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Transmembrane Helical Interactions and the Assembly of the T Cell Receptor Complex

NICHOLAS MANOLIOS, JUAN S. BONIFACINO, RICHARD D. KLAUSNER

Studies of the subunit interactions of the multicomponent T cell antigen receptor (TCR) revealed that specific pairs of chains have the ability to assemble after transfection into fibroblasts. For one such pair, TCR-α and CD3-δ, their ability to assemble was encoded by their transmembrane domains. The specificity of this interaction suggests that well-defined helical interactions in the membrane can explain the assembly of some multichain membrane complexes.

MANY MEMBRANE PROTEINS EXIST as part of multicomponent complexes (*1*). These range in complexity from the homodimeric transferrin receptor to the T cell antigen receptor (TCR), which is comprised of at least seven transmembrane subunits encoded by six different genes (*2*). These complexes may be held together by either covalent (disulfide) bonds or noncovalent interactions. Correct assembly is most likely required for function. To prevent the cell surface expression of unassembled subunits or partially assembled complexes, the cell imposes an effective set of mechanisms that prevent the transport or survival or both of all but completely assembled complexes (*3*). Transmembrane proteins can conveniently be divided into three domains; extracellular, membrane-spanning, and intracellular. For proteins that cross the membrane once, these domains are arranged as uninterrupted segments along the primary sequence.

This is the situation for the seven chains of the TCR, which are all single membrane-spanning proteins with their amino termini facing the external milieu (*2*). The external domains of the TCR-α and TCR-β subunits are responsible for antigen recognition (*4*) whereas the cytoplasmic domain of ζ has been directly implicated in signal transduction (*5*). The hydrophobic transmembrane domains of the TCR chains and other membrane proteins anchor them in the membrane and negotiate the hydrophobic lipid bilayer. The transmembrane domains of each of the TCR subunits have the unusual characteristic of possessing single (or in the case of TCR-α, two) charged amino acids (*2*). Mutation of the positively charged amino acid residues of TCR-α and TCR-β transmembrane domains failed to allow reconstitution of surface expression of the TCR in α- or β-deficient T cells (*6*). It has been proposed that these charged amino acids may be involved in subunit assembly. These observations along with the high degree of sequence conservation of these domains between mouse and human suggest more specific structural information resides in the membrane-spanning regions than merely the ability to sit in a lipid environment. That the transmembrane domain of TCR-α contains specific sequence and structural information is supported by the recent identification of this region as containing information that causes targeting for rapid degradation in the endoplasmic reticulum (*7*). In this study we demonstrate that the transmembrane domains of the TCR-α and

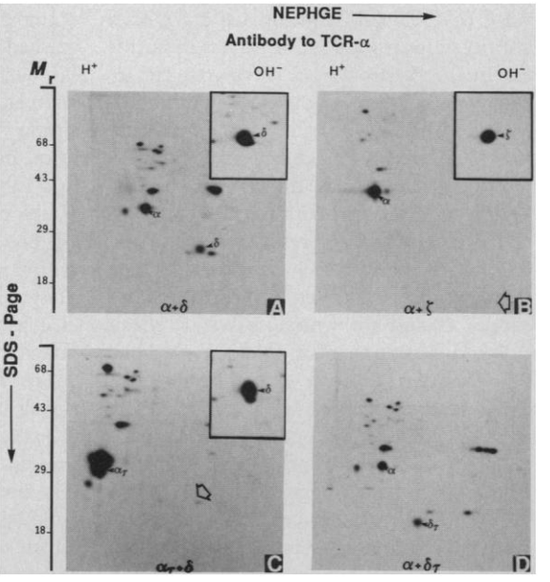

Fig. 1. Assembly of TCR-α (α) with CD3-δ (δ). COS-1 cells were cotransfected (*12*) with expression plasmids encoding: (**A**) full-length TCR-α and CD3-δ; (**B**) full-length TCR-α and TCR-ζ (ζ); (**C**) truncated TCR-α (α_t) lacking 41 carboxyl-terminal amino acid residues and full-length CD3-δ; and (**D**) full-length TCR-α with a truncated CD3-δ lacking 41 carboxyl-terminal amino acid residues (δ_t) (*13*). At 48 hours after transfection, cells were treated for 16 hours with 5 mM sodium butyrate and metabolically labeled for 2 hours at 37°C (*14*). Cells were removed from the plates, solubilized with lysis buffer containing 0.5% Triton X-100, and supernatants were added to antibody beads (*15*). The immunoprecipitated TCR chains were resolved by 2-D nonequilibrium pH gel electrophoresis (NEPHGE)/SDS-PAGE, as previously described (*2, 16*). Panels (A) through (D) show immunoprecipitates with antibody to TCR-α. Insets in (A) and (C) show the amount of total CD3-δ expressed in the same cells as analyzed by direct immunoprecipitation with antibodies to CD3-δ. The inset in (B) shows total TCR-ζ immunoprecipitated with antibodies to TCR-ζ. The positions of molecular weight (M_r) markers (kilodaltons) are indicated on the left. The positions of TCR-α, CD3-δ, and TCR-ζ are indicated. In (B), the expected position of TCR-ζ is marked by the open arrow. In (C), the expected position of CD3-δ is shown by the open arrow. The spot just below the arrowhead in (C) is not TCR-δ, but a nonspecific band. A truncated TCR-α lacking the transmembrane and cytoplasmic domains (TCR-α_t) failed to assemble with CD3-δ (C). However, a truncated CD3-δ lacking the cytoplasmic domain assembled normally with full-length TCR-α (D). Truncated CD3-δ could not be directly assessed by immunoprecipitation.

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892.

CD3-δ subunits contain all of the information necessary for subunit assembly.

The ability of individual pairs of TCR subunits to assemble with each other was tested by transfecting COS cells with complementary DNAs (cDNAs) encoding each subunit. In this report we focus on the interaction of TCR-α with one of the invariant chains, CD3-δ. When TCR-α plus CD3-δ cDNAs are cotransfected into COS cells, the association of these chains can be demonstrated by immunoprecipitation of CD3-δ with a clonotypic monoclonal antibody that recognizes the TCR-α chain (Fig. 1A). Likewise, TCR-α can be precipitated with a specific antibody against the cotransfected CD3-δ subunits. The absolute specificity of the antibodies was established by the failure to precipitate any TCR subunits when the target chains were not included in the transfection. In contrast, cotransfection of TCR-α with either TCR-ζ (Fig. 1B) or CD3-γ resulted in no association, despite high levels of expression of all of these chains. Antibodies to TCR-α could coprecipitate CD3-γ and TCR-ζ when the cDNAs encoding all six TCR genes were cotransfected. Thus, failure to assemble was not due to the failure to coexpress chains in the same cells in these transient transfections.

We localized these interactions to specific domains of the subunits by examining the assembly of truncated TCR-α (α_t) chain that lacked the transmembrane and cytoplasmic domains with CD3-δ. This lumenal protein was not aggregated and could assemble with full-length TCR-β, but no interaction with CD3-δ could be detected (Fig. 1C). In contrast, when the CD3-δ cDNA was engineered to produce a protein whose cytoplasmic tail was removed (CD3-δ_t), it assembled well with full-length TCR-α (Fig. 1D). These results suggested that the cytoplasmic tail of CD3-δ was not necessary for its interaction with TCR-α and that the extracellular domain of TCR-α did not contain sufficient information to mediate the assembly with CD3-δ. One possible interpretation of this was that the transmembrane domain or short cytoplasmic tail or both of TCR-α were required for this assembly. To test this possibility chimeric proteins were constructed in which the extracellular domains of CD4 or the interleukin-2 receptor α chain (Tac antigen) were fused to the putative transmembrane domain and five–amino acid cytoplasmic tail of the TCR-α chain. When full-length, normal CD4 or Tac were cotransfected with CD3-δ, little if any CD4 or Tac could be precipitated with antibodies to CD3-δ, nor could antibodies against either CD4 or Tac coprecipitate CD3-δ (Figs. 2 and 3). In contrast, when the chimeric proteins containing the transmembrane and cytoplasmic domains of α were used, significant assembly was apparent (Figs. 2 and 3). Coprecipitation of CD3-δ with the chimeric proteins was readily detected with antibodies to CD3-δ or antibodies directed against either CD4 or Tac, as appropriate.

To further define the specificity of this interaction, a series of chimeric proteins were constructed that included variable amounts of the transmembrane region or cytoplasmic tail or both of TCR-α placed in the context of the Tac protein (Fig. 4). Assembly of most of the chimeric proteins was analyzed with an immunoblotting protocol that affords a more rapid and quantitative assay than the two-dimensional (2-D) gels used to initially demonstrate interactions. Cells were transiently transfected with these chimeras along with the cDNA encoding full-length CD3-δ. Cell lysates were subjected to immunoprecipitation with either antibodies to CD3-δ or Tac covalently bound to Sepharose, and the precipitates were immunoblotted with antibody to CD3-δ after resolution on 1-D SDS gels (Fig. 4). The chimeric protein containing the transmembrane and cytoplasmic domains of TCR-α (Tac–TCR-α_1) assembled well with CD3-δ (30% of the total CD3-δ could be precipitated with antibody to Tac). Deletion of the five carboxyl-terminal residues of TCR-α and replacement with the cytoplasmic sequences of Tac (Tac–TCR-α_2) had little effect on the level of association. When the five amino acids at the amino-terminal portion of the TCR-α transmembrane domain were replaced with the corresponding sequence of Tac (Tac–TCR-α_3), assembly was observed, although it occurred to a lesser extent. In this switch only three of the five TCR-α amino acids are altered. However, when the next eight residues of the transmembrane domain of TCR-α, including the two transmembrane-charged amino acid residues were replaced, essentially all assembly was lost (Tac–TCR-α_4, Fig. 4). The cytoplasmic tail of TCR-α (Tac–TCR-α_5) was insufficient to transfer the ability to assemble with CD3-δ (Fig. 4). Differences in the level of assembly could not be explained as a consequence of varying levels of Tac expression as assessed by direct precipitation and quantitative immunoblotting of the Tac protein. To further localize the region responsible for assembly, a construct (see dotted box in Fig. 4), containing only eight amino acids of the TCR-α transmembrane domain (L-R-I-L-L-L-K-V) (*8*), was tested (Tac–TCR-α_6). As shown in Fig. 3, E and F, this limited region was still capable of mediating assembly with CD3-δ. As previously suggested (*2*), the positive charges in TCR-α and negative charges in the transmembrane domains of TCR invari-

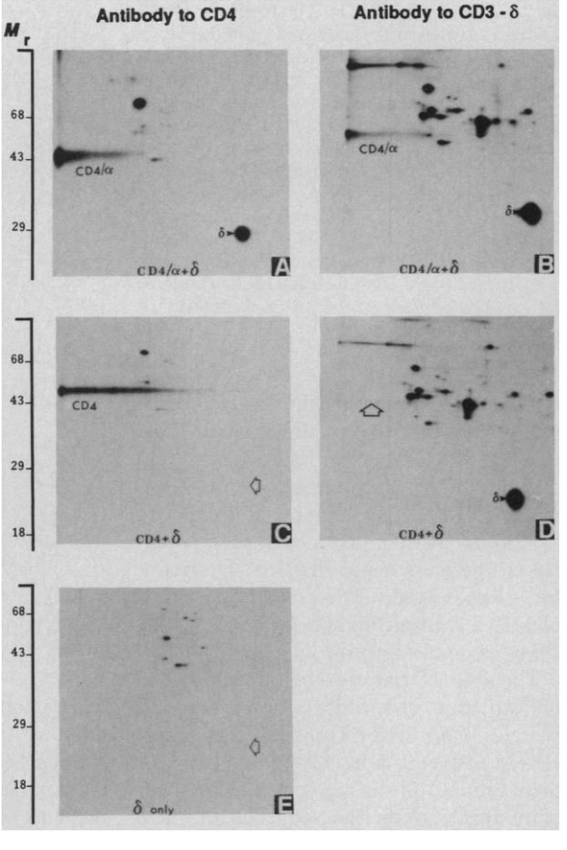

Fig. 2. Assembly of CD3-δ with a chimeric CD4 containing substituted TCR-α transmembrane and cytoplasmic regions (CD4/α) (*17*). COS cells were transfected with CD4–TCR-α plus CD3-δ (**A** and **B**), full-length CD4 plus CD3-δ (**C** and **D**) or CD3-δ alone (**E**). Lysates were subjected to immunoprecipitation with either antibody to CD4 (OKT4a, Ortho Pharmaceutical) (A, C, and E) or antibody to CD3-δ (B and D). Immunoprecipitates were analyzed by 2-D NEPHGE/SDS-PAGE. The positions of M_r markers (kilodaltons) are indicated on the left. CD4 containing TCR-α transmembrane domain assembled with CD3-δ (A and B). In contrast, normal full-length CD4 did not assemble with CD3-δ (C and D). Open arrows point to the expected location of CD3-δ (C and E) or CD4 (D).

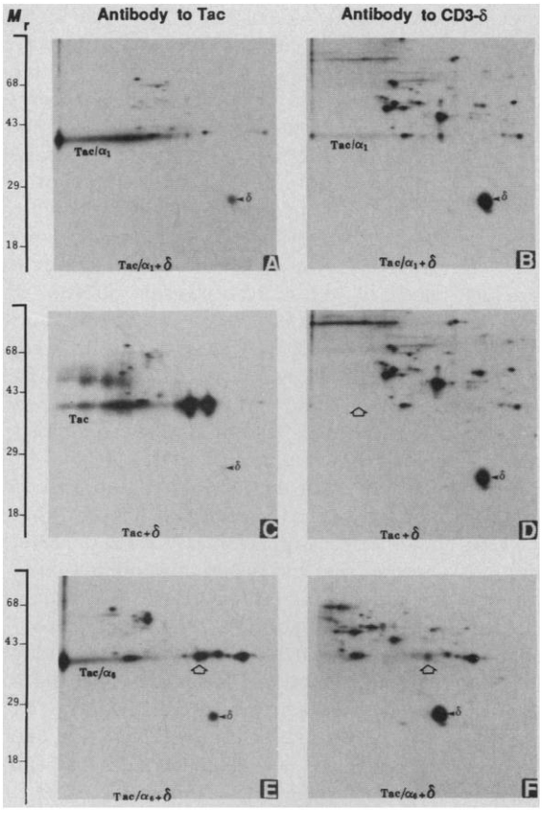

Fig. 3. Interaction between CD3-δ with a chimeric Tac containing TCR-α transmembrane and cytoplasmic amino acid sequences (Tac/α_1) (*7*). The antibody used to immunoprecipitate Tac was 7G7 (*18*). The positions of M_r markers (kilodaltons) are indicated on the left. When the Tac transmembrane region was replaced with TCR-α transmembrane and cytoplasmic amino acid sequence, antibody to Tac (**A**) and antibody to CD3-δ (**B**) were able to coprecipitate CD3-δ and Tac, respectively. In contrast, CD3-δ cotransfected with full-length Tac could not be coprecipitated by antibody to Tac (**C**) nor could full-length Tac be coprecipitated by antibody to CD3-δ (**D**). Chimeric construct Tac–TCR-α_6 (Tac/α_6), containing only eight amino acids of the TCR-α transmembrane domain (shown as dotted box in Fig. 4) assembled with CD3-δ (**E**). This was evidenced by coprecipitation of CD3-δ by antibody to Tac (E). Similarly, Tac–TCR-α_6 was coprecipitated by antibody to CD3-δ (**F**). The open arrow in (F) shows the focused Tac–TCR-α_6 protein. Note that the Tac proteins in general were poorly focused in this gel system.

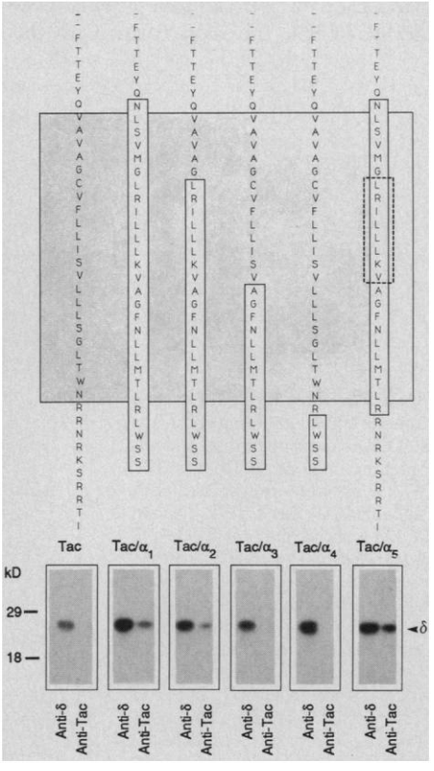

Fig. 4. Localizing the segment of the TCR-α transmembrane domain responsible for CD3-δ assembly. In the schematic representation of the sequence of either Tac or Tac/α chimerae around the transmembrane region (*19*) (**top**), the boxed sequences represent those derived from the TCR-α chain. Each of these chimerae were cotransfected into COS cells along with the cDNA encoding CD3-δ. Lysates were subject to immune precipitation with either antibodies to CD3-δ or Tac (**bottom**). Those immune precipitates were resolved by SDS-PAGE and analyzed by immunoblotting with an antibody to CD3-δ (*20*) antipeptide antibody. The autoradiograms demonstrate the total amount of CD3-δ (left lane) and the amount of CD3-δ precipitated with the antibody to Tac (right lane) for each of the chimerae shown. The position of the M_r markers (kilodaltons) are indicated on the left.

ant chains are likely to be involved in assembly. When the two transmembrane basic residues of TCR-α were replaced with leucine by site-directed mutagenesis, no binding to CD3-δ was observed. It should be mentioned that the presence of such potential charge pairs is not sufficient for assembly, since ζ and CD3-γ, both of which possess a transmembrane negative charge, fail to assemble with the TCR-α chain.

The role of transmembrane domains in the structure and function of membrane proteins is not well established, and mainly viewed as serving as hydrophobic anchors of these proteins in the membrane. The possibility that the specific sequence of these hydrophobic domains is of functional significance, however, has been raised. The striking presence of conserved charged amino acids in the predicted transmembrane domains of all of the subunits of the TCR complex has led to the suggestion that these residues may be involved in subunit interactions. Indeed, mutation of the transmembrane positively charged residues of the α and β chains of TCR to a variety of other amino acids abrogated the ability of transfected chains to reconstitute surface expression of α- or β-negative T cells (*6*). One recent example where protein-protein interaction has been similarly pinpointed to a transmembrane domain is in the dimerization of glycophorin A (*9*). In this study peptides corresponding to the transmembrane region of this protein specifically bound to glycophorin A in detergent micelles and artificial bilayers. Peptides corresponding to the transmembrane domains of two other proteins were inactive. Another recent study has suggested that specific sequences of the transmembrane domain of membrane-bound immunoglobulin M (IgM) are required for retention of this protein in the endoplasmic reticulum of plasma cells (*10*). Sequence-specific interactions between transmembrane domains have multiple implications for the biology and study of membrane proteins. There is good experimental and theoretical evidence that these domains are α helical (*11*). Thus our ability to understand the molecular basis of these interactions will be greatly aided by this structural constraint. The localization of assembly between membrane proteins to this limited region will allow us to describe, in detail, the basis of specific assembly interactions. At these sites of interaction become even more refined, the possibility of designing peptides that could specifically compete for assembly or induce disassembly may provide powerful tools for probing and perturbing membrane proteins.

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13. Full-length 2B4-α [D. M. Becker *et al.*, *Nature* **317**, 430 (1985)] and mouse CD3-δ [P. van den Elsen, B. A. Shepley, M. Cho, C. Terhorst, *ibid.* **314**, 542 (1987)] cDNAs were cloned into the expression vectors pCDM8 [B. Seed, *ibid.* **329**, 840 (1987)] and pcD (Pharmacia), respectively. A truncated TCR-α was generated by insertion of a translation stop codon after the codon for Thr^{207} of the mature protein, as previously described (7). The cytoplasmic domain of the CD3-δ chain was deleted by insertion of a stop codon after the codon for Arg^{111} of the mature protein with the polymerase chain reaction. Truncated TCR-α and truncated CD3-δ were cloned into the expression vectors pCDM8 and pCDL-SRα [Y. Takabe *et al.*, *Mol. Cell Biol.* **8**, 466 (1988)], respectively.
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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17. In the CD4–TCR-α chimeric protein, the extracellular domain of human CD4 (Glu^1 to Met^{376}) was fused to the transmembrane and cytoplasmic domains of mouse TCR-α (Asn^{221} to Ser^{248}). To construct a cDNA encoding the CD4–TCR-α chimeric protein, pGEM 4 containing a full-length human CD4 cDNA [P. J. Maddon *et al.*, *Cell* **42**, 93 (1985)] cloned into the Eco RI and Bam HI sites of the polylinker was cut with Ava I and Bam HI. Phosphorylated synthetic oligonucleotides encoding part of the CD4 extracellular domain and the transmembrane and cytoplasmic domains of the TCR-α were then ligated. The CD4-TCR–α and a full-length human CD4 cDNAs were excised from pGEM 4 and cloned into pCDM8.
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19. Construction of Tac–TCR-α_1 and Tac–TCR-α_2 has been described (7). The other chimeric proteins shown in Fig. 4 (Tac–TCR-α_3, Tac–TCR-α_4, Tac–TCR-α_5, and Tac–TCR-α_6; or Tac/α_3, Tac/α_4, Tac/α_5, Tac/α_6) were constructed by cutting a pUC 18-Tac plasmid [W. J. Leonard *et al.*, *Nature* **312**, 626 (1984)] with Nae I and Xba I, which deleted sequences corresponding to the transmembrane and cytoplasmic domains of the Tac protein. Phosphorylated synthetic oligonucleotides encoding the sequences shown in Fig. 4 were ligated. The hybrid genes were cut from pUC18 and cloned into pCDM8.
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).
21. We thank C. Suzuki, T. Rutledge, T. Wileman, C. Terhorst, D. Nelson, W. Leonard, E. Berger, and D. Littman for reagents used in this study; J. Harford for critical review of this manuscript; and E. Perry and T. Koomson for secretarial assistance. N.M. is supported by the National Health and Medical Research Council (Australia) Neil Hamilton Fairley Fellowship.

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The Structure of a Complex of Recombinant Hirudin and Human α-Thrombin

TIMOTHY J. RYDEL, K. G. RAVICHANDRAN, A. TULINSKY,* WOLFRAM BODE, ROBERT HUBER,* CAROLYN ROITSCH, JOHN W. FENTON II

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

T. J. Rydel, K. G. Ravichandran, A. Tulinsky, Department of Chemistry, Michigan State University, East Lansing, MI 48824.
W. Bode and R. Huber, Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany.
C. Roitsch, Transgene S.A., 11 rue de Molsheim, 67082 Strasbourg Cedex, France.
J. W. Fenton II, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201, and Departments of Physiology and Biochemistry, The Albany Medical College of Union University, Albany, NY 12208.

*To whom correspondence should be addressed.