X-ray and spectrophotometric studies of the binding of proflavin to the S1 specificity pocket of human α-thrombin

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Received 9 February 1998

Abstract Proflavin can be used to study the interactions of inhibitors and substrates with thrombin by monitoring the changes in the visible absorption spectrum that occur on dye displacement. We have used microspectrophotometric methods to investigate the binding of proflavin to crystals of an α-thrombin-hirugen complex and have determined the structure by X-ray crystallography. The proflavin molecule binds in the S1 pocket of the enzyme with one of the amino groups hydrogen bonded to the carboxylate of Asp-189 while the protonated ring nitrogen is hydrogen bonded to the carbonyl of Gly-219. This result indicates that the proflavin displacement assay can be used to specifically monitor the binding of inhibitors to the S1 pocket.

Key words: Thrombin; Proflavin; X-ray crystallography

1. Introduction

The thrombin sequence numbering based on topological similarities with chymotrypsinogen [1]. PPACK residue numbers are primed. S*n* and P*n* represent inhibitor (or substrate) and proteinase sites prior to the cleavage site [31].

The design of specific, non-peptidic, mechanism-based inhibitors of human α-thrombin is currently of great interest for the development of potential antithrombotic drugs. Thrombin is a serine proteinase with trypsin-like specificity towards basic residues, and has the typical structural features of this class of enzymes. The three-dimensional structure of human α-thrombin in various complexes with substrates and inhibitors have been reported, allowing the details of the enzyme active site to be elucidated [1–5]. The principal determinant of substrate specificity is the S1 pocket, an elongated channel with Asp-189 at the bottom, able to make salt bridges with arginyl and more rarely lysyl P1 residues of the physiological substrates. Near the entrance of the specificity pocket lies a hydrophobic region, identified as the S2 site, ideally suited to the binding of P2 proline and other medium-sized non-polar residues. The S2 cavity forms a single common hydrophobic surface with the adjacent 'aryl binding site', found to bind the phenylalanine P9 residue of the fibrinopeptide A [6] and the D-phenylalanine P3 residue of the non-physiological inhibitor PPACK [1].

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Abbreviations: hirugen, *N*-acetylhirudin 53′–64′ with sulfato-Tyr-63′; PPACK, D-Phe-Pro-Arg chloromethylketone

The equilibrium and kinetic interactions of inhibitors and substrates to thrombin can be studied spectrophotometrically by monitoring the change in the visible absorption spectrum that occurs on displacing proflavin. Proflavin is an acridine dye that shows large absorption spectrum changes on binding to the active sites of α-thrombin [7] and other proteolytic enzymes such as trypsin [8] and chymotrypsin [9]. In the case of chymotrypsin, proflavin competes with substrates such as indole and tryptophan derivatives, consistent with the binding to the large hydrophobic S1 pocket of this proteolytic enzyme. Analogously, the dye binds to a single specific site on thrombin with micromolar affinity ($K_d \sim 20$ μM), competing both with the charged inhibitor *p*-aminobenzamidine [7] and with the neutral indole and related compounds [10]. The binding of benzamidine to the specificity pocket of thrombin [11] and the displacement of proflavin by indole [12] lead to the idea that the acridine moiety binds adjacent to the catalytic centre in an apolar region of the active site, in agreement with the strong preference of thrombin for charged P1 side chains. On the other hand, proflavin binding has been used as a probe of the integrity of the trypsin S1 binding pocket which has a very similar architecture to that of thrombin [13].

Here we report crystallographic and microspectrophotometric binding studies of the proflavin dye to the active site of human α-thrombin. These studies were carried out with crystals of α-thrombin complexed with hirugen. The hirugen-thrombin complex is the only crystal structure determined so far with an unobstructed active site; the presence of hirudin-type peptides at the exosite of the enzyme has been shown not to prevent the binding of active site directed inhibitors [4]. The results show that proflavin binds in the S1 specificity pocket and suggest that the spectrophotometric displacement assay can be used to screen for binding of compounds in the S1 pocket.

2. Materials and methods

2.1. Crystallisation

Human α-thrombin was produced by activating prothrombin with taipan-snake venom [14,15]. Activated α-thrombin was assayed by measuring its amidolytic activity towards a chromogenic substrate and purified by taking advantage of its highly specific affinity to heparin, which was immobilised on a matrix support of sepharose [16]. Both prothrombin and snake venom V-3129 were purchased from Sigma, as was the chromogenic compound S2238 (H-D-Phe-Pip-Arg-*p*-nitroanilide·2HCl). The heparin-sepharose CL-6B affinity column was obtained from Pharmacia LKB Biotechnology. From about 4.5 μmoles of prothrombin, around 1.5 μmoles of pure active α-thrombin were recovered and stored at a concentration of 10 mg/ml (0.27 μM) in 20 mM Tris buffer at pH 6.0 and 750 mM NaCl. The hirugen peptide was purchased from Bachem.

PII S0014-5793(98)00235-X

An approximately 10-fold excess of hirugen was added to the α-thrombin sample. The thrombin-hirugen complex solution was concentrated with a Centricon10 to 20 mg/ml in 20 mM Tris pH 6.0 and 375 mM NaCl. Monoclinic thrombin-hirugen crystals belonging to space group C2 were grown according to Skrzypczak-Jankun et al. [3] with polyethylene glycol (PEG) as precipitant. The crystals used in this study were obtained using the vapour diffusion method and micro-seeding techniques in 6 μl hanging drops containing 10 mg/ml thrombin-hirugen, 0.05 M HEPES buffer at pH 7.0, 0.25 M NaCl, 10–12% w/v PEG-4000 and by equilibrating the drops for one day against 0.1 M HEPES pH 7.0, 0.2 M NaCl, 20–24% PEG-4000 at 18°C. Crystals larger than 100 μm in the smallest dimension usually appeared in 10 days. Crystals were stabilised in 25% w/v PEG-4000, 0.2 M NaCl and 0.1 M HEPES pH 7.0. For diffraction measurements the crystals were soaked for 24 h into a stabilising solution containing 1.4 mM proflavin. Proflavin was purchased from Sigma.

2.2. X-ray data collection

X-ray data were collected at room temperature on a CAD4 Enraf-Nonius diffractometer equipped with a FAST television area detector, using an Enraf-Nonius FR571 rotating anode X-ray generator operated at 42 kV and 99 mÅ. Oscillation frames with a width of 0.15 degree rotation were collected with exposure times of 60 s per frame. The data from the FAST area detector were evaluated on-line using the program MADNES [17]. Subsequent data processing and reduction were carried out using the CCP4 suite of programmes [18]. Statistics regarding the diffraction data are summarised in Table 1.

2.3. Crystallographic refinement

The coordinates of the thrombin-hirugen complex [3] have been used as starting model. Refinement was carried out using energy restrained least-squares refinement with the program X-PLOR [19]. Several cycles of positional and isotropic temperature factors refinement were followed by model inspection and manual intervention using the program FRODO [20]. After this first round of refinement, the proflavin model [21] was fitted into the electron density map, which clearly showed the presence of the bound inhibitor in the active site. The refinement proceeded until all uninterpretable difference electron density was less than four times the r.m.s. deviation from mean density. A total of three rounds of refinement were carried out giving a crystallographic *R*-factor of 18.8%. The refinement parameters and the quality of the final model are presented in Table 1.

2.4. Spectroscopic measurements

Spectroscopic data have been obtained by single crystal polarised absorption microspectrophotometry [22,23]. For spectral measurements, single crystals mounted in a flow cell were placed on the stage of a Zeiss MPM03 microspectrophotometer, equipped with a Glan-Thompson prism to produce linearly polarised light. The absorbance from the monoclinic crystals of thrombin-hirugen was measured with light polarised parallel to the *x* and *y* optical axes, which were experimentally determined by observing the extinction directions. Spectra were recorded between 300 and 550 nm.

Small crystals of the thrombin-hirugen complex (about 50 μm thick) were stabilised and incubated with 0.1 mM proflavin. The reference spectrum of proflavin in the suspending medium was recorded and the kinetics of the complex formation were monitored by collecting polarised absorption spectra at different times (Fig. 1A). The kinetics are diffusion limited and dependent on crystal size. The dependence on concentration was also investigated by the incubation of a crystal with proflavin over the range 0.05 mM to 0.5 mM (Fig. 1B). Spectra were recorded after equilibrium was reached. From a least-squares fit of these data to a binding isotherm, the dissociation constant (K_d) was determined as 0.53 ± 0.07 mM. To study the time dependence of the displacement of proflavin by PPACK, polarised absorption spectra were recorded from a crystal preincubated in a 0.1 mM proflavin solution and then transferred to a solution containing proflavin (0.1 mM) and PPACK (0.1 mM) (Fig. 1C).

3. Results and discussion

3.1. Absorption spectra

Proflavin absorption spectra display different characteristics depending on the polarity of the surrounding medium. The 444 nm absorption maximum of the free dye in saline solutions [12,24] shifts to longer wavelength when polyethylene glycol (PEG) is present. A red shift to 458 nm is observed as 25% w/v PEG-4000 is added to the proflavin solution (data not shown).

Perturbation of the proflavin spectrum occurs upon binding to the serine proteinases trypsin [8], α-chymotrypsin [9] and α-thrombin [7,10]. The acridine dye spectrum undergoes a large increase in absorbance at about 465 nm, which accounts for the enzyme-proflavin complex. The spectrum of proflavin bound to the thrombin-hirugen complex in the crystal exhibits a large increase in absorbance at about 465 nm (Fig. 1A), similar to the value reported for the proflavin-thrombin complex in saline solution. This suggests that the acridine binding mode in the crystal is consistent with the binding in solution and is not significantly affected by the presence of hirugen at the exosite of the enzyme nor by the PEG content of the solution used to stabilise the crystal.

The binding of proflavin to the thrombin-hirugen crystal is slow, though clearly specific, as indicated by the high polarisation of the absorption bands (Fig. 1A). Incubation with proflavin at higher concentrations (Fig. 1B) shows a significant increase in the amount of proflavin bound compared with the 0.1 mM incubation. The polarisation ratio is concentration independent, indicating that the binding is still specific at higher chromophore concentrations (data not shown). In principle one can estimate the stoichiometry of proflavin binding from the relative isotropic absorbance at 280 nm and 465 nm by measuring the components in the three principal optical directions. In practice it was not possible to make measurements along the *z* direction in these monoclinic crystals. The value of the dissociation constant for the binding of proflavin to the crystals ($K_d = 0.53$ mM) (Fig. 1C) is very much larger than that reported for the binding of the dye to thrombin in aqueous solution at similar pH conditions ($K_d \sim 10$ μM) [7] suggesting that either there is competition between components of the stabilising solution and proflavin for the

Table 1
Crystallographic statistics

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Space group	C2
Cell parameters:	
a	71.15 Å
b	72.25 Å
c	72.67 Å
β	100.61°
Resolution range	20.0–2.0 Å
Measured reflections	56491
Unique reflections	24042
R_{merge}[a]	5.1% (15.8%)[b]
Completeness	98.1% (94.9%)[b]
Refinement:	
R_{factor}[c]	18.9%
R_{free}[d]	21.2%
R.m.s. deviation from ideal bond lengths	0.009 Å
R.m.s. deviation from ideal bond angles	1.84°

[a] $R_{merge} = 100 \times \sum_h \sum_j |I_{hj} - I_h| / \sum_h \sum_j I_{hj}$ where I_h is the weighted mean intensity of the symmetry related reflections I_{hj}.
[b] Values for the outermost resolution shell are given in parentheses.
[c] $R_{factor} = 100 \times \sum |F_{obs} - F_{calc}| / \sum |F_{obs}|$ where F_{obs} and F_{calc} are the observed and calculated structure factors respectively.
[d] R_{free} is the R_{factor} calculated using a random 5% sample of reflection data omitted from refinement.

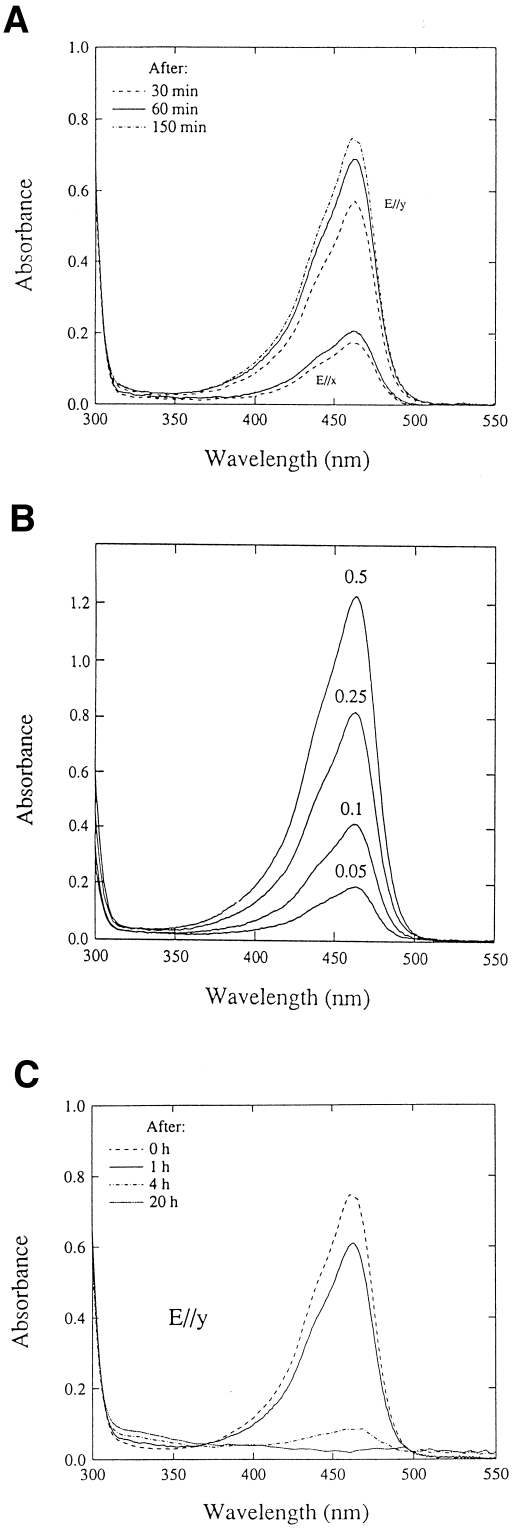

Fig. 1. Spectra of proflavin bound to crystals of the thrombin-hirugen complex stabilised in a solution containing 25% w/v PEG-4000, 0.2 M NaCl, HEPES 0.1 M pH 7.0. A: Time dependence of the polarised absorption spectra of a crystal soaked in 0.1 mM proflavin. The spectra were recorded with electric vector of the polarised light parallel to either the *x* or *y* crystal optical directions. B: The effect on the polarised absorption spectra of varying the proflavin concentration in the range 0.05 to 0.5 mM. Spectra were recorded with light polarised along the *y* direction. C: Spectral changes of the thrombin-hirugen-proflavin complex occurring upon PPACK binding. A crystal was soaked in a solution of proflavin 0.1 mM and then transferred to a solution containing both proflavin 0.1 mM and PPACK 0.1 mM. Spectra were recorded over 20 h with the electric vector of polarised light parallel to the *y* optical axis of the crystal.
←

binding site or the binding is intrinsically weaker in the crystalline state. Addition of PPACK to the thrombin-hirugen-proflavin crystals results in the progressive disappearance over 20 h of the 465 nm peak (Fig. 1C), indicating the presence of overlapping binding sites for proflavin and PPACK.

3.2. Crystallographic studies

The thrombin-hirugen model of proflavin complex is essentially identical to the native thrombin-hirugen structure. The amino terminus (Thr-1H-Glu-1C) and the carboxy terminus (Ile-14K-Arg-15) of the thrombin A chain are not defined in the electron density map. There is also no electron density for the carboxy terminal end (Gly-246-Glu-247) of the thrombin B chain and the Thr-147-Lys-149E polypeptide segment, which is part of the 148 insertion loop.

Despite the large heat capacity change reported upon binding of proflavin to thrombin [25], the presence of proflavin does not result in significant displacements of the polypeptide chains with respect to the native structure. Thus the proflavin bound and native structures can be superposed with an r.m.s. deviation of 0.1 Å. The proflavin molecule binds in the S1 specificity pocket of the protease (Figs. 2 and 3) with one of the two amino groups of the inhibitor interacting with the carboxylate of Asp-189 at the bottom of the specificity pocket. In addition, this amino group forms a hydrogen bond to solvent molecule W2, which bridges to the carbonyl group of Phe-227. The buried solvent molecule together with W1 (Figs. 2 and 3) are present in other thrombin structures [5,11,26], as well as in the trypsin pocket [27].

Although the proflavin amino group interacting with Asp-189 is located in much the same position as the Arg-3′ NH1 in the PPACK-thrombin structure [1] with the plane of the acridine ring matching that of the guanidinium moiety, the orientation of the proflavin molecule is quite different. The planar proflavin interacts with the hydrophobic sides of the S1 pocket, directing one amino group towards Asp-189 and the other towards the surface of the thrombin molecule rather than towards the active site Ser-195. This amino group is not involved in hydrogen bonds and has a larger temperature factor than the amino group involved in ionic interactions at the bottom of the pocket. The orientation of the acridine molecule may be favoured by the hydrogen bond interaction between the protonated aromatic nitrogen of proflavin and the carbonyl group of Gly-219. In the PPACK structure [1], this carbonyl group forms hydrogen bonds with both the Arg-3′ NH2 and with a water molecule located at the position occupied by the ring nitrogen of proflavin. Glu-192 has the same conformation as in the native model and does not participate in the binding of the P1 residue as observed in the PPACK complex. The active site cleft is occupied by a string of ordered water molecules that extend from the carbonyl group of Gly-216.

Proflavin binding to thrombin is specific and occurs at the S1 pocket of the enzyme, which physiologically accommodates basic P1 residues with a strong preference for arginine

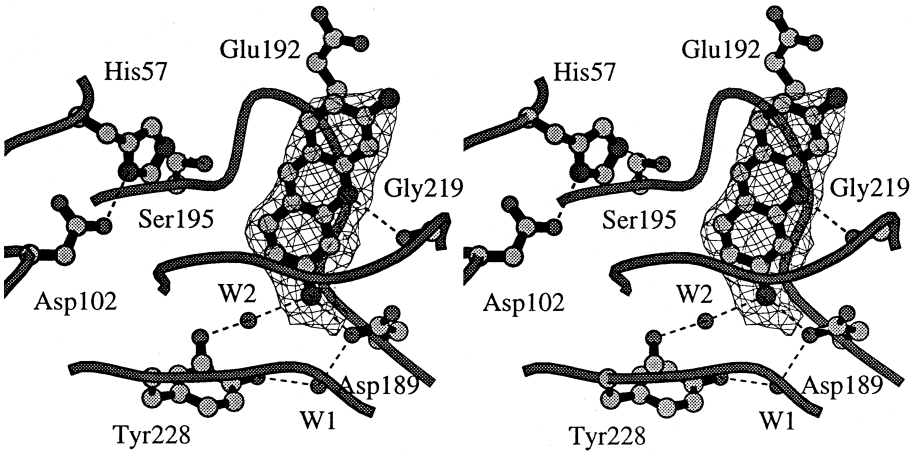

Fig. 2. Stereo diagram showing the proflavin molecule bound in the S1 specificity pocket of the α-thrombin-hirugen complex. The residues of the catalytic triad are on the left of the figure while the water molecules W1 and W2 are represented by small spheres. The diagram includes the difference electron density contoured at 2σ and calculated using data between 20–2.0 Å with the proflavin molecule omitted from model. (Generated using the program MOLSCRIPT [32] with modifications by R. Esnouf [33].)

side chains. Benzamidine is a stronger base ($pK_a = 11.6$) [28] than proflavin ($pK_a = 9.5$) [29] but at neutral pH both are positively charged. In solution, the proflavin molecule binds more tightly ($K_d \sim 10$ μM) [7] than benzamidine ($K_i = 343$ μM) [7] indicating that the presence of a highly basic group is not an overriding requirement for tight specific binding to the S1 pocket of thrombin. The proflavin complex is partly stabilised by hydrophobic interactions with the upper section of the specificity pocket in a manner similar to that observed for some peptide boronic acid inhibitors which lack a positive change at the P1 position [30].

PPACK, which has an extended binding region that includes the apolar S2 and aryl binding sites as well as the S1 pocket, competes directly with proflavin as seen in the loss of the 465 nm absorption peak (Fig. 1C). The crystallographic results reveal that proflavin competes with the PPACK Arg-3′ residue for the S1 pocket. It follows that proflavin displacement can be used to monitor binding of other low molecular weight inhibitors to the S1 pocket.

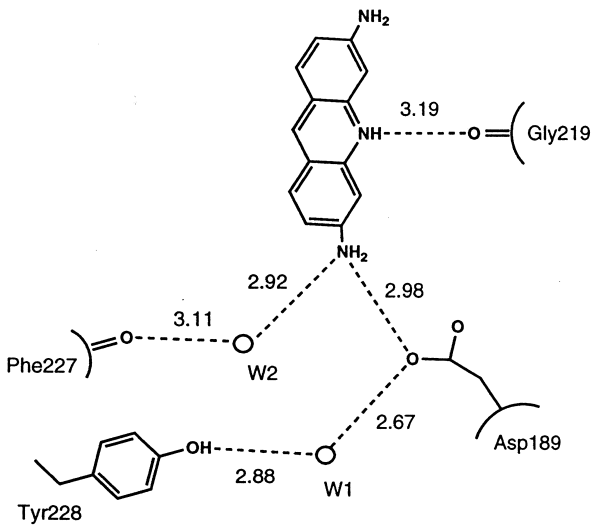

Fig. 3. Schematic representation of the hydrogen bonding between the proflavin molecule and the α-thrombin-hirugen complex. Distances are given in angstroms.

Acknowledgements: We are very grateful to Gian Luigi Rossi and Andrea Mozzarelli for their advice and discussion regarding the spectrophotometric measurements, Susanne Bethell for the thrombin activation and purification experiments, Scott Reid for providing the initial crystals and help in the thrombin-hirugen crystallisation, and Tadeusz Skarzynski for assistance during data collection. E.C. wishes to acknowledge the support of a Glaxo studentship.

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