact with thrombin through water-mediated contacts.

The tripeptide segment h-Pro[46]-Lys[47]-Pro[48] appears to be important in facilitating the hirudin NH_2-terminal active site interaction but not because of its thrombin substrate-like sequence (*13*) or its similarity to the fragment 1 cleavage site of prothrombin. The Nε group of h-Lys[47] helps orient the NH_2-terminal by forming hydrogen bonds with the carbonyl oxygen of h-Asp[5] (2.5 Å) and h-Thr[4] Oγ (3.0 Å), thus also terminating the folding of the compact NH_2-terminal domain. Moreover, the position of the h-Lys[47] side chain appears to be maintained by the two Pro residues that flank it through a polyproline II conformation. By forming a close contact with Pro[60c], h-Pro[46] also aids in anchoring the domain for the NH_2-terminus to penetrate into the active site (Fig. 3). The h-Lys[47] has often been viewed as the P1 residue of substrate in the hirudin-thrombin complex (*14, 15*). However, the Lys side chain does not occupy the specificity pocket of thrombin and is located about 11 Å away; nonetheless, it appears to be a factor in stabilizing the hirudin active site interaction.

The COOH-terminal tail of hirudin adopts a long extended conformation in the complex and can be viewed as two stretches of peptide (18 and 19 Å long) with a bend at h-Asp[55]. By means of the extended conformation, the tail can interact with many residues on the thrombin surface. This binding region of thrombin, the anion binding exosite (*2*), is an extension of the active site cleft dominated by positively charged side chains (*4*). Two of the first three residues of

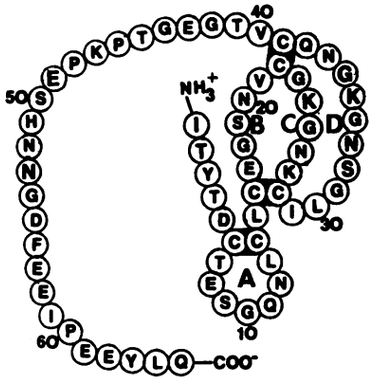

Fig. 1. Sequence of recombinant hirudin variant 2-Lys[47] (rHV2-Lys[47]). Hirudin forms a stable noncovalent stoichiometric 1:1 complex with thrombin [inhibition constants $K_i \approx 10^{-11}$ to 10^{-14} M (*12*)] that does not require active enzyme, although kinetic and equilibrium studies indicate that hirudin interacts simultaneously with the catalytic site and the fibrinogen binding exosite (*2, 14*). Studies with synthetic peptides have shown that the thrombin exosite contains a number of Lys residues (*22*) and interacts with the COOH-terminal undecapeptide of hirudin (*23, 24*). Natural hirudins are mixtures of variants, but recombinant techniques produce homogeneous preparations. The latter lack a sulfated h-Tyr[63] but have K_i values in the picomolar range (*16*). Human α-thrombin (*25*) and rHV2-Lys[47] (*26*) were used to prepare the complex in this work. Crystals were grown by the vapor diffusion method with 28 to 30% PEG 4000, 0.2 M $MgCl_2$, 0.1 M sodium acetate buffer, pH 4.5, and 1 mM NaN_3. The crystals are tetragonal ($a = b = 90.39 \pm 0.02$ and $c = 132.97 \pm 0.07$ Å), space group $P4_32_12$, with eight molecules per unit cell (protein volume fraction ~39%).

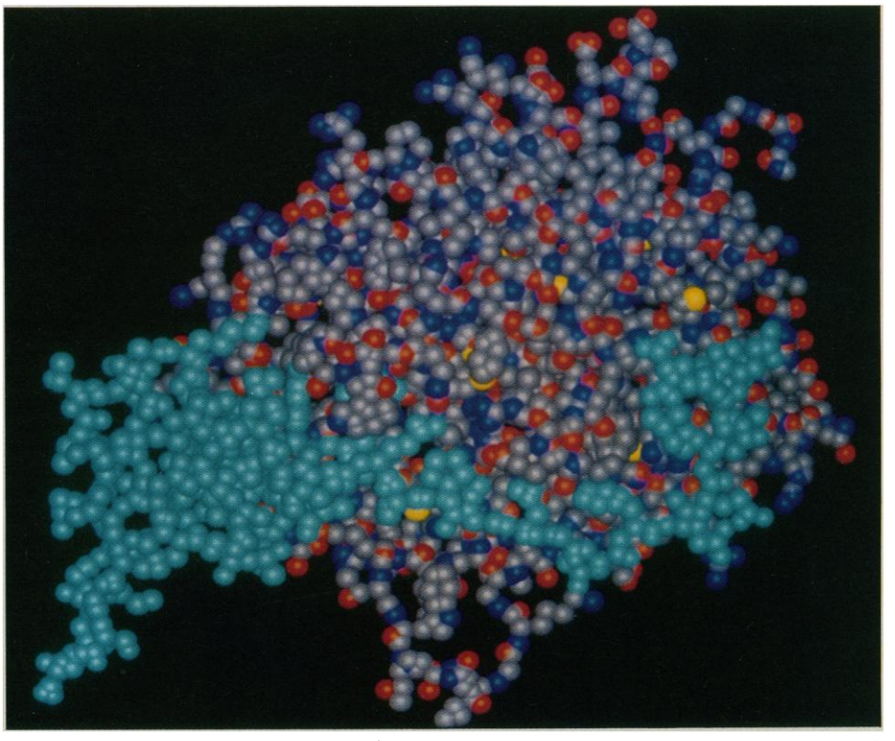

Fig. 2. Space-filling drawing of rHV2-Lys[47] human α-thrombin complex. Hirudin is in light blue and thrombin is multicolored: carbons, gray; nitrogens, blue; oxygens, red; and sulfurs, yellow. The hirudin NH_2-terminal domain is on the left and the COOH-terminal 3_{10} helix is on the right. Penetration of active site by hirudin NH_2-terminal can be noted left of center. The upper left surface of thrombin is highly electropositive and consists of Lys[87], Arg[93], Arg[97], Lys[169], Arg[175], His[230], Lys[233], Lys[236], and Lys[240], which may be the heparin binding site since the latter binds at an exosite different from that of fibrinogen (*27*). The orientation of the hirudin-thrombin complex is the same as in Fig. 3.

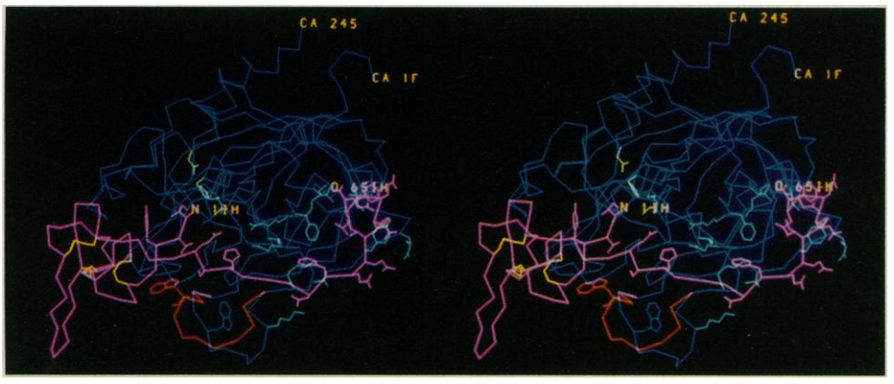

Fig. 3. Stereoview of Cα structure of the rHV2-Lys[47] human α-thrombin complex. Orientation is the same as in Fig. 2; the hirudin main chain and NH_2- and COOH-terminal side chains that interact with thrombin are in pink and the hirudin disulfides are yellow. For thrombin, the main chain is in dark blue, the side chains interacting with hirudin are in light blue, and the active site is in yellow-blue. The conformation of Glu[146]-Gly[150] insertion loop of PPACK thrombin is shown in orange. The NH_2- and COOH-terminals of hirudin are labeled. The NH_2-terminal domain is compactly folded (h-Ile[1] to Pro[48]), and the COOH-terminal exists as two extended stretches of peptide (h-Glu[49] to Gly[54] and h-Asp[55] to Pro[60]) that terminate with a near-3_{10} helical type III reverse turn (h-Glu[61] to Leu[64]) (*24*). The h-Pro[46] to His[51] segment, with two prolines, is almost in a perfect polyproline II conformation. The side chains of 12 of the 17 extended tail residues make salt bridges or hydrophobic interactions with thrombin. The salt bridges are: h-Glu[49], Lys[60f]; h-Glu[49], Arg[35]; h-His[51], Glu[39]; h-Asp[55], Arg[73], and Lys[149e]; h-Glu[58], Arg[77a]; and h-Glu[65], CO_2^-, and Lys[36].

the tail are involved in ion pair interactions (Fig. 3), whereas h-Ser[50] Oγ makes a bifurcated hydrogen bond with Glu[192]. The next three residues of the segment are in poorly defined electron density.

Polar interactions between the second segment of the tail and thrombin persist but hydrophobic interactions dominate the 3_{10} helical region. A total of 9 of the final 11 COOH-terminal residues are involved in exosite interactions: h-Glu[57] forms a hydrogen bond with the amide of Tyr[76] and also makes an ion pair with Arg[75] of a twofold symmetry-related molecule in the crystal although this interaction could possibly transpire in solution intramolecularly; h-Glu[57] also interacts with Arg[77a] through a mediating water molecule. Both h-Glu[61] to Glu[62] appear to be directed toward the solvent and are not involved with the exosite (Fig. 3).

A number of hydrophobic interactions also reside between the end of the extended tail and the exosite; h-Phe[56] penetrates into a depression on the thrombin surface (Fig. 2) and makes contacts with Phe[34], Leu[40], the methylene groups of Arg[73], and Thr[74] Cγ. The planes of h-Phe[56] and Phe[34] are nearly perpendicular and give a favorable edge-on aromatic stacking interaction (Fig. 3). In addition, h-Ile[59] is close to Leu[65] and Ile[82], and h-Pro[60] abuts with Tyr[76] (3.3 Å) and Ile[82] (4.1 Å). The important h-Tyr[63] residue, which is sulfated in native hirudin, is also involved in hydrophobic interactions with Ile[82] and h-Pro[60]. Although this Tyr is unsulfated in rHV2-K47, Lys[81] and Lys[109] to Lys[110] are nearby and could form ion pairs with a sulfated residue by free bond rotations. Lastly, the 3_{10} helical turn puts h-Leu[64] close to h-Ile[59] (3.3 Å), and they in turn adjoin Leu[65] and Phe[34]. Thus the COOH-terminal of hirudin is firmly anchored at the end of the thrombin exosite by hydrophobic contacts of the helical turn (Fig. 2) and the salt bridge formed by the carboxylate of h-Gln[65] and Lys[36]. Removal of h-Gln[65] has little effect on the apparent inhibition constant (K_{iapp} increases by a factor of 1.3), but deletion of the next two residues increases K_{iapp} by a factor of 40 (*16*).

An unexpected aspect of the hirudin-thrombin interaction in the exosite is the extent of the nonpolar contribution. In the terminal part of the COOH-terminal tail, nearly half of the residues (5 of 11) are hydrophobic or aromatic or both and all five participate in nonpolar hirudin-thrombin interactions. The importance of hydrophobic interactions in the anticoagulant activity of the tail has been suggested in studies with synthetic polypeptides (*17*), which indicate that the minimal peptide necessary for the detection of activity is h-Phe[56] to Gln[65] and that it is sensitive to modification of residues h-Phe[56], h-Ile[59], h-Pro[60], h-Leu[64], as well as h-Glu[57]. The hydrophobic contribution in the tail is as important as the polar interactions. In all, 27 of the 65 residues of hirudin and 36 of the 259 residues of the B chain of thrombin are involved in 212 contacts of less than 4.0 Å, of which 10 are ion pairs and 23 are hydrogen bonds. Residues h-Ile[1] to Tyr[3], h-Ser[19] to Val[21], h-Glu[49] to His[51], and h-Asp[55] to Pro[60] make 46, 34, 25, and 65 of the contacts, respectively.

The structures of thrombin in the hirudin complex and in PPACK-thrombin are practically the same even to the disorder of the carbohydrate attached to Asn[60g]. An optimal superposition of 266 Cα atoms between the two gives a root-mean-square (rms) deviation of 0.46 Å. The only large difference occurs in the decapeptide insertion loop between Glu[146] to Gly[150] (Fig. 3), which has a Cα rms deviation of 6.3 Å. In PPACK-thrombin, Trp[148] is positioned close to the active site. A conformational change occurs in the insertion loop of the complex at Glu[146] and Gly[150], which results in a totally different position for Trp[148]: the loop now encircles the indole of this residue.

Hirudin is basically a bivalent inhibitor consisting of two domains that could trigger the conformational change. The side chain of Trp[148] of PPACK-thrombin occupies the space of the main chain of h-Thr[4] to Asp[5] (Fig. 3), suggesting that a conformational change is required of the binding of the NH_2-terminal three residues in the active site. The binding of the COOH-terminal dodecapeptide of hirudin to thrombin also produces a change in circular dichroism (CD); however, PPACK inhibition of thrombin does not (*18*). The CD results thus suggest that the COOH-terminal tail might be responsible for the conformational change. The salt bridge between h-Asp[55] and Lys[149e] is a consequence of the conformational change. However, the hirudin thrombin and COOH-terminal peptide thrombin CD changes may correspond to different thrombin structural transitions. Studies of the kinetics of thrombin inhibition by hirudin (*19*) further implicate the tail: the rate of the first step in the formation of the complex is decreased by increased ionic strength which is consistent with the COOH-terminal tail inducing the conformational change that then allows access of the active site to the NH_2-terminus of hirudin. The initial step of the process may center about the binding of the bulky COOH-terminal 3_{10} helix in the exosite.

Although h-Ile[1] and h-Tyr[3] occupy approximately the same positions as Pro and D-Phe of PPACK, the chain direction of PPACK is antiparallel with Ser[214] to Glu[217] whereas that of h-Ile[1] to Tyr[3] runs parallel to it. Moreover, the Arg of PPACK occupies the specificity pocket fully in a substrate-like manner but h-Thr[2] of hirudin does not. All serine proteinase-inhibitor complexes show antiparallel alignment of a rigid loop segment in substrate-like conformation and contain a residue corresponding to the specificity of the enzyme at the P1 site (*11, 20*). These generalities, coupled with hirudin displaying no close sequence or topological homology to the ten existing classes of

serine proteinase inhibitors, suggest that hirudin is a representative of a new family of inhibitors (*21*).

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Calcium-Induced Peptide Association to Form an Intact Protein Domain: ^{1}H NMR Structural Evidence

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The 70-residue carboxyl-terminal domain of the muscle contractile protein troponin-C contains two helix-loop-helix calcium (Ca)-binding sites that are related to each other by approximate twofold rotational symmetry. Hydrophobic residues from the helices and a short three residue β sheet at the interface of the two sites act to stabilize the protein domain in the presence of Ca. A synthetic 34-residue peptide representing one of these sites (site III) has been synthesized and studied by H-1 nuclear magnetic resonance (NMR) spectroscopy. In solution this peptide undergoes a Ca-induced conformational change to form the helix-loop-helix Ca-binding motif. Two-dimensional nuclear Overhauser effect spectra have provided evidence for the formation of a β sheet and interactions between several hydrophobic residues from opposing helices as found in troponin-C. It is proposed that a symmetric two-site dimer similar in tertiary structure to the carboxyl-terminal domain of troponin-C forms from the assembly of two site III peptides in the Ca-bound form.

A FUNDAMENTAL ASPECT OF SUCCESSFUL protein design is an understanding of the processes that stabilize the self-assembly of proteins. Although the correct folding of a protein is ultimately determined by its amino acid sequence, the most important energy contributions that determine protein assembly are derived from hydrophobic interactions, hydrogen bonding, and electrostatic forces (*1*). In many cases, specific information about these interactions can be obtained from the three-dimensional (3-D) structure of a protein as determined by 2-D NMR spectroscopy or x-ray crystallography.

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One approach to study the effects that hydrophobic and electrostatic interactions have on protein structure is to use synthetic peptides to represent specific portions of a protein (*2*). Peptides are much smaller than proteins and generally exist in random coil conformations. However, under suitable conditions peptides may adopt well-defined conformations more representative of their structures found in the native protein (*3*). We used this approach to study the effects that metal-ion binding has on the conformational changes that occur in the Ca-binding protein troponin-C (TnC). This muscle protein contains four highly homologous Ca-binding sites each arranged in a helix-loop-helix motif about 30 amino acids in length (*4*). These sites are organized pairwise to