The Anticoagulant Thrombin Mutant W215A/E217A Has a Collapsed Primary Specificity Pocket*

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The thrombin mutant W215A/E217A features a drastically impaired catalytic activity toward chromogenic and natural substrates but efficiently activates the anticoagulant protein C in the presence of thrombomodulin. As the remarkable anticoagulant properties of this mutant continue to be unraveled in preclinical studies, we solved the x-ray crystal structures of its free form and its complex with the active site inhibitor H-o-Phe-Pro-Arg-CH₂Cl (PPACK). The PPACK-bound structure of W215A/E217A is identical to the structure of the PPACK-bound slow form of thrombin. On the other hand, the structure of the free form reveals a collapse of the 215–217 strand that crushes the primary specificity pocket. The collapse results from abrogation of the stacking interaction between Phe-227 and Trp-215 and the polar interactions of Glu-217 with Thr-172 and Lys-224. Other notable changes are a rotation of the carboxylate group of Asp-189, breakage of the H-bond between the catalytic residues Ser-195 and His-57, breakage of the ion pair between Asp-222 and Arg-187, and significant disorder in the 186- and 220-loops that define the Na⁺ site. These findings explain the impaired catalytic activity of W215A/E217A and demonstrate that the analysis of the molecular basis of substrate recognition by thrombin and other proteases requires crystallization of both the free and bound forms of the enzyme.

Thrombin possesses a paradoxical combination of procoagulant and anticoagulant roles (1). The procoagulant role involves the cleavage of fibrinogen and the platelet receptor PAR1, leading, respectively, to fibrin polymerization and platelet aggre-

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The atomic coordinates and structure factors (code ITQ0 and 1TQ7) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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The abbreviations used are: WE, mutant W215A/E217A; PPACK, H-o-Phe-Pro-Arg-CH₂Cl; MES, 4-morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; FPR, H-o-Phe-Pro-Arg-p-nitroanilide; r.m.s., root mean square; SL, PPACK-bound slow form of wild type thrombin.

MATERIALS AND METHODS

The mutant WE was expressed, purified, and tested for activity as described previously (8). The mutant was concentrated to 10 mg/ml in 50 mM choline chloride, 20 mM MES, pH 6.0. For crystallization with PPACK, the mutant was mixed with the inhibitor at a molar ratio of 1:15 and incubated at room temperature for 1 h. Crystallization was achieved at 25 °C by vapor diffusion, with each crystallization well containing 500 μl of reservoir solution. Equal volumes of the protein sample and reservoir solution (1 μl each) were mixed to prepare the hanging drops. The reservoir solution consisted of 18% polyethylene glycol 8000, 0.2 M lithium acetate, and 0.1 M sodium cacodylate, pH 6.5. The reservoir solution for the free form of WE was composed of 0.1 M CAPS, 0.2 M lithium sulfate, 0.12 M sodium didehydrophosphate, and 0.53 M diopotassium hydrogen phosphate, pH 7.9. Diffraction quality crystals of both PPACK-inhibited and free WE were grown within 2 weeks. Crystals were cryoprotected in a solution similar to the reservoir solution but containing 25% glycerol prior to flash freezing. X-ray diffraction data for the WE-PPACK complex were recorded on an R axis image plate detector and processed and scaled with Denzo and Scalepack (10).
We have been deposited in the Protein Data Bank (accession codes 1TQ0 for free WE and 1TQ7 for WE-PPACK).

RESULTS

The backbone traces of the structures of WE free or bound to PPACK are shown in Fig. 1. The structure of WE bound to the active site inhibitor PPACK is practically identical to that of the wild type (12, 15) and especially to the PPACK-bound slow form (SL) reported recently (15). The autolysis loop is quite disordered, as is usually found in thrombin structures at this resolution (2.4 Å). Remarkably, the backbone region of the 215–217 strand hosting the mutated residues 215 and 217 retains the wild-type architecture of the SL structure (Fig. 2). Valence screening of the solvent molecules using WASP (17) returns no values > 0.5 valence units for possible bound Na⁺.

The structure of free WE shows notable changes relative to the wild type in its free form (15) and provides a molecular basis for understanding the devastating effects of the Ala replacement of Trp-215 and Glu-217 on substrate recognition. Because of the modest resolution of the crystal structure (2.8 Å), the autolysis loop is not visible nor are water molecules in the primary specificity pocket, the Na⁺ site, or the active site region. Also subject to considerable disorder are the 186- and 220-loops that define the Na⁺ site. The 220-loop is ordered only in one monomer in the asymmetric unit, whereas the 186-loop is ordered only in the other monomer. Disorder in these loops has never been reported in > 150 structures of thrombin deposited in the Protein Data Bank. The disorder is a result of the WE mutation and is likely exacerbated by the low resolution of the structure. As a consequence of the disorder in the 186- and 220-loops, the ion pair between Asp-222 and Arg-187 cannot be defined. Formation of this ion pair is a hallmark of the Na⁺-induced conformational transition of thrombin from the slow to the fast form (15). In contrast to the disorder documented in the 186- and 220-loops, well defined density exists for other important regions of the enzyme. The 215–217 strand hosting the replaced residues Trp-215 and Glu-217 collapses into the primary specificity site and obliterates access of the Arg of substrate in the S1 cavity (Fig. 3). The collapse leaves behind Phe-227, normally in stacking interaction with Trp-215, and causes a shift in the position of its benzene ring. Contributing to the collapse also is the abrogation of important polar interactions of Glu-217 with Thr-172 and Lys-224, which stabilize the fast form of thrombin (15). The WE mutant shows a 35,000-fold drop in the kcat/Km for the hydrolysis of the chromogenic substrate H₂-O-Phe-Pro-Arg-p-nitroanilide (FPR), which is the cleavable analog of PPACK (8). This drastic perturbation of substrate recognition is explained by the collapse of the 215–217 strand that comes in close contact (within 3 Å) with the 189–192 strand. Coupled to this change, the carboxylate group of Asp-189 in the primary specificity pocket reorients almost 90° and aligns parallel to the backbone of the 189–192 strand instead of being perpendicular to it as in the wild type. The orientation of Asp-189 precludes ionic interaction with the guanidinium group of the incoming Arg of substrate. The side chain of Glu-192 is oriented as in the PPACK-inhibited form of WE and is in the same orientation as the PPACK-bound fast form (FL) of the wild type (15). The catalytic Ser-195, on the other hand, experiences a main chain movement of 0.5 Å and a rotation in its side chain of approximately 70° relative to the

### Table I

| Crystallographic data of the free and PPACK-inhibited forms of WE |
|----------------------|----------------------|
|                      | WE               | WE-PPACK          |
| Data collection      | 1TQ0             | 1TQ7              |
| Wavelength (Å)       | 2.4              | 2.4               |
| Space group          | P2₁2₁2₁         | P2₁2₁2₁          |
| Unit cell dimensions (Å) | a = 131.93  | a = 131.93       |
|                      | b = 131.93     | b = 131.93       |
|                      | c = 131.93     | c = 131.93       |
| Resolution range (Å) | 3.00 – 2.8    | 3.00 – 2.4       |
| Observations         | 134,869         | 68,871           |
| Unique observations  | 18,400          | 13,234           |
| Completeness         | 98.0 (99.7)     | 98.1 (90.6)      |
| R(mean) (%)          | 7.9 (45.5)      | 5.9 (21.8)       |
| I/σ(I)               | 22.3 (3.6)      | 23.2 (6.3)       |

- R.m.s.d., root mean squared deviation from ideal bond lengths and angles and r.m.s. deviation in B factors of bonded atoms.
- m.c., main chain; s.c., side chain.

Data for the free form were collected at the Advanced Photon Source (beamline 14-BMC, Argonne National Laboratory) and processed using the HKL2000 software package (11). The WE-PPACK crystal was orthorhombic of space group P2₁2₁2₁, and contained one molecule per asymmetric unit. The free WE crystal was cubic of space group P2₁2₁2₁ and contained two molecules per asymmetric unit. Both structures were solved by molecular replacement using the coordinates of the thrombin-PPACK complex (12) as a search model and the program package Crystallography NMR System (13). Crystallographic refinement was carried out by simulated annealing and conjugated gradient minimization using Crystallography NMR System, and model building was performed with the program "O" (14). The autolysis loop could not be resolved in either structure. In the free WE structure, part of the 220-loop in the first monomer and a portion of the 186-loop in the second monomer were not included in the model because of their weak electron density. This observation is notable and underscores disorder in two critical loop regions of the molecule that are always well ordered in the wild type (12, 15). Except for this difference, the two monomers in the asymmetric units could be refined without constraints to nearly identical conformations, showing a r.m.s. deviation of 0.42 Å over 270 equivalent Ca atoms and no regions of significant difference in backbone configuration. Refinement carried out with noncrystallographic symmetry restraints produced essentially the same results, with no improvement in Rcryst and Rfree. Monomer B, with the 220-loop intact, was chosen for structural comparisons. Weak densities were also detected in the N- and C-terminal regions, which were not included in subsequent refinement. Crystal contacts involved the e xo site I region of one monomer and e xo site II region of the second monomer in which the closest distance was 6.0 Å. The contacts did not involve any of the regions showing significant structural changes compared with the PPACK-bound form. The two monomers of free WE in the asymmetric unit make lattice contact with seven molecules related crystallographically, but the closest contact to the mutated residues 215 and 217 in either monomer is a distance of approximately 25 Å. Water molecules were added in the final stage of the refinement process. They were subjected to visual inspection to check their positioning in electron density maps and allowed to refine freely. Water molecules with a temperature factor (B factor) > 80 Å² were excluded from subsequent refinement. Structural comparisons were computed using LSQMAN (16). The final refinement and model quality statistics are presented in Table I. Coordinates of the structures of the free and PPACK-bound forms of WE reveal no evidence of bound Na⁺, even though the complex was crystallized in the presence of 0.1 M sodium cacodylate. Remarkably, the water molecules in this region overlap 1:1 with those identified in the SL structure, recently solved at a resolution of 1.55 Å (15). These findings are consistent with the lack of Na⁺ binding and allosteric transduction in the WE mutant (8).
The PPACK-inhibited form, causing a breakage of the H-bond with the catalytic His-57. This orientation is similar to that seen in the free slow form(s) of the wild type (15).

Some of the structural changes in the free form of WE are reminiscent of those observed in the S structure of the wild type reported recently (15). Specifically, the absence of the Asp-222: Arg-187 ion pair, the breakage of the H-bond interaction between Ser-195 and His-57, and the reorientation of the side chain of Asp-189 are also seen in the S structure. It is possible that these changes are a direct manifestation of the lack of Na\(^+\) binding in the WE mutant. In addition to these structural signatures that pertain to the slow form of thrombin, the structure of WE contains features that are entirely the result of the double mutation. The replacement of Trp-215 and Glu-217 with Ala causes a detachment of the 215–217 strand that crushes the primary specificity pocket. The movement likely causes disorder in the 186- and 220-loops that define much of the Na\(^+\) site and the entry point of the cation (18). These additional changes explain why the catalytic properties of WE are compromised >1,000-fold relative to those of the slow form of the wild type.

DISCUSSION

The WE mutant of thrombin is currently being evaluated in preclinical studies as an effective anticoagulant and antithrombotic drug (9). A remarkable property of this mutant is that it shows very little catalytic activity toward chromogenic or natural substrates of thrombin. The \(k_{cat}/K_m\) values for substrate hydrolysis are compromised 1000-fold for PAR1, 20,000-fold for fibrinogen, and up to 35,000-fold for the chromogenic substrate FPR (8). The \(k_{cat}/K_m\) value for the hydrolysis of protein C in the absence of thrombomodulin is \(<1\ \text{m}^{-1}\ \text{s}^{-1}\) (8). The mutation involves two residues that play a critical role in substrate recognition. Trp-215 is highly conserved in the entire realm of serine proteases, and both Trp-215 and Glu-217 are absolutely conserved in thrombins from hagfish to human (19). The crystal structure of the free form of WE provides a striking explanation for the drastic drop in catalytic activity. Mutation of
Trp-215 abrogates the important stacking interaction with the benzene ring of Phe-227, and mutation of Glu-217 abrogates polar interactions of its side chain with Thr-172 and Lys-224. As a result, the 215–217 strand detaches from its position as a wall of the primary specificity pocket and crashes on the neighboring 189–192 strand. This prevents the Arg at the P1 position of incoming substrate to access the side chain of Asp-189 at the bottom of the S1 site. The compromised catalytic activity of WE is advantageous in vivo because it ensures that the mutant can circulate in the blood without causing fibrinogen clotting or platelet aggregation. When the mutant WE interacts with thrombomodulin, however, its activity toward protein C increases 60,000-fold, to a level comparable with that of the wild type (8). The mutant thus acts as a molecular switch that turns on its catalytic activity only in the presence of thrombomodulin and protein C. This is the basis of its potent anticoagulant effect in vivo (9).

What is the mechanism that rescues the catalytic activity of WE toward protein C in the presence of thrombomodulin? It is tempting to speculate that thrombomodulin corrects the collapse of the 215–217 strand and restores access to the primary specificity pocket. If so, thrombomodulin should be able to significantly enhance the catalytic activity of WE toward FPR. However, the k_{cat}/K_m value for FPR hydrolysis by WE improves only 3-fold in the presence of saturating (100 nM) concentrations of thrombomodulin (Fig. 4). The 3-fold increase is also observed in the presence of saturating (100 µM) concentrations of hirugen (Fig. 4). These effects are analogous to those observed with the wild type and argue against thrombomodulin being an allosteric effector of thrombin (20). It is therefore unlikely that thrombomodulin induces significant changes in the primary specificity pocket of WE. An alternative hypothesis is that thrombomodulin acts as a scaffold for presenting protein C to the thrombin active site in a correct orientation for cleavage (20, 21). This hypothesis explains the effects of thrombomodulin in the wild type and does not call for conformational changes in the mutant WE, consistent with the data in Fig. 4; however, the hypothesis fails to explain how the collapsed active site of WE is functionally restored to interact with protein C. A plausible scenario is that formation of the ternary complex WE-thrombomodulin-protein C causes a large induced fit transition in the active site of the enzyme that cannot take place when either protein C or thrombomodulin are separately bound. The concerted action of protein C and thrombomodulin turns WE into a molecular switch that only delivers its anticoagulant function.

An important implication of the results reported in this study is that no relevant information on the molecular properties of WE would have emerged from the structure of the mutant bound to PPACK. The thermodynamic definition of a binding constant in the ground state or the transition state involves the chemical potentials of the bound and free species; hence, structural analysis of substrate recognition cannot be based exclusively on information from the enzyme-substrate complex. This becomes even more obvious in the case of mutant enzymes, in which the effect of the structural perturbation may be confused to the free form. A direct demonstration of this possibility is offered by the thrombin mutant Y225I. Although its catalytic activity toward FPR and fibrinogen is compromised >10,000-fold, its crystal structure bound to PPACK is identical to that of PPACK-inhibited wild type (15, 22). Crystallization of thrombin in its free form is beset by many difficulties, including the need to prevent autodigestion. In a recent
study (23), removal of the site of autocatalytic cleavage in exosite I ensured crystallization of the free form of thrombin for the first time. In the case of thrombin mutants with severely compromised catalytic activity, there is in principle no need to remove this site of cleavage. The crystal structure of the anticoagulant mutant E217K has recently been solved in the free form (24). As for the WE mutant presented in this study, the structure of free E217K reveals changes in the active site region, resulting from the drastic charge reversal at residue 217, that explain the perturbed functional properties of the mutant. The structure of E217K bound to PPACK was not presented in the study, but it would have been a critical comparison with those of Y225I-PPACK (22) and WE-PPACK reported here.

The recent structure of thrombin in its free slow form (15, 23) has fostered new interest in the crystallographic investigation of the molecular basis of thrombin function and regulation. We have now demonstrated that it is possible, and indeed highly desirable, to crystallize thrombin mutants with compromised catalytic activity in both their free and PPACK-bound forms. The information on the free form is highly relevant to the biochemical properties of such mutants. Comparison of the free and bound forms affords a deeper understanding of the molecular basis of substrate recognition, which has a bearing on the study of protease specificity in general.

REFERENCES

1. Griffin, J. H. (1995) Nature 376, 337–338
2. Di Cera, E. (2003) Chest 124, (suppl.) 11–17
3. Esmon, C. T. (2003) Chest 124, (suppl.) 26–32
4. Wu, Q. Y., Sheehan, J. P., Tsian, M., Lenta, S. R., Birktoft, J. J., and Sadler, J. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 88, 6775–6779
5. Le Bonnecie, B. F., and Esmon, C. T. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7371–7375
6. Gibbs, C. S., Coutre, S. E., Tsian, M., Li, W. X., Jain, A. K., Dunn, K. E., Law, V. S., Mao, C. T., Matsumura, S. Y., Megna, S. J., Paborsky, L. R., and Leung, L. L. K. (1995) Nature 376, 413–416L. K. L.
7. Dang, Q. D., Guinto, E. R., and Di Cera, E. (1997) Nat. Biotechnol. 15, 146–149
8. Cantwell, A. M., and Di Cera, E. (2000) J. Biol. Chem. 275, 39827–39830
9. Graber, A., Cantwell, A. M., Di Cera, E., and Hanson, S. R. (2002) J. Biol. Chem. 277, 27581–27584
10. Otwinowski, Z. (1993) in Proceedings of the CCP4 Study Weekend: Data Collection and Processing (Sawyers, L., Isaacs, N., and Bailey, S., eds) pp. 56–62, SERC Daresbury Laboratory, Warrington, U. K.
11. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
12. Bode, W., Turk, D., and Karshikov, A. (1992) Protein Sci. 1, 426–471
13. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–912
14. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard. (1991) Acta Crystallogr. Sect. A 47, 110–119
15. Pineda, A. O., Carrell, C. J., Bush, L. A., Prasad, S., Caccia, S., Chen, Z.-W., Mathews, F. S., and Di Cera, E. (2004) J. Biol. Chem. 279, 31842–31853
16. Kleywegt, G. J. (1999) Acta Crystalllogr. Sect. D Biol. Crystallogr. 55, 1878–1884
17. Naylor, M., and Di Cera, E. (1996) J. Mol. Biol. 256, 228–234
18. Prasad, S., Wright, K. J., Roy, D. B., Bush, L. A., Cantwell, A. M., and Di Cera, E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15785–15790
19. Banfield, D. K., and MacGillivray, R. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2779–2783
20. Vindigni, A., White, C. E., Komives, E. A., and Di Cera, E. (1997) Biochemistry 36, 6674–6681
21. Fuentes-Prior, P., Iwanaga, Y., Huber, R., Pagila, R., Rumennik, G., Seto, M., Morser, J., Light, D. R., and Bode, W. (2000) Nature 404, 518–525
22. Guinto, E. R., Caccia, S., Rose, T., Putzer, R., Waksman, G., and Di Cera, E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1852–1857
23. Pineda, A. O., Savvides, S. N., Waksman, G., and Di Cera, E. (2002) J. Biol. Chem. 277, 40177–40180
24. Carter, W. J., Myles, T., Gibbs, C. S., Leung, L. L., and Huntington, J. A. (2004) J. Biol. Chem. 279, 26387–26394
25. Krem, M. M., and Di Cera, E. (2003) Biophys. Chem. 100, 315–323
26. Morris, A. L., MacArthur, M. W., Hutchinson, E. G., and Thornton, J. M. (1992) Proteins 12, 345–364
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