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Structure of the Ser195Ala mutant of human α-thrombin complexed with fibrinopeptide A(7–16): evidence for residual catalytic activity

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The Ser195Ala mutant of human α-thrombin was complexed with fibrinopeptide A(7–22) (FPA) in an effort to describe the (P1′–P6′) post-cleavage binding subsites of the fibrinogen-recognition exosite and define more clearly the nature of the Michaelis complex and the scissile peptide bond bound at the catalytic site. The thrombin mutant, however, has residual catalytic activity and proteolysis occurred at the Arg16–Gly17 bond. Thus, the structure of the thrombin complex determined was that of FPA(7–16) bound at the active site, which is very similar to the ternary FPA(7–16)cmk–human thrombin–hirugen complex (r.m.s.d. $\simeq$ 0.4 Å; Stubbs *et al.*, 1992). It is further shown by subsidiary experiments that the cleavage is the result of residual catalytic activity of the altered catalytic machinery.

1. Introduction

One of the most important functions of thrombin in hemostasis and thrombosis is to convert fibrinogen to fibrin. Fibrin is a major component of a blood clot and is responsible for encapsulating platelets and some plasma proteins with a network of fibers to block the flow of blood at the site of injury. Fibrin assembly begins with the thrombin cleavage of the two Aα chains of fibrinogen at Arg16–Gly17, followed by the release of two molecules of FPA(1–16)[1] (Blomback, 1967); this is then followed by further cleavage of Bβ chains at Arg14–Gly15 with the subsequent release of FPB(1–14). Thrombin exhibits a remarkable specificity in the activation process by cleaving only two Arg–Gly bonds of the 181 Arg/Lys-amino acid peptide bonds in fibrinogen (Blomback, 1967). This is accomplished with the aid of a fibrinogen-recognition exosite distinct from the active site but generally operating in concert with it.

The structure of a ternary human thrombin complex with hirugen bound at the fibrinogen-recognition site and FPA(7–16)cmk bound to the active site has been determined (Stubbs *et al.*, 1992), as well as FPA(7–16) bound to bovine thrombin (Martin *et al.*, 1992). The binding of the (P1′–P3′) residues to the fibrinogen-recognition exosite C-terminal to the scissile peptide bond in bovine thrombin has been described with a FPA(7–19) analogue replacing the amide N atom between Arg16 and Gly17 with a methylene C atom to produce a non-cleavable –$COCH_2$– linkage (Martin *et al.*,

[1] Abbreviations: FPA(1–16), fibrinopeptide A; FPB(1–14), fibrinopeptide B; hirugen, *N*-acetyl-sulfato-Tyr63-hirudin(55–65); FPA(7–16)cmk, residues 7–16 of *N*-acetyl-fibrinopeptide A chloromethylketone; FPA(7–22), N-acetyl-DFLAEGGGVRGPRVVE-amide; PPACK, D-Phe-Pro-Arg chloromethylketone; MALDI–MS, matrix-assisted laser desorption ionization mass spectroscopy; PMSF, phenylmethanesulfonyl fluoride.

1996). The bovine thrombin structures have three molecules in the asymmetric unit, one of which is ε-thrombin cleaved at Thr149A (chymotrypsinogen numbering; Bode *et al.*, 1992), while the other two are α-thrombin. The structures of the P1′ and P2′ residues of FPA(7–19) are the same in all three complexes, but Arg19 is disordered in one and has two alternate binding sites in one of the other two complexes (Martin *et al.*, 1996).

We undertook the structure determination of FPA(7–22) bound to the Ser195Ala point mutant of human α-thrombin in order to describe more extensively the (P1′–P6′) post-cleavage sites of the fibrinogen-recognition exosite and to define more precisely the nature of the Michaelis complex and the scissile peptide bond bound at the catalytic site. The thrombin mutant, however, proved to have residual catalytic activity like similar mutants of subtilisin (Carter & Wells, 1988) and trypsin (Corey & Craik, 1992): over the time required to crystallize the FPA(7–22) mutant–thrombin complex, hydrolysis occurred at the Arg16–Gly17 peptide bond. Thus, we report here the first structure of a Ser195Ala mutant serine proteinase and confirm the structure of FPA(7–16) bound to thrombin (Stubbs *et al.*, 1992) and show that the cleavage of FPA(7–22) is the result of residual activity of the altered catalytic machinery of the mutant thrombin.

2. Experimental

The FPA(7–22) of human fibrinogen was synthesized with an ABI Model 430 peptide synthesizer using *t*-butyloxycarbonyl chemistry and standard cycle conditions and was purified by reverse-phase HPLC. The sequence was confirmed by Edman degradation (prior to acetylation) and the peptide was >99% pure by analytical reverse-phase HPLC. The M_r of 1698.98 Da by electrospray ionization mass spectrometry was consistent with the calculated M_r of 1698.90 Da. The mutant thrombin was prepared and purified to homogeneity as described previously (Sheehan & Sadler, 1994) and was stored frozen at 193 K in HEPES buffer pH 7.5 and 50 m*M* NaCl. Linear MALDI mass spectra were obtained using a PerSeptive Biosystems (Framingham, MA) Voyager Elite delayed extraction time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm, 3 nsec pulse).

An approximately 20-fold molar excess of FPA(7–22) was placed over 0.25 ml of frozen mutant thrombin (2.24 mg ml^{-1}), which was allowed to thaw and stand overnight, forming the binary FPA(7–22)–mutant thrombin complex. The solution of the complex was concentrated at 277 K to about 5 mg ml^{-1} using a Microcon filter with a cutoff of 10 kDa.[2] Crystallization was carried out using the hanging-drop vapor-diffusion technique. Interleaved crystals appear in about two weeks from 2 µl drops consisting of 1 µl of FPA(7–22)–mutant thrombin solution and 1 µl of well solution (1.4 *M* sodium citrate, 0.1 *M* HEPES buffer pH 7.5). Of various additives examined to improve crystal morphology, 1–2% 2-propanol in the well worked best and gave single crystals with good cubic face development.

[2] The complex was also prepared by first concentrating the mutant thrombin followed by treatment with a 20-fold molar excess of FPA(7–22), producing identical crystallization results.

Table 1
Refinement statistics of FPA(7-16)–mutant thrombin.

Total number of atoms	3536
Number of solvent atoms	161
Number of observations	8330
Mean isotropic B (Å^2)	28.2
R factor (%)	16.9
R.m.s.d.†	
Bond distances (Å)	0.016
Angle distances (Å)	0.042
1–4 distances (Å)	0.057
Chiral volume (Å^3)	0.18
Single torsion contacts (Å)	0.25
Multiple torsion contacts (Å)	0.36

† Root-mean-square deviation from ideal values.

The X-ray intensity data collection was carried out with an R-AXIS II imaging-plate detector system equipped with Molecular Structure Corporation focusing mirrors, using Cu $K\alpha$ radiation from an RU-200 rotating-anode generator employing a fine focus filament (0.3 × 3.0 mm) at a power setting of 5 kW. A crystal cryoprotectant was used for collecting diffraction data at 123 K: the crystals were flash-frozen in a solution of 1:1 2,4-methyl-pentanediol to crystallization well solution. The crystal-to-detector distance was set at 140 mm with the detector swing angle at 10°. A total of 19 frames were measured using a 2.5° oscillation per frame with 15 min exposure per frame. The unit-cell parameters were determined by autoindexing (Higashi, 1990) and processing of the diffraction data was carried out with the Rigaku *R-AXIS* data-processing software package. Crystals of the mutant thrombin complex are cubic, space group $I2_13$ or $I23$, 12 complexes per unit cell, with a = 135.1 Å (V_m = 3.02 Å^3 Da^{-1}; protein fraction 40%). A total of 21 833 observations to 2.5 Å resolution gave 9561 independent reflections [$I/\sigma(I) > 2.0$] having an $R_{\rm merge}$ = 9.3%, with 61% of the reflections observable in the 2.75–2.50 Å resolution range ($R_{\rm merge}$ = 19.2%). Monoclinic (Vijayalakshmi *et al.*, 1994) and orthorhombic (Tabernero *et al.*, 1995) crystals of thrombin–hirugen display comparable or better diffraction but also have a 50% protein fraction.

The molecular orientation of the thrombin molecule in the crystal was determined using the program *AMoRe* (Navaza, 1994) and the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). The thrombin coordinates were those of the CVS995–thrombin complex (Brookhaven Protein Data Bank entry 1dit; Krishnan *et al.*, 1996) stripped of solvent, the divalent CVS995 inhibitor and the Ser195 OG atom. The rotation search had a unique solution of 6.5σ above the mean in the 8.0–3.5 Å resolution range. Translation searches were performed in space groups $I2_13$ and $I23$ (indistinguishable by systematic absences), with $I2_13$ giving outstanding solutions (correlation of 0.67 *versus* 0.20; R factor of 37% *versus* 50%). Rigid-body fitting improved these values to 0.69 and 36%, respectively. This model was then refined using restrained least-squares methods with the

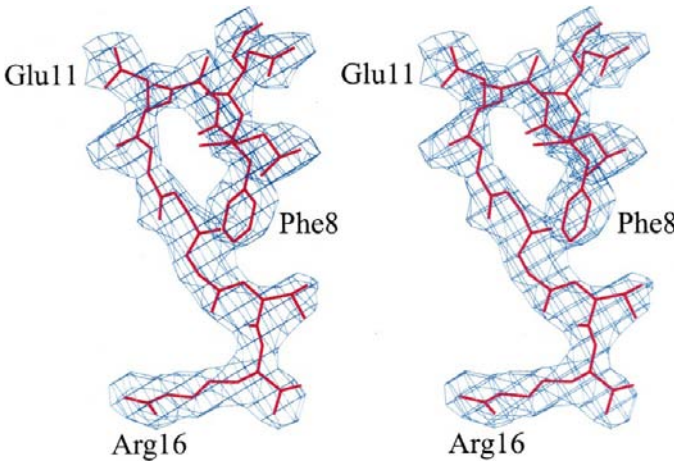

Figure 1
Stereoview of $2F_o - F_c$ electron density of FPA(7–16) as bound to Ser195Ala mutant thrombin. Contoured at the 1σ level.

program *PROFFT* (Finzel, 1987). After 15 cycles of overall *B* refinement at 2.8 Å resolution, the *R* factor converged to 25.5%. Electron- and difference-density maps clearly indicated the presence of residues FPA(7–16); however, there was no electron density for FPA(17–22), indicating either flexible disorder or possibly cleavage of FPA(7–22) at the P1 site. Furthermore, the electron density of Ala195 confirmed the absence of a serine OG atom. Water molecules were added at positions that were within 2.5–3.5 Å of a hydrogen-bonding donor or acceptor and that had positive electron density in both $F_o - F_c$ and $2F_o - F_c$ maps. The final structure converged to an *R* factor of 16.9% containing 161 water molecules. The refinement statistics are summarized in Table 1. The coordinates of FPA(7–16)–mutant thrombin have been deposited in the Protein Data Bank (PDB code 1dm4).

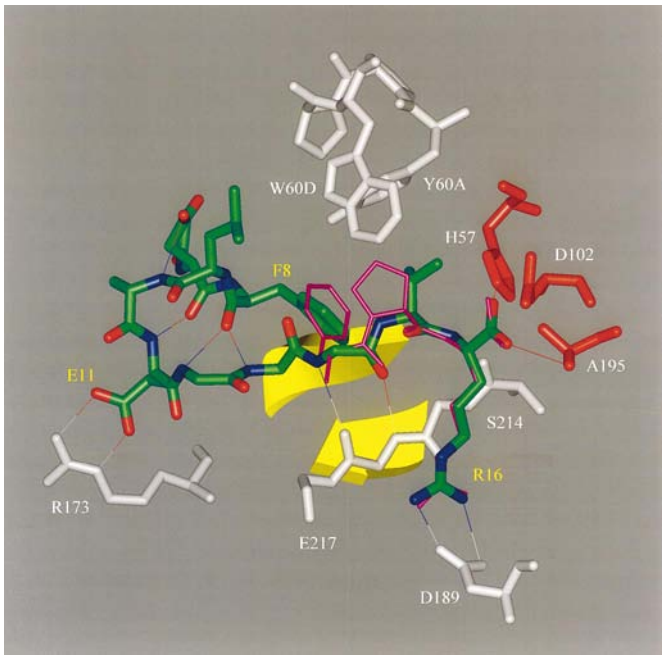

Figure 2
Structure of FPA(7–16) bound in the active site of Ser195Ala mutant thrombin. FPA(7–16) in atom colors: carbon, green; nitrogen, blue; oxygen, red. Thrombin, gray; His57, Asp102, Ala195 of catalytic site, red. PPACK of PPACK–thrombin superposed on FPA(7–16) in magenta. Hydrogen bonds are dual-colored thin lines (except those of catalytic site residues). The β-strand schematic is in yellow. Gly193 of the oxyanion hole, Asp194 and the Trp215 side group are omitted for clarity. The positions of Asp194 and the oxyanion hole (Gly193 N, Ala195 N) can be seen in Fig. 4.

3. Results

The structure of the mutant thrombin is the first Ser195Ala mutant structure to be reported of a serine proteinase. Some parts of the A chain (Thr1H–Glu1C, Asp14L–Arg15) as well as the autolysis loop between Glu146 and Lys149E in the B chain carrying the γ-cleavage site (Lys149E–Gly150) did not have good electron density. This loop has only been found to be ordered when involved in close crystal packing contacts, as in the orthorhombic form of PPACK–thrombin (Rydel *et al.*, 1991) or active-site inhibited hirugen–thrombin complexes crystallizing in the space group $P2_12_12$ (Priestle *et al.*, 1993; Tabernero *et al.*, 1995); disorder in the A chain is also common in wild-type structures.

The molecular structure of mutant thrombin is practically the same as the wild type in the ternary FPA(7–16)cmk–human thrombin–hirugen complex (r.m.s.Δ = 0.35 Å for 245 CA atoms of the B chain; Stubbs *et al.*, 1992) and other wild-type thrombin structures (<0.5 Å). The same applies to the FPA(7–16) peptide [r.m.s.d. = 0.50 Å for all atoms of FPA(7–16)]. The residues of the catalytic triad of FPA(7–16)–mutant thrombin have the same orientations as those of the wild-type hirugen–thrombin structure [Vijayalakshmi *et al.*, 1994; r.m.s.d. (all atoms included): His57 = 0.34 Å, Asp102 = 0.01 Å, Ala(Ser)195 = 0.35 Å]. The close congruence of the orientations of the catalytic triads of the native and mutated enzymes suggests that the catalytic machinery is still well placed and prepared for catalysis even with Ser195 changed to alanine.

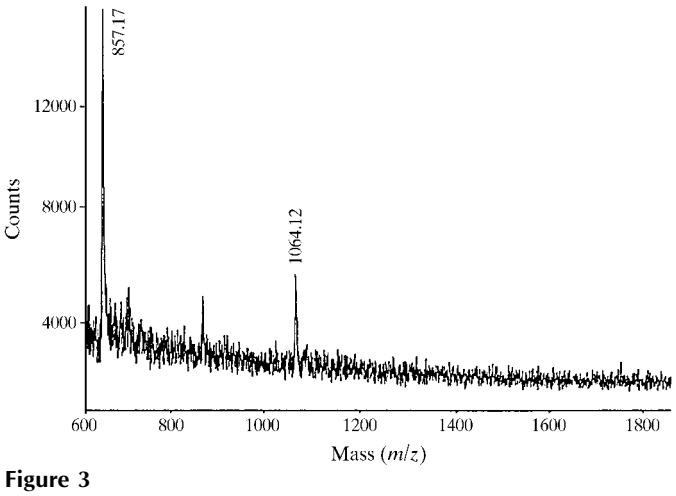

Figure 3
MALDI–MS of a crystal of FPA(7–16)–mutant thrombin used in structure determination. The M_r of *N*-acetyl-FPA(7–16) is calculated to be 1062 Da and that of FPA(17–22)-amide to be 657 Da.

The structure of FPA(7–16) is well defined in the electron density (Fig. 1) but there is no density corresponding to FPA(17–22). The decapeptide folds halfway back on itself at Leu9–Glu11 and contains a number of important intramolecular hydrogen bonds maintaining the conformation, beginning with two from Asp7 O and OD2 to the amides of Glu11 and Ala10, respectively, which lead to an irregular helical turn that is further stabilized by additional intramolecular hydrogen bonds to Phe8 O from Gly12 N and Gly13 N (Fig. 2). The phenyl side group of Phe8 clusters with Val15; this intramolecular hydrophobic pair, along with Leu9, interacts with Tyr60A and Trp60D of the thrombin 60-insertion loop (Fig. 2). The Glu11–Arg16 stretch of FPA(7–16) is in an extended β-strand conformation that forms two intermolecular hydrogen bonds and an antiparallel β-strand with Ser214–Gly216 of thrombin terminating at Arg16 O and O_T, hydrogen bonding, respectively, with Ala195 N of the oxyanion hole (Arg16 O–Gly193 N = 3.5 Å) and His57 NE of the catalytic site. Two doubly hydrogen-bonded salt bridges complete the intermolecular interactions, one between the guanidinium group of Arg16 and the carboxylate of Asp189 of the S1 specificity site, the other between the side chains of Glu11 and Arg173 of thrombin (Fig. 2). Val15 is close to the S2 site, while the phenyl group of Phe8 is in the D-enantiomorphic S3 site found in PPACK-bound thrombin and Arg16 C of the scissile bond is 3.4 Å from Ala195 CB (Fig. 2). Thus, intramolecular hydrogen bonds are important in producing the complementary binding conformation of FPA(7–16) that fits the active-site region immediate to the catalytic site, whereas the two doubly hydrogen-bonded intermolecular salt bridges and four other intermolecular hydrogen bonds bind FPA(7–16) convincingly to thrombin. Considering the extent of the intermolecular interactions and that the solvent structure of the active site is typical of arginyl substrates and inhibitors (Zhang & Tulinsky, 1997), it is somewhat surprising that FPA(7–16) is such a good leaving group (K_m = 310 μM; Marsh *et al.*, 1983).

The absence of electron density beyond Arg16 suggested that Gly17–Glu22 of FPA(7–22) was either disordered or FPA(7–22) was cleaved at Arg16 of the P1 site. In order to resolve the ambiguity, MALDI–MS measurements were carried out. These were made on: (i) the crystal of the complex used to determine the structure dissolved in water, (ii) the FPA(7–22)–mutant thrombin solution used for crystallization and (iii) FPA(7–22) dissolved in 1.4 *M* sodium citrate and 0.1 *M* HEPES buffer pH 7.5 (crystallization solution less mutant thrombin). The MALDI spectrum of the crystal of the complex used for diffraction measurements is shown in Fig. 3. The mass spectrum clearly shows that FPA(7–22) is cleaved at Arg16: the two major peaks correspond to the FPA(7–16) and FPA(17–22) peptides.[3] A similar spectrum was obtained with a two-week-old solution of the FPA(7–22)–mutant thrombin complex, which is about the time required for crystals to grow. The FPA(7–22) alone in a crystallization solution, in the absence of mutant thrombin, remains uncleaved for this same time period.

If the activity arises from contaminating wild-type thrombin or some other thrombin-like serine proteinase, it should be removed by PMSF treatment (Gold, 1965). This led us to incubate a mutant thrombin solution with a 100 *M* excess of PMSF for 4 h prior to addition of a 20 *M* excess of FPA(7–22), followed by crystallization in hanging drops as previously described. The diffraction pattern of a crystal was measured and its structure analyzed, which proved to be the same as that of crystals grown without prior PMSF treatment. The mutant thrombin, therefore, must have residual catalytic activity. Even if this is 10^{-6}-fold less than wild type [as with subtilisin (Carter & Wells, 1988) and trypsin (Corey & Craik, 1992)], it is nonetheless sufficient to convert all the FPA(7–22) used in crystallization to FPA(7–16) in the two-week crystallization period.

Residues Trp60D, Lys60F and Asn60G of the 60-insertion loop of thrombin show the largest deviations (about 1.0 Å) of CA atoms when superposed on the FPA(7–16)cmk–thrombin–hirugen complex. These deviations are most likely to result from the different crystal packing arrangements with the approach of the 60-insertion loop of a symmetry-related molecule in the cubic crystals, since the position of FPA(7–16) is otherwise the same in both complexes. This insertion loop, which aids thrombin specificity for substrates and inhibitors, is generally fairly rigid. In the mutant structure, Tyr60A and Trp60D of the β-hairpin turn pack against the phenyl of Phe8 and the side group of Val15 to form a hydrophobic lid on the latter (Fig. 2) also seen in the other active-site occupied thrombin structures.

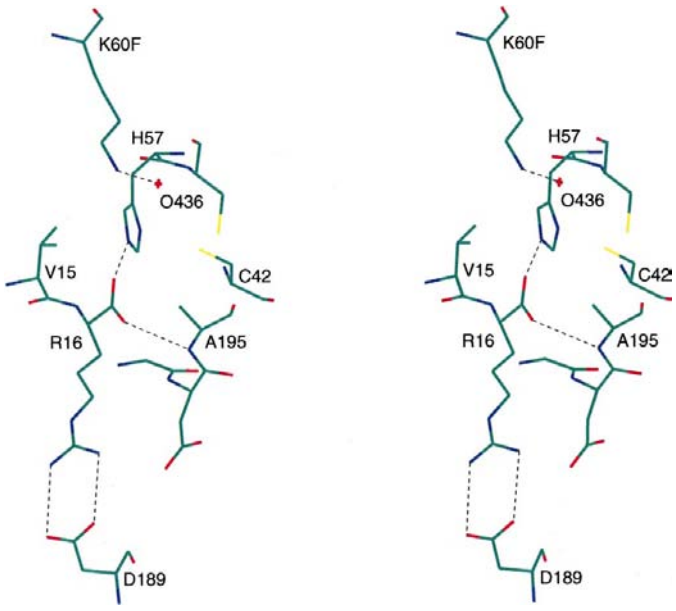

Figure 4
Stereoview of catalytic site of mutant thrombin. Only Val15–Arg16 of FPA(7–16) shown. FPA(15–16) and mutant thrombin are in atom colors as in Fig. 2. Hydrogen bonds are shown as dashed lines.

[3] The presence of FPA(17–22) could be the result of insufficient washing of the crystal or the presence of trapped hexapeptide in interstitial solvent space of the crystal or possibly FPA(17–22) partially bound to thrombin.

Another new crystal packing arrangement involves the β-cleavage site of thrombin (Tyr76–Arg77A) located in the fibrinogen-recognition exosite (Rydel *et al.*, 1994) interacting with a symmetry-related molecule. The carboxylate of Glu77 makes a doubly hydrogen-bonded salt bridge with the guanidinium of Arg77A of an adjacent molecule, while Tyr76 and Tyr117 of two different molecules are engaged in a π-stacking interaction.

4. Discussion

The catalysis reaction of Ser195Ala mutant thrombin cannot proceed by the usually accepted acylenzyme intermediate. To account for the residual catalytic activity of such mutants, an alternate mechanism has been described (Carter & Wells, 1988) where, in the absence of the Ser195 hydroxyl, a water molecule directly attacks the scissile peptide bond. Only a single intermediate is then formed that converts to the hydrolyzed products. In this process, a factor of about 10^{-6} is lost in the catalytic efficiency of subtilisin (Carter & Wells, 1988) and trypsin (Corey & Craik, 1992), which probably also applies more or less to mutant thrombin.

Examining the structure of the catalytic site of FPA(7–16)–mutant thrombin reveals the presence of a triangular-shaped depression formed by the atoms His57 NE, Ala195 CB and Arg16 C of FPA(7–16), which is occupied by either the carbonyl O atom or the carboxyl O atom of the terminal carboxylate of Arg16 (Fig. 4). In ordinary catalysis, the carbonyl O atom of the scissile bond is in the oxyanion hole, hydrogen bonding with Gly193 N and Ser195 N. Since the latter interaction is also found in the FPA(7–16)–mutant thrombin structure (Figs. 2 and 4), the triangular cavity is likely to contain the carboxyl O atom of Arg16 (hydrogen bonding with His57 NE), which comes from a water molecule that hydrolyzes the scissile peptide bond of FPA(7–22). The cavity is further defined by the disulfide Cys42–Cys58 and the side group of Val15 of FPA(7–16) (Fig. 4), so that a water molecule approaching the region could be directed by repulsive hydrophobic steering toward Arg16 C of the scissile amide, leading to cleavage through a single intermediate (Carter & Wells, 1988). A channel along the arginyl of Lys60F of thrombin leads to the cavity from behind His57 (Fig. 4). This possible path for a water molecule to the scissile bond is from within rather than frontal to the active site. Both of the approaches appear to be viable possibilities; however, the frontal one seems the more likely.

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