The Single-stranded DNA Aptamer-binding Site of Human Thrombin*

Lisa R. Paborsky†, Sarah N. McCurdy, Linda C. Griffin, John J. Toole, and Lawrence L. K. Leung
From Gilead Sciences, Inc., Foster City, California 94404

A new class of thrombin inhibitors based on sequence-specific single-stranded DNA oligonucleotides (thrombin aptamer) has recently been identified. The aptamer-binding site on thrombin was examined by a solid-phase plate binding assay and by chemical modification. Binding assay results demonstrated that the thrombin aptamer bound specifically to α-thrombin but not to γ-thrombin and that hirudin competed with aptamer binding, suggesting that thrombin's anion-binding exosite was important for aptamer-thrombin interactions. To identify lysine residues of thrombin that participated in the binding of the thrombin aptamer, thrombin was modified with fluorescein 5'-isothiocyanate in the presence or absence of the thrombin aptamer, reduced, carboxymethylated, and digested with endoproteinase Arg-C. The digestion products were analyzed by reversed-phase high performance liquid chromatography and the peptide maps compared. Four peptides with significantly decreased modification in the presence of the aptamer were identified and subjected to N-terminal sequence analysis. Results indicated that B chain Lys-21 and Lys-65, both located in close proximity to the aptamer-binding site of human α-thrombin. The thrombin aptamer binds to the anion-binding exosite and inhibits thrombin's function by competing with exosite binding substrates fibrinogen and the platelet thrombin receptor.

Thrombin is a serine protease responsible for the conversion of fibrinogen to fibrin, platelet activation, and the cleavage of coagulation factors V, VIII, XI, and XIII (Fenton, 1981; Shuman, 1986; Furie and Furie, 1988; Mann et al., 1990; Gailani and Broze, 1991). In addition to its role in blood coagulation, thrombin can act as a potent mitogen (Chen and Buchanan, 1975; Carney et al., 1985) and can also exert a chemotactic effect on monocytes (Bar Shavit et al., 1983). Because of its pivotal role in both thrombosis and hemostasis, thrombin is a major target for anticoagulation and cardiovascular disease therapy. Using a novel in vitro selection/amplification technique, a new class of thrombin inhibitors based on single-stranded DNA (ssDNA)1 oligonucleotides has been recently identified (Bock et al., 1992). These thrombin inhibitors are the first example of the use of this technique to obtain ssDNA oligonucleotides that bind a target protein with no known specificity for nucleic acids. One oligonucleotide GGTTGGTGTTGGTTGG (thrombin aptamer) was capable of nanomolar inhibition of fibrinogen cleavage in vitro (Bock et al., 1992) and was shown to inhibit clot-bound thrombin and reduce arterial thrombus formation in an ex vivo whole artery angioplasty model.2 Recent in vivo studies in cynomolgus monkeys have shown the thrombin aptamer to be a potent antithrombotic agent with a rapid onset of action and a short half-life (Griffin et al., 1993). The three-dimensional structure of thrombin has been solved by x-ray crystallography (Bode et al., 1989), and, recently, the tertiary structure of the thrombin aptamer has been elucidated by NMR spectroscopy (Macaya et al., 1993; Wang et al., 1993). Identification of the aptamer binding site on thrombin will help define the structural basis of this novel ssDNA-protein interaction and guide further development of a thrombin aptamer with an improved therapeutic profile.

Unlike hirudin and other active site thrombin inhibitors, the thrombin aptamer does not inhibit the cleavage of small chromogenic amidase substrates3 (Wu et al., 1992), indicating that the aptamer does not bind directly to the active site of thrombin. However, distinct from the catalytic center of thrombin are two highly basic regions that form secondary interactions with several macromolecular substrates and receptors. One site, the anion-binding exosite, contributes to the formation of a tight specific complex with fibrinogen (Fenton et al., 1988; Church et al., 1989), thrombomodulin (Wu et al., 1991), hirudin (Chang, 1989; Grutter et al., 1990; Rydel et al., 1990), the platelet thrombin receptor (Vu et al., 1991), and heparin cofactor II (Sheehan et al., 1991; Rogers et al., 1992). The second site, the putative heparin recognition site, contributes to the significant increase of thrombin inactivation by antithrombin III in the presence of heparin (Chang et al., 1979; Messmore et al., 1979). Considering the polyanionic nature of the nuclear acid phosphodiester backbone, these basic sites on thrombin, shown to interact with acidic regions of other thrombin-binding molecules, are likely targets for thrombin aptamer binding.

In this paper the area of thrombin involved in aptamer binding was investigated by both a solid-phase binding assay and by chemical modification of the thrombin-aptamer complex. The thrombin aptamer was shown to bind within the anion-binding exosite, and two of the lysine residues1 that participate in aptamer binding were identified.

EXPERIMENTAL PROCEDURES

Materials—Human α-thrombin and human γ-thrombin were from Haematologic Technologies (Essex Junction, VT). Deoxyoligonucleo-

---

1. The abbreviations used are: ss, single-stranded; FITC, fluorescein 5'-isothiocyanate; HPLC, high performance liquid chromatography.

2. W. X. Li and L. L. K. Leung, personal communication.

3. Louis Bock, personal communication.

4. Amino acid residues are numbered sequentially beginning with the first residue of the human α-thrombin B chain.
Aptamer-binding Site of Thrombin

20809

tides, GGTGCGTGTGCGTTGG (thrombin aptamer) and GGTGCGTGCGTGTGCGTTGG (15-mer scramble sequence) were prepared by solid-phase phosphoramidite chemistry on a Biosearch 8800 synthesizer using standard methods and assayed as described previously (Bock et al., 1992). Biotinylated oligonucleotides were prepared by coupling biotin-X-X- NHS ester (Glen Research) to the 5' 'amine of both the 5' modified (hydrol oxamine) thrombin aptamer and the 15-mer scramble sequence. Avidin DN and anti-avidin alkaline phosphatase were from Vector Laboratories. Hirudin was from American Diagnostica, Inc. Fluorescein 5'-isothiocyanate (isomer 1) (FITC) was from Molecular Probes, Endoproteinase Arg-C was from Boehringer Mannheim, NICK (Sephadex G-50) and NAP-5 (Sephadex G-25) disposable gel filtration columns were from Pharmacia LKB Biotechnology Inc.

Thrombin Aptamer Binding Assay—A 96-well enzyme-linked immunosorbent assay plate (Corning) was coated overnight at 4 °C with 250 nM thrombin, human albumin, or chymotrypsin in selection buffer (Bock et al., 1992). The excess sites on the plates were blocked with 1% bovine serum albumin, 0.05% Tween 20 in selection buffer. Biotinylated thrombin aptamer or the biotinylated 15-mer scramble sequence were added at increasing concentrations and incubated for 2 h at room temperature. The plate was washed with 0.1% bovine serum albumin, 0.05% Tween 20 in selection buffer. Binding was detected by incubating the plates with Avidin DN (250 µg/ml) for 1 h at room temperature. Avidin DN and anti-avidin alkaline phosphatase (1:250) for 1 h at 4 °C. The plate was washed in between each incubation step. The substrate, p-nitrophenyl phosphate, was added and incubated at 37 °C for an additional hour. The absorbance at 405 nm was measured using a Molecular Devices plate reader.

Results and Discussion

Competition of Thrombin Aptamer Binding to Immobilized α-Thrombin by γ-Thrombin and Hirudin—To characterize the aptamer-thrombin interaction, a solid-phase plate binding assay was developed using the biotinylated thrombin aptamer and a biotinylated 15-mer scramble sequence. To assess the effect of biotinylation on thrombin inhibitory activity, these oligonucleotides were assayed for their anticoagulant activity in a plasma thrombin time clotting assay. The biotinylated thrombin aptamer was only slightly less active than the unmodified thrombin aptamer, requiring approximately 1.7-fold higher concentration to double the thrombin clotting time. The biotinylated 15-mer scramble sequence, like the unmodified 15-mer scramble sequence, was completely inactive (data not shown). Increasing amounts of the biotinylated thrombin aptamer or the control biotinylated 15-mer scramble sequence were added to immobilized α-thrombin (Fig. 1A). The thrombin aptamer bound α-thrombin in a dose-dependent manner, whereas the 15-mer scramble sequence did not bind. The thrombin aptamer was a potent and specific thrombin anion-binding exosite inhibitor, as it was used for its ability to compete with immobilized α-thrombin for aptamer binding. In addition, hirudin, a potent and specific thrombin anion-binding exosite inhibitor, was tested for its ability to compete with the aptamer for thrombin binding. The results are displayed in Fig. 1B. The unmodified thrombin aptamer competed with the biotinylated thrombin aptamer for thrombin binding, whereas no competition was observed with the unmodified 15-mer scramble sequence. α-Thrombin effectively competed with the immobilized α-thrombin with maximum inhibition observed at 500 nM. However, even at a 2000-fold excess concentration, γ-thrombin, which contains a cleavage within the anion-binding exosite as well as two additional cleavages to produce four noncovalently associated fragments, did not compete with immobilized α-thrombin for aptamer binding. Therefore, the regions that differ between α-thrombin and γ-thrombin may participate in thrombin aptamer binding. Furthermore, hirudin, which is known to bind in the anion-binding exosite, effectively competed with the aptamer for thrombin binding. Taken together, these data suggest that the anion-binding exosite is important for aptamer-thrombin interactions.

Identification of Thrombin Lysine Residues Involved in Aptamer-Thrombin Complex by FITC Derivatization and Microsequencing—To identify the essential lysyl residues in human α-thrombin that participate in thrombin aptamer binding, both the aptamer-thrombin complex and thrombin alone were derivatized with FITC, a reagent for ε-amino group modification. α-Thrombin was incubated in the presence or absence of an excess of the thrombin aptamer. The extent of modification was 3.9 mol of FITC/mol of thrombin in the absence of aptamer. In the presence of the aptamer, the derivatization was decreased approximately 30% to yield 2.7 mol of FITC/mol of thrombin.

Following the FITC modification, the two samples were reduced, carboxymethylated, and digested with endopeptidase Arg-C. The digestion products were analyzed by reversed-phase HPLC with detection at 440 nm and identified the lysine residues in thrombin that were protected by the aptamer from FITC modification (Fig. 2). In the absence of the aptamer, approximately 20 FITC-labeled peptides were generated (Fig. 2A). As a result of aptamer binding, the recovery subjected to N-terminal sequence analysis using a Hewlett Packard G101A system.
of unbiotinylated thrombin aptamer well enzyme-linked immunosorbent assay plates and then incubated at 250 nM was coated onto 96-well enzyme-linked immunosorbent assay plates and then incubated with increasing concentrations of either the biotinylated thrombin aptamer (solid lines) or the biotinylated 15-mer scramble sequence (dashed lines). Binding was detected as described under “Experimental Procedures.” Each data point represents an average of duplicate experiments. This incubation mix was then added to the coated plate and incubated for an additional hour at room temperature. Binding was detected as described under “Experimental Procedures.”

FIG. 2. Endoproteinase Arg-C peptide mapping of α-thrombin. Thrombin and the thrombin-aptamer complex were derivatized with FITC and digested with endoproteinase Arg-C as described under “Experimental Procedures.” The digestion products were separated on a Vydac C18 (250 × 4.6 mm) column at 40 °C equilibrated in solvent A (0.1% trifluoroacetic acid in 5% acetonitrile). A linear gradient from 7 to 65% solvent B (0.08% trifluoroacetic acid in 90% acetonitrile) over 80 min was developed by a Hewlett Packard Model 1090 HPLC. The flow rate was 1 ml/min. FITC-labeled peptides were monitored at 440 nm at a sensitivity setting of 100 milliabsorbance units at full scale. A, thrombin only; B, thrombin-aptamer complex.

TABLE I

| Peak | Peptide sequence |
|------|----------------|
| P1   | Ile(23)-Gly(18)-Lys(0)-His(8)-Ser(2)-Arg(9) |
| P2   | Ile(31)-Gly(25)-Lys(0)-His(30)-Ser(1)-Arg(ND) |
| P3   | Lys(5)-Ser(16)-Pro(44)-Pro(22)-Leu(29)-Leu(33)-Cys(NQ)-Gly(36)-Ala(29)-Ser(6)-Leu(18)-Ile(11)-Ser(5)-Asp(15)-Arg(15) |
| P4   | Lys(0)-Ser(18)-Pro(45)-Pro(45)-Leu(29)-Leu(59)-Cys(NQ)-Gly(27)-Ala(23)-Ser(4)-Leu(12)-Ile(8)-Ser(4)-Asp(8)-Arg(10) |

* The underlined residues represent fluoresceinated lysyl residues. Quantitative yields of amino acid residues in pmol are given in parentheses following the identified residue. NQ, not quantitated; ND, not detected.

* This peak also included a coeluting peptide, 113-123, which contains no lysyl residues.

peptides. The identification of Lys-21 as the second protected residue was confirmed by the sequencing results obtained with peak 4, which contained no contaminating peptides and no detectable Lys at the N-terminal position of Arg-C peptide 21–36.

The major peptides present in peaks P1 and P2 have identical amino acid sequences and compositions but different retention times, as do the major peptide components of peaks P3 and P4. This may be due to a rearrangement of a portion of the fluorescein group, which alters the hydrophobic properties of the peptides and, hence, their retention times. This phenomenon has been observed for several other FITC-labeled peptides (Mitchinson et al., 1982; Farley et al., 1984; Phillips, 1988).

A basic premise of these experiments is that in the aptamer-thrombin complex, amino acids in the thrombin binding site are shielded and become relatively inaccessible to FITC modification. These results indicate that both Lys-21 and Lys-65, which are in the anion-binding exosite of α-thrombin, are located either within or in close proximity to the thrombin aptamer binding site. The possibility exists that dsDNA binding induces conformation changes that interfere with the chemical modification of amino acid residues that are not in the combining site. However, this is unlikely, since the lack of competition by γ-thrombin and the effective competition of four peaks was drastically reduced (Fig. 2B). These peaks, P1–P4, were selected for further analysis, because they represented significant absorbance at 214 nm and consistently showed a greater than 50% decrease in absorbance at 440 nm. The four peaks were collected and subjected to N-terminal sequence analysis (Table I). Peaks P1 and P2 both contain peptides with the identical sequence, Ile-Gly-(Lys)-His-Ser-Arg, which corresponds to Arg-C peptide 63–68. FITC-labeled lysine is not detected in the sequencer; therefore, the lack of lysine at the third position in these peptides demonstrated that Lys-65 was modified in the absence of the aptamer and protected by the aptamer from FITC derivatization. Peaks P3 and P4 were both found to contain the Arg-C peptide 21–36 (Table I). The extremely low recovery of Lys at the N-terminal position of this peptide indicated that Lys-21 was partially protected by the aptamer, but interpretation of these results was complicated by the presence of unrelated coeluting proteins.

![Aptamer-binding Site of Thrombin](image)

**Fig. 1.** Thrombin aptamer binding. A, purified α-thrombin (○), albumin (■), or chymotrypsin (▲) at 250 nM was coated onto 96-well enzyme-linked immunosorbent assay plates and then incubated with increasing concentrations of either the biotinylated thrombin aptamer (solid lines) or the biotinylated 15-mer scramble sequence (dashed lines). Binding was detected as described under “Experimental Procedures.” Each data point represents an average of duplicate wells. The absorbance value with no DNA added was subtracted from each data point. B, purified α-thrombin at 250 nM was coated onto the bottom of a 96-well enzyme-linked immunosorbent assay plate. Five hundred nanomolar biotinylated thrombin aptamer was preincubated for 1.5 h at room temperature with increasing concentrations of unbiotinylated thrombin aptamer (○), unbiotinylated 15-mer scramble sequence (▲), α-thrombin (○), hirudin (■) or γ-thrombin (□). This incubation mix was then added to the coated plate and incubated for an additional hour at room temperature. Binding was detected as described under “Experimental Procedures.”
of aptamer binding to immobilized thrombin by hirudin indicated that the anion-binding exosite is important for aptamer binding (Fig. 1).

Lys-21 and Lys-65 also participate in fibrinogen-thrombin interactions (Church et al., 1989). These residues, along with four other lysyl residues of α-thrombin, were shown to be protected from chemical modification following complex formation with hirudin (Chang, 1989). Recently, a thrombin mutant that contained an Arg to Glu substitution at position 70 (Arg-70 → Glu) in the anion-binding exosite was shown to no longer bind the thrombin aptamer (Wu et al., 1992). Using a complementary approach, our results extend this observation that the thrombin aptamer binds to the anion-binding exosite to include that Lys-21 and Lys-65 play a role in that interaction. Finally, Griffin et al. (1993) and others (Wu et al., 1992) have observed that the thrombin aptamer inhibits thrombin-induced platelet activation. Binding of thrombin’s anion-binding exosite by the platelet thrombin receptor has been shown to be involved in platelet activation (Vu et al., 1991). Thus, the thrombin aptamer binding site appears to overlap with the binding sites of hirudin, fibrinogen, thrombomodulin, and the platelet thrombin receptor.

Recently, the tertiary structure of the thrombin aptamer has been elucidated by NMR spectroscopy (Macaya et al., 1993; Wang et al., 1993). The ssDNA molecule forms a highly compact, partially symmetrical structure in solution containing two syn-anti-syn-anti G-tetrads, a loop which is partially folded over one tetrad and two T-T loops folded under the other tetrad. The stability and rigidity of this sequence-dependent structure probably contributes to the formation of a tight complex with thrombin. With the identification of the protected lysines in the anion-binding exosite, the thrombin aptamer can be “docked” onto the surface of thrombin by molecular modeling. Elucidation of the structural basis of aptamer-thrombin interaction will greatly aid medicinal chemistry efforts to improve the therapeutic profile of the thrombin aptamer.

Acknowledgements—We thank the Protein Structure Laboratory at University of California, Davis, for the N-terminal sequence analysis and Veronica Law for performing the clotting time assays.

REFERENCES

Bar Shavit, R., Kahn, A., Wiener, G. D., and Fenton, J. W., II (1983) Science 220, 729–730
Bock, L. C., Griffin, L. C., Latham, J. A., Vermass, E. H., and Toole, J. J. (1992) Nature 355, 564–566
Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) EMBO J. 8, 3467–3475
Carney, D. H., Scott, D. L., Gordon, E. A., and LaBelle, E. F. (1985) Cell 42, 479–488
Chang, J.-Y. (1989) J. Biol. Chem. 264, 7141–7146
Chang, T.-L., Feinman, R. D., Landis, B. H., and Fenton, J. W., II (1979) Biochemistry 18, 113–119
Chen, L. B., and Buchanan, J. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 131–135
Church, F. C., Pratt, C. W., Noyes, C. M., Kalayanarnit, T., Sherrill, G. B., Tobin, R. B., and Meade, J. B. (1989) J. Biol. Chem. 264, 18419–18425
Farley, R. A., Tsin, C. M., Carrill, C. T., Hawke, D., and Shively, J. E. (1984) J. Biol. Chem. 259, 9532–9535
Fenton, J. W., II (1981) Ann. N. Y. Acad. Sci. 370, 468–485
Fenton, J. W., II, Olsen, T. A., Zabinski, M. P., and Wilner, G. D. (1988) Biochemistry 27, 7106–7112
Furie, B., and Furie, B. C. (1988) Cell 53, 505–518
Galani, D., and Broze, G. J. (1991) Science 253, 308–312
Griffin, L. C., Tidmarsh, G. F., Bock, L. C., Toole, J. J., and Leung, L. L. K. (1983) Blood 81, 3271–3279
Grutter, M. G., Priestle, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., and Stone, S. R. (1990) EMBO J. 9, 2361–2365
Macaya, R. F., Schultze, P., Smith, F. W., Roe, J. A., and Feigon, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3745–3749
Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Kriss-krissawamy, S. (1986) Blood 66, 1–16
Mesamore, H. L., Fareed, J., Zabinski, M. P., Orfei, P., Kniffin, J., and Fenton, J. W., II (1979) Fed. Proc. 38, 756
Mitchinson, C., Wildenson, A. F., Trimmerman, B. J., and Green, N. M. (1982) FEBS Lett. 149, 87–90
Phillips, N. F. B. (1988) Biochemistry 27, 3514–3520
Rogers, S. J., Pratt, C. W., Whinne, H. C., and Church, F. C. (1992) J. Biol. Chem. 267, 3613–3617
Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., and Fenton, J. W., II (1986) Science 234, 277–280
Sheehan, J. P., Wu, Q., and Sadler, J. E. (1991) Blood 78, Suppl. 1, 277a
Shuman, M. A. (1986) Ann. N. Y. Acad. Sci. 486, 228–239
Vu, T. K. H., Wheaston, V. I., Hung, D. T., Charo, I., and Couchlin, S. R. (1991) Nature 353, 674–677
Wang, K. Y., McCurdy, S., Shees, R. G., Swaminathan, S., and Bolton, P. H. (1993) Biochemistry 32, 1892–1904
Wu, Q., Sheehan, J. P., Tsang, M., Lantz, R. B., Birktoft, J. J., and Sadler, J. E. (1991) Proc. Natl. Acad. Sci. 88, 6775–6779
Wu, Q., Tsang, M., and Sadler, J. E. (1992) J. Biol. Chem. 267, 24408–24412