pH-Dependent Binding Modes Observed in Trypsin Crystals: Lessons for Structure-Based Drug Design

Milton T. Stubbs,*[a] Sabine Reyda,[a] Frank Dullweber,[a] Maren Möller,[a] Gerhard Klebe,[a] Dieter Dorsch,[b] Werner W. K. R. Mederski,[b] and Hanns Wurziger[b]

Dedicated to Professor Robert Huber on the occasion of his 65th birthday

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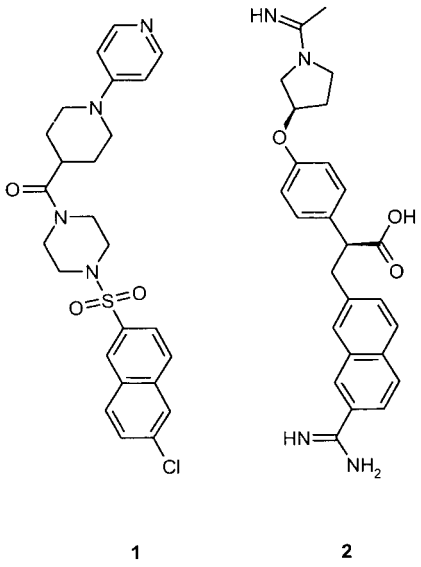
Advances in the various genome sequencing projects promise to provide fresh impetus to the target-oriented search for novel drugs to treat human ailments.[1] Crucial to this pursuit is the fast and efficient identification and optimization of new lead compounds. Current approaches to identification rely heavily on high-throughput screening and increasingly on virtual computer screening. Optimization is driven largely by chemical intuition complemented by the translation of structural constraints (for example, pharmacophore patterns) into molecular skeletons. Typical compounds arising from experimental screening exhibit binding constants of the order of 10^{-5} M with respect to their target receptors or enzymes. To further develop any hits, it is necessary to coordinate the experimental binding data with chemical features of the ensemble of ligands found to be responsible and determinant for receptor binding. Thus, a frequent assumption for lead development is that the hits found by screening can be associated with a definitive binding mode common to all inhibitors (pharmacophore hypothesis). Here we explore the link between affinity and binding mode and the extent to which lead development might be influenced by choice of experimental system.

We investigated the structural requirements for the inhibition of Factor Xa, a serine protease involved in blood clotting.[2–4] Most synthetic inhibitors for this enzyme contain a basic group that binds in the primary specificity (S1) pocket, although such moieties are generally detrimental to oral uptake and systemic bioavailability.[5] Zeneca have patented a series of Factor Xa inhibitors[6] that contain a weakly basic pyridine group such as that in **1** (Scheme 1). We investigated the interaction of **1** with the archaetypal serine proteinase trypsin to try to understand how **1** inhibits Factor Xa. Compound **1** inhibits bovine β-trypsin in the μM range; therefore, this interaction may serve as a paradigm for initial lead compound – receptor interactions.



1 **2**

Scheme 1. *Chemical formulae of two Factor Xa inhibitors for which structural data are available: compound **1** (Zeneca) = the subject of this investigation; compound **2** (Daiichi) = DX9065a.[2, 14]*

Compound **1** shows IC_{50} values of 3 nM and 34 μM for human Factor Xa and human thrombin, respectively (IC_{50} = concentration required for 50% inhibition).[6] Potentiometric pH titration[7] of **1** indicates that the compound possesses a pK_a value of 7.5, which we assign to the pyridinyl group. The inhibition constant K_i (dissociation constant for the enzyme – inhibitor complex in the presence of the enzyme substrate) of **1** for bovine trypsin, measured in a photometric assay[8] at pH 8.0, was 13.4 μM (compare with benzamidine, K_i = 21.3 μM). K_i values at lower pH could not be measured reliably due to the reduced activity of bovine β-trypsin (optimum pH value = 9.5). In addition, the dissociation constant if the enzyme—inhibitor complex was measured with isothermal titration calorimetry[9] and exhibited a slight pH dependency: K_d(pH 5.0) = 50 ± 10 μM, K_d(pH 7.8) = 15 ± 5 μM.

Cocrystallization of **1** with bovine trypsin[10] resulted in two clearly distinguishable crystal forms: a cubic A form at pH 7 and an orthorhombic B form at pH 8 (Figure 1). Analysis of the A crystals reveals that the pyridine ring of **1** occupies the specificity pocket of the enzyme (Figure 2a). Contrary to the situation in inhibitor complexes based on thrombin—pyridine interactions,[11] the pyridinyl nitrogen atom of **1** does not interact

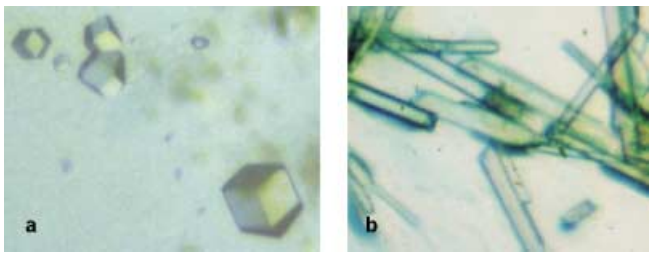


Figure 1. *Cocrystals of **1** with bovine trypsin grown at a) pH 7 (cubic form A) and b) pH 8 (orthorhombic form B).*

[a] *Dr. M. T. Stubbs, Dr. S. Reyda, Dr. F. Dullweber, M. Möller, Prof. Dr. G. Klebe*
Institut für Pharmazeutische Chemie
der Philipps-Universität Marburg
Marbacher Weg 6, 35032 Marburg (Germany)
Fax: (+49) 6421-28-28994
E-mail: stubbs@mailer.uni-marburg.de

[b] *Dr. D. Dorsch, Dr. W. W. K. R. Mederski, Dr. H. Wurziger*
Preclinical Pharmaceutical Research, Merck KgaA
Frankfurter Strasse 250, 64271 Darmstadt (Germany)

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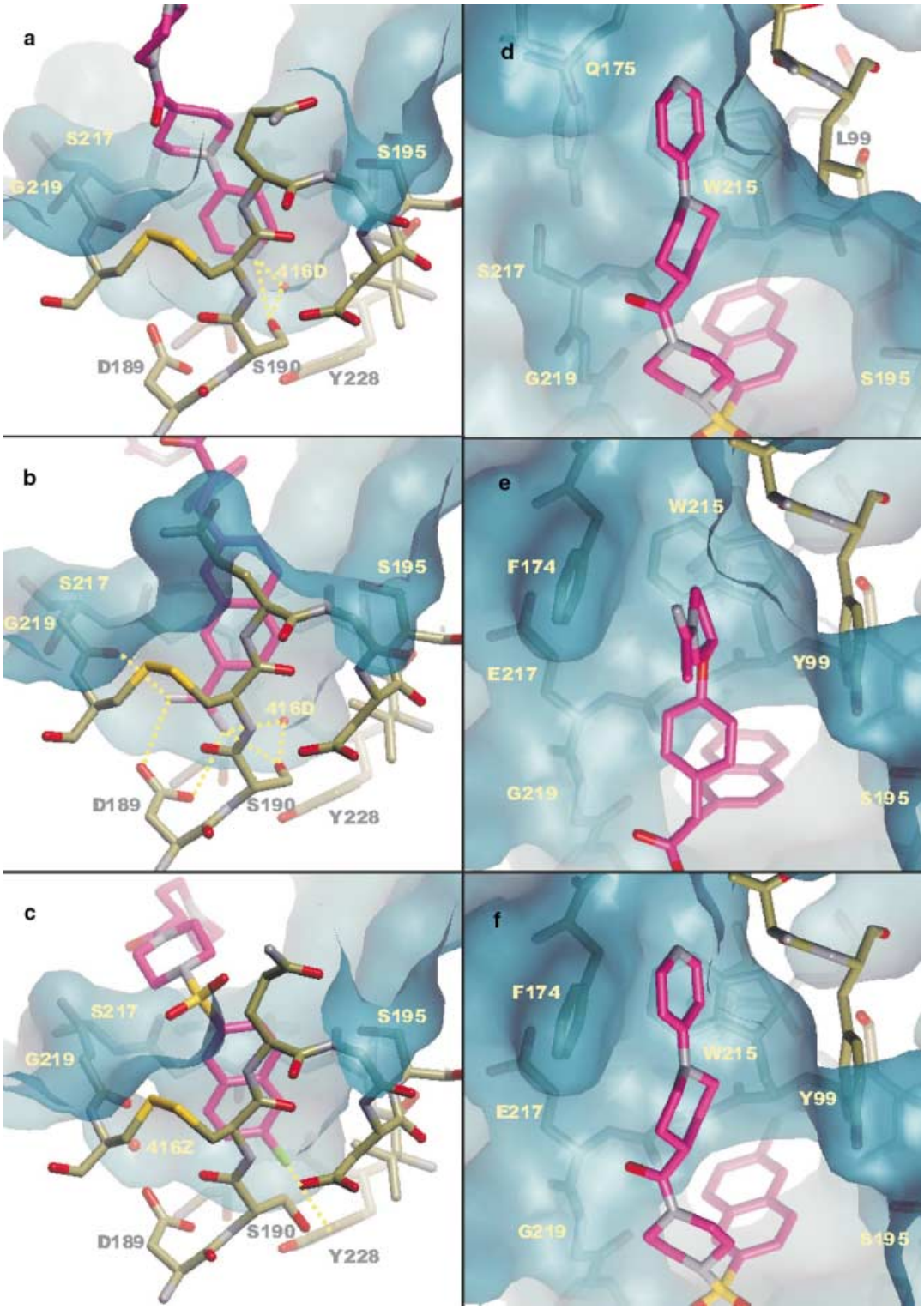

Figure 2. *a) Binding mode of **1** in crystal form A. The pyridine moiety occupies the S1 pocket, with its nitrogen atom interacting with the S190 Oγ atom and water molecule 416D (termed Wat416 in the text). This is in contrast to the binding of **2** in trypsin (b),[2] where there is a salt bridge to the D189 carboxylate group. In crystal form B, **1** binds with the chloronaphthyl function deep in the pocket (c). The Cl atom displaces the "conserved" water molecule 416D, which in turn relocates to the external surface of trypsin (416Z). The hydroxy group of the S190 residue rotates by 120°, so that the inhibitor Cl atom sees only the Cβ atom of the S190 residue and the aromatic ring of the amino acid Y228 (yellow dashed line). The peptide bond S217–G219 rotates around 120°, such that its hydrophobic surface abuts that of the piperazinyl group of the inhibitor. Rotation of the **1**B structure by 90° about a horizontal axis (d) reveals that the pyridinyl group reaches over the side chain of W215, reminiscent of the acetimidoyl group of **2** in Factor Xa (e).[14] The three typical point interactions seen for other Factor Xa inhibitors[2–4, 14–16] are displayed for **2** in (e): occupation of the proximal S1, the intermediate aromatic, and the distal electrophilic pockets.[16] Transfer of **1** to Factor Xa (f) shows that piperidinyl – pyridinyl moieties would be ideally suited to occupy the hydrophobic box (Y99, F174, and W215) of Factor Xa in the B crystal form. Three-letter nomenclature is used for residues in the text.*

directly with the carboxylate group of the Asp 189 residue in bovine trypsin. Instead, it shows a short contact (distance = 2.77 Å) to the Ser 190 Oγ atom that we assume acts as a weak hydrogen-bond acceptor for the pyridine nitrogen atom. In addition, the interaction is bridged by a "conserved" water molecule, Wat 416 (see below). All other intermolecular contacts occur over distances greater than 3.2 Å. Outside the S1 pocket, the rest of the inhibitor wraps around the side chain of Gln 192 (without contacting it), so that the chloronaphthyl group reaches towards the S1′ site (S′ is the region normally occupied by the substrate residue on the C-terminal side of the cleavage bond).

The inhibitor binds to the orthorhombic B crystal form in a completely different way to that described for the A crystals. The hydrophobic chloronapthyl group of the inhibitor is found deep in the S1 pocket (Figure 2c), with its C—Cl bond pointing towards the Tyr 228 residue. The chlorine atom occupies the position of water molecule Wat 416 (conserved in all trypsin structures solved to date). There is no hydrogen-bonding partner in the inhibitor molecule for the side chain of the Asp 189 residue. A new water molecule (provocatively also termed Wat 416) appears on the external surface of the specificity pocket, coordinated by the Asp 189 Oδ2 (Oδ2 = one of the two carboxylate oxygen atoms in the side chain), Lys 224 O, and Gly 219 O atoms and the Wat 415 molecule. Concomitant with the displacement of the Wat 416 molecule, the side chain of the Ser 190 residue (hydrogen-bonded to the Wat 416 molecule and the Tyr 228 Oη atom in the A form crystals) rotates through 120°, to form a new hydrogen bond with the Tyr 228 Oη atom and make a van der Waals' contact (3.15 Å) with the Ile 138 Cγ2 atom. Finally, the Ser 217—Gly 219 peptide bond[12] rotates such that the hydrophobic surface (parallel to the amide plane) is presented to the edge of the inhibitor, and the hydroxy group of the Ser 217 residue rotates through 120°. An almost 90° bend at the sulphonamide moiety causes the remaining piperidinyl/pyridinyl portions to extend over the side chain of the Trp 215 residue at the base of the S2/S3 pockets (Figure 2d).

Two structures and two binding modes appear possible. Which one is "correct", and which one corresponds to the situation in Factor Xa? To answer the former question, we must address the significance of individual crystal forms. To date, we have investigated 32 different compounds with bovine trypsin ($K_i = 10^{-5} - 10^{-9}$ M) by using a narrow crystallization screening method(pH 7 – 8, 0.1 – 0.3 M ammonium sulphate, 20 – 30 % polyethylene glycol (PEG6/8k); Table 1).[2, 4] The most frequently observed crystal forms are either the B or the trigonal T modifications, obtained with roughly equal frequency at both pH 7 and pH 8. The B form also makes up 50 % of bovine trypsin – ligand complexes found in the Protein Databank,[13] while the T form comprises a further 15 %. In contrast, the A form obtained here has been observed only once before.[4] Hence, the most likely outcome of our crystallization screening system is either form B or T, regardless of pH, so that pH effects can be ruled out as the driving force behind the formation of the various crystal forms. The fact that the rare cubic A form results only at pH 7 indicates that the binding mode found in these crystals predominates under the applied crystallization conditions. Our structural data indicate that the A form occurs only if the space occupied by the bound inhibitor precludes molecular packing in the B or T lattices because of collision with symmetry-related molecules. The binding mode observed in the A crystal form when the crystals are grown at lower pH suggests that a protonated pyridinyl group is favored in the S1 pocket of trypsin under these conditions. The remarkable lack of specific secondary interactions outside this pocket suggests that the heterocycle contributes little to the observed affinity, at least for trypsin.

The reverse binding mode observed in the B crystal form is highly reminiscent of the three-point interactions seen for other Factor Xa inhibitors[2–4, 14–16] (Figure 2e). Comparison with DX9065a (**2**; Scheme 1) reveals a deeper penetration of the specificity pocket by **1** (Figure 2b, c).[2] This penetration involves displacing the "conserved" water molecule Wat 416 and relocating the hydroxy function of the Ser 190 residue into a hydrophobic environment. This rearrangement is the most likely cause for the low affinity of **1** for trypsin. On the other hand, residue 190 is an alanine in both Factor Xa and thrombin—the only difference within the S1 pocket between trypsin and these two coagulation enzymes. The more hydrophobic environment, which leads to less tight binding of Wat 416 in the case of thrombin,[11, 17] is conducive to favorable burial of the chloronaphthyl group.

Similar interactions to those described for the B crystal form have been observed between a haloaromatic compound and thrombin,[18] compensated by favorable contacts outside the

Table 1. *Crystal forms and pH conditions for bovine trypsin – ligand complexes.*

Form[a]	Cell constants [Å]			[°]			Space group	No. of crystal structures at pH:[b]				Total no. of crystal forms[b]	PDB[d]
	a	*b*	*c*	α	β	γ		6.5	7.0	7.5	8.0		
A	125.8	125.8	125.8	90	90	90	$I2_13$		1	1		2	0
B	54.1	58.6	63.1	90	90	90	$P2_12_12_1$		3 + 3e		6 + 3e	9 + 6e	24
T	55.1	55.1	109.2	90	90	120	$P3_12_1$	1	7		7 + 1e	15 + 1e	7
O									4	1	1	6	15[c]
Total								1	18	2	18	39	46
N												5	

[a] A and B: the cubic and orthorhombic forms observed here, T: the frequently observed trigonal form, O: five other crystal forms different from those given explicitly, N: no crystals obtained. Forms B and T have closed active sites (the inhibitors are enclosed by symmetry contacts), while the inhibitors in form A and some of the O form compounds are freely accessible to bulk solvent. [b] Suffix "e" implies that the crystal was "empty" (disordered or no density for the inhibitor). [c] These crystals represent the open orthorhombic form, which is often used for soaking experiments. [d] No. of crystal forms found in the Protein Data Bank.

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primary specificity pocket. In Factor Xa, the aromatic pocket formed by the side chains of the Tyr 99, Trp 215, and Phe 174 residues would be occupied by the piperidinyl and pyridinyl portions of the inhibitor (Figure 2 f). The distal electrophilic pocket of Factor Xa, formed by the carbonyl groups of the amino acids Glu 97, Thr 98, and Ile 175 (with a possible contribution from the side chain of the Glu 97 residue), acts as a receptor for weakly basic groups, in this case pyridine.

The complexes presented here highlight some of the problems associated with structure-based drug design; in particular, the existence of distinct protonation dependent binding modes and the importance of multiple polymorphic forms. Weak binding, as typically observed for initial lead compounds, may result from unfavorable interactions and mutual adaptations of an as yet not optimized ligand to its receptor. Although multiple near-isoenergetic binding orientations adopted by a single ligand might appear to be a hindrance to drug discovery, observation of these arrangements can also serve as a guideline to map favorable alternative sites in a binding pocket. Consideration of these sites can then be used to increase the chances of obtaining ligands with improved affinity during the optimization process.

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[7] The inhibitor was synthesized as described in ref. [6] and then 4 mg were dissolved in potassium chloride solution (13 mL, 150 mM) at 25 °C and titrated with 0.5 M potassium hydroxide solution between pH 3 and pH 11. The ionization constants (pK_a) of the inhibitor were measured potentiometrically with a pH-sensitive electrode and the PCA 101 analyzer (Sirius Analytical Instruments, England) and the data was analyzed with the pK_a log P software (Sirius). For details of this process, see: a) J. Comer, *Chem. Br.* **1994**, 983 – 986; b) A. Avdeef, *Quant. Struct. Act. Relat.* **1992**, *11*, 510 – 517.
[8] K_i values for bovine β-trypsin were determined photometrically under the following conditions: 0.05 M tris(hydroxymethyl)aminomethane/HCl (tris/HCl; pH 8.0) 0.154 M NaCl, and 5% ethanol, with three different concentrations of the Factor Xa substrate Pefachrome tPA (Pentapharm, Basel) at 25 °C. For the standard protocols used, see: J. Stürzebecher, U. Stürzebecher, H. Vieweg, G. Wagner, J. Hauptmann, F. Markwardt, *Thromb. Res.* **1989**, *54*, 245 – 252.
[9] Dissociation constants K_d were determined by isothermal titration experiments with an MCS-ITC instrument (Microcal Inc., USA) as described in: T. Wiseman, S. Williston, J. F. Brandts, L. N. Lin, *Anal. Biochem.* **1989**, *179*, 131 – 137. The inhibitor (0.5 mM) was titrated into the trypsin solution (0.04 mM) in the reaction cell with a 250 µL syringe at 25 °C. Experiments were carried out at pH 5.0 in 50 mM β-morpholinoethanesulfonic acid buffer solution and at pH 7.8 in 50 mM tris buffer solution, in each case with 100 mM sodium chloride and 5% ethanol. Injection volumes were 15 µL and 4 min equilibration time was allowed between each injection. Observations were corrected for the heats of dilution of the inhibitor in the buffer solution.
[10] The inhibitor was preincubated with bovine β-trypsin in 1 mM HCl and 10 mM $CaCl_2$ for 1 hour. Cocrystallization was carried out at room temperature by using vapor diffusion, with a protein concentration of 10 mg mL^{-1} and an inhibitor concentration of 2 mg mL^{-1}. A cubic $I2_13$ crystal (form A, $a = b = c = 125.8$ Å, grown in 0.1 M imidazole (pH 7) from 0.3 M ammonium sulphate and 30% polyethyleneglycol 8000), and an orthorhombic $P2_12_12_1$ crystal (form B, $a = 54.1$, $b = 58.6$, $c = 63.1$ Å, grown in 0.1 M imidazole (pH 8) from 0.1 M ammonium sulphate and 20% polyethyleneglycol 8000) were mounted in capillaries at room temperature. Data were collected in-house by using an R-AxisIV image plate system (MSC) installed on a Rigaku rotating anode generator and evaluated with the DENZO program.[10a] Starting coordinates were taken from those published by Renatus et al.,[4] as were refinement protocols for using the X-PLOR software,[10b] and model-building was performed by using the O program.[10c] The final model for A corresponds to an *R* factor of 19.0% for reflections between 10 and 3.0 Å (99.9% completeness); the model for B yields an *R* factor of 20.3% from 10 to 2.1 Å (96.6% completeness). Coordinates, structure factors, and all relevant statistical data have been deposited at the Protein Data Bank (accession codes: 1ql8 and 1ql7 for the A and B forms, respectively). a) Z. Otkinowski, W. Minor, *Methods Enzymol. A* **1997**, *276*, 307 – 326; b) A. Brünger, X-PLOR (Version 3.1), Yale University Press, New Haven, **1992**; c) T. A. Jones, J. Y. Zou, S. W. Cowan, M. Kjeelgaard, *Acta Crystallogr.* **1991**, *47*, 110 – 119.
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