

The Many Faces of Platelet Glycoprotein Ib α - Thrombin Interaction

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Abstract: The platelet glycoprotein receptor regulates the adhesion of blood platelets to damaged blood vessel walls and the subsequent platelet aggregation. One of the subunits, platelet glycoprotein Ib α (GpIb α), binds thrombin, a serine protease with both procoagulant and anticoagulant activities. Two groups reported the crystal structures of the complex between thrombin and the N-terminal extracellular domain (leucine-rich repeat [LRR] domain) of GpIb α . In both these structures, GpIb α was reported to bind two thrombin molecules, but both the primary and secondary thrombin binding sites differed between them. We performed a detailed comparison of the two structures to look for insights that may explain the differences. Our results show that the 1:1 GpIb α -thrombin complex detected in solution between the crystallized proteins is likely the only strong interaction. The anionic sequence (residues 268-284) of GpIb α is likely responsible for the initial interaction with thrombin and the interaction with the rest of LRR domain of GpIb α occurs subsequently and may alternate between two or more different binding modes. Our modelling suggests the interaction between GpIb α and thrombin is highly pH-dependent and a small change in pH is likely to contribute to the formation of alternate binding modes. The differences in the crystal structures reported for the GpIb α -thrombin complex suggest a fascinating plasticity in this protein-protein interaction that may be biologically significant.

Keywords: Crystal structure, platelet glycoprotein Ib α , protein-protein interactions, thrombin.

INTRODUCTION

It has become quite common in the structural biology field that several research groups target the same biological system and therefore end up independently determining the structures of the same protein or protein complex. Differences in experimental approaches and experimental conditions can shed light on interesting functional details that may have been overlooked if only one structure was determined. Generally, structural differences in similar proteins are minor and local over a range of near-physiological conditions. However, the discrepancy of the structures of the complex between the N-terminal extracellular domain (leucine-rich repeat [LRR] domain) of platelet glycoprotein Ib α (GpIb α) and thrombin, reported by two different groups [1,2], is quite unprecedented [3-5].

The platelet glycoprotein (Gp) receptor regulates the adhesion of blood platelets to damaged blood vessel walls and the subsequent platelet aggregation [6-8]. The complex is composed of four transmembrane subunits, GpIb α , GpIb β , GpIX and GpV. GpIb α binds von Willebrand factor (vWF), a major regulator of platelet adhesion [9]. GpIb α also binds thrombin, a serine protease with both procoagulant and anticoagulant activities [5,10,11]. The binding of thrombin to GpIb α results in procoagulant activities, causing platelet aggregation and activation [12,13].

Based on their crystals structures of the GpIb α -thrombin complex, the two groups [1,2] produced a number of similar interpretations. In both cases, one GpIb α molecule was reported to interact with two thrombin molecules (Fig. 1). The binding sites in both complexes mapped to similar regions of the GpIb α surface, and the thrombin molecules used a similar portion of their surfaces in binding (Fig. 2). In both cases there was one "primary" binding site (where the interacting surface was largest; the corresponding thrombin molecule is termed here thrombin 1), while the interface in the other pair was smaller. However, a closer inspection revealed that both GpIb α -thrombin binding interfaces in both complexes were in fact different. The contacts made between GpIb α and thrombin did not match in any of the interfaces. It is quite extraordinary that despite the differences, both structures managed to reconcile much of the conflicting biochemical data in the literature describing the interaction, and to lead the authors of both publications to offer similar interpretations of the biological implications of the structural results. The functional interpretations with regards to thrombin inducing crosslinking of GpIb α molecules on the same platelet [1] or causing aggregation of platelets [2] could conceivably be made by either structure.

The disparity of the reported GpIb α -thrombin complex structures is one of the first examples of two independently determined structures being different to such an extent. Interestingly, little in terms of follow-up structural and functional studies attempting to clarify the discrepancies has been reported to date. In an attempt to understand the molecular basis of the discrepancies, we performed a detailed comparison of the two structures; we looked for clues that may explain the structural variation with changing conditions and

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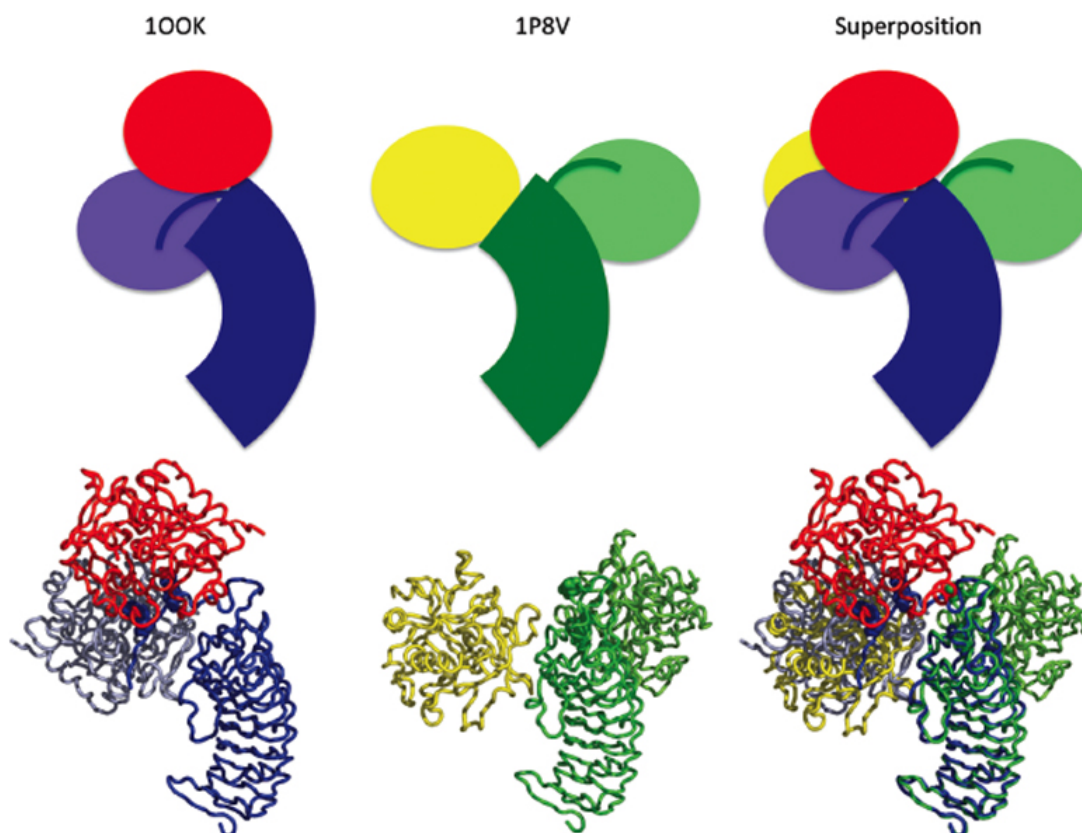


Fig. (1). GpIb α -thrombin complexes in 100K and 1P8V crystal structures. Top row, schematic diagrams; thrombin is represented by an oval, and GpIb α by a block arc, with its C-terminal region indicated as a thin protrusion. Bottom row, ribbon diagrams. Left column, 100K: blue, GpIb α -1; light blue, thrombin 1; red, thrombin 2. Middle column, 1P8V; green, GpIb α -1; light green, thrombin 1; yellow, thrombin 2. Right column, superposition of 100K and 1P8V structures. The structures are shown in the same orientation after superposition of GpIb α residues 1-265 (rmsd 0.871 Å). The molecules present in the asymmetric unit of the respective crystals structures, as defined in the deposited coordinate files, are termed GpIb α 1 and thrombin 1. The structure figures were produced using the program PyMOL (DeLano Scientific LLC).

shed light on the biological implications of the divergent structures.

COMPARISON OF INTERFACES BETWEEN NEIGHBOURING MOLECULES IN THE CRYSTALS

A complication inherent with analyses of protein-protein complexes in crystal structures is that individual molecules form contacts not only with their binding partners, but also contact the neighbouring molecules in the crystal. In the majority of cases, the biologically relevant interactions can be distinguished from the non-relevant ones by comparing the solvent-accessible surface areas buried in the interface; the biologically relevant buried surface areas are generally larger [14]. “Standard-size” protein-protein interfaces in stable protein-protein complexes bury around $1,600 \pm 400 \text{ Å}^2$, and the smallest interfaces considered to be biologically relevant around $1,150 \text{ Å}^2$ [15]. Table 1 shows a comparison of the interfaces corresponding to all molecules contacting one GpIb α molecule in the crystals (Fig. 3), in terms of the number of atom-atom contacts in the interfaces, buried surface areas, and complementarity values.

In the crystal structure 100K [1], one GpIb α molecule contacts four different thrombin molecules (Table 1, Fig.

3A-B). One GpIb α -thrombin interface buries a large surface area (GpIb α 1 - thrombin 1; Table 1) exceeding $2,000 \text{ Å}^2$. Two symmetry-related thrombin molecules form further GpIb α -thrombin interfaces of $>1,000 \text{ Å}^2$, and there is one additional smaller interface. Two symmetry-related thrombin molecules that contact each other bury a surface area of almost $1,500 \text{ Å}^2$.

In the crystal structure 1P8V [2], there are three GpIb α - thrombin interfaces (Table 1, Fig. **3C-D**). The largest interface (GpIb α 1 - thrombin 1) buries close to $2,000 \text{ Å}^2$ of surface area; the corresponding number for an alternative interface with thrombin 2 is $1,400 \text{ Å}^2$, while the third interface (with thrombin 3) is very small. There is a small thrombin-thrombin interface in the crystals, and two different GpIb α -GpIb α interfaces, one exceeding $1,000 \text{ Å}^2$.

Thrombin-thrombin and GpIb α -GpIb α interactions are likely non-specific, as these proteins have not been reported to form homo-oligomeric structures. However, these “non-specific” interfaces can be as large as $1,500 \text{ Å}^2$ for the largest thrombin-thrombin interface in 100K, and $1,000 \text{ Å}^2$ for the largest GpIb α -GpIb α interaction in 1P8V (Table 1). Notably, there are no GpIb α -GpIb α contacts in the 100K crystals, and only a very small thrombin-thrombin contact in the

100K GPIb α	HPICEVSKVASHLEVNC DKRNL TALPPDL PKD TTILH LSEN LLYTFSLAT	50
1P8V GPIb α	HPICEVSKVASHLEVNC DKRDL TALPPDL PKD TTILH LSEN LLYTFSLAT	50
100K GPIb α	LMPYTRLTQ LNLDR AELTK LQVDG TLPVL GTLD LSHNQLQSL PLLGQ TLP	100
1P8V GPIb α	LMPYTRLTQ LNLDR CELTK LQVDG TLPVL GTLD LSHNQLQSL PLLGQ TLP	100
100K GPIb α	ALTVDV SFNRLTSL PLGAL RGLGELQ ELYLKG NELKTLP PGLLTPTPKL	150
1P8V GPIb α	ALTVDV SFNRLTSL PLGAL RGLGELQ ELYLKG NELKTLP PGLLTPTPKL	150
100K GPIb α	<u>E</u> KLSLANNNLTEL PAGLLNGLE <u>N</u> LD TLL LQENS LYTIPK GFFG <u>S</u> HLLPFA	200
1P8V GPIb α	<u>E</u> KLSLANNNLTEL PAGLLNGLE <u>N</u> LD TLL LQENS LYTIPK GFFG <u>S</u> HLLPFA	200
100K GPIb α	FLHGNP WLCNCEILY FRRWLQ <u>D</u> NAE <u>N</u> VYVWKQGV DVKAMTSN VASVQCDN	250
1P8V GPIb α	FLHGNP WLCNCEILY FRRWLQ <u>D</u> NAE <u>N</u> VYVWKQGV DVKAMTSN VASVQCDN	250
100K GPIb α	SDKFPVYKYPGKG CPTLGDEGDT <u>T</u> DL <u>Y</u> DY <u>Y</u> PEEDT <u>E</u> GDKVR	290
1P8V GPIb α	SDKFPVYKYPGKG CPTLGDEGDT <u>T</u> DL <u>Y</u> DY <u>Y</u> PEEDT <u>E</u> GDK	288
100K thrombin (heavy chain)	IVEGSDAEIGMSPWQV MLFR <u>K</u> SPQELLCGAS LISDRWVLTAAHCLLYPPW	50
1P8V thrombin (heavy chain)	IVEGSDAEIGMSPWQV MLFR <u>K</u> SPQELLCGAS LISDRWVLTAAHCLLYPPW	50
100K thrombin (heavy chain)	DKNFTENDLLVRIGKHSRTRY <u>E</u> RNIEKISMLEKIYIHPRYNWRENLD <u>R</u> DI	100
1P8V thrombin (heavy chain)	DKNFTENDLLVRIGKHSRTRY <u>E</u> RNIEKISMLEKIYIHPRYNWRENLD <u>R</u> DI	100
100K thrombin (heavy chain)	ALMKLK <u>K</u> PVAFSDYIHPVCLPD <u>R</u> ETAASLLQAGYKGRVTGWGNL <u>K</u> ETWTA	150
1P8V thrombin (heavy chain)	ALMKLK <u>K</u> PVAFSDYIHPVCLPD <u>R</u> ETAASLLQAGYKGRVTGWGNL <u>K</u> ETWTA	150
100K thrombin (heavy chain)	NVGKGQPSVLQV VNLPIVERPVCKDSTRIRITD <u>N</u> MFCAGYKPDEGKRGDA	200
1P8V thrombin (heavy chain)	NVGKGQPSVLQV VNLPIVERPVCKDSTRIRITD <u>N</u> MFCAGYKPDEGKRGDA	200
100K thrombin (heavy chain)	CEGDSGGPFVMKSPFNRRWYQMGIVSWGEGCDRDGKYGFYTHV <u>F</u> RLK <u>K</u> WI	250
1P8V thrombin (heavy chain)	CEGDSGGPFVMKSPFNRRWYQMGIVSWGEGCDRDGKYGFYTHV <u>F</u> RLK <u>K</u> WI	250
100K thrombin (heavy chain)	<u>Q</u> KVIDQFGE	259
1P8V thrombin (heavy chain)	<u>Q</u> KVIDQFGE	259

Fig. (2). GPIb α -thrombin contacts. The residues forming contacts at distances < 4 Å are coloured as in Fig. 1 (100K: GPIb α -thrombin 1, light blue; GPIb α -thrombin 2, red; 1P8V: GPIb α -thrombin 1, light green; GPIb α -thrombin 2, yellow), and the residues forming polar contacts at distances < 3.5 Å are underlined. 100K GPIb α residues 276, 278 and 279 (highlighted with “*” above the sequence) interact at distances < 4 Å with both thrombin 1 and 2, making polar contacts at distances < 3.5 Å with one of the thrombin molecules (indicated by appropriate colour and underlined) but not the other. Thrombin residues are numbered from the first residue of the heavy chain throughout the manuscript (only the heavy chain interacts with GPIb α).

Table 1. Comparison of All Protein-Protein Contacts in GPIb α -Thrombin Crystals

Protein-protein pair	Number of contacts (< 4 Å)	Buried surface area (Å ²)	Shape complementarity	RPScore	Electrostatic energy (kcal/mol)
<i>Crystal structure 100K</i>					
GPIb α 1-thrombin 1	323	2155	0.717	+2.20	-43.74
GPIb α 1-thrombin 2	73	1157	0.723	+0.30	-26.74
GPIb α 1-thrombin 3	52	1133	0.521	-0.80	-7.07
GPIb α 1-thrombin 4	55	671	0.803	+0.10	-6.43
thrombin 1-thrombin 2	113	1479	0.643	+1.40	-6.58
<i>Crystal structure 1P8V</i>					
GPIb α 1-thrombin 1	237	1940	0.680	-0.20	-43.72
GPIb α 1-thrombin 2	71	1396	0.573	+3.10	-29.37
GPIb α 1-thrombin 3	1	45	0.683	-0.20	-3.16
thrombin 1-thrombin 4	12	259	0.757	+0.30	1.85

(Table 1) contd...

Protein-protein pair	Number of contacts (<4 Å)	Buried surface area (Å ²)	Shape complementarity	RPScore	Electrostatic energy (kcal/mol)
GPIbα 1-GPIbα 2	76	1048	0.671	+0.70	-12.73
GPIbα 1-GPIbα 3	11	413	0.512	+0.30	-5.17

All interacting pairs of molecules in the crystals were generated using the crystallographic symmetry operations. Carbohydrates and thrombin inhibitors were considered a part of the associated molecule. Contacts were analyzed with the program CONTACT [26]. Buried surface areas were calculated using the program SURFACE [26]. Shape complementarities were calculated using the program SC [27]. RPScore is the residue pair potential score [28] and indicates the likelihood of trans-interface pairs of residues in the interface. Electrostatic energy refers to the electrostatic contribution to the free energy of binding as calculated by the program FastContact [24], which is based on Coulomb electrostatics utilizing a distance-dependent dielectric constant. CHARMM19 parameters were used to assign partial charges and van der Waals radii of the atoms. Each sulfated tyrosine residue was assigned a total charge of -1.0. Calculations were then performed with FastContact default parameters for all pairs of proteins as they are found in the crystal structures.

1P8V crystals. The primary GPIbα-thrombin interfaces in both crystals are larger than an average “standard-size” interface [15], and extremely unlikely to be non-specific; all other contacts observed in the crystals would be considered questionable in terms of biological relevance, considering their size; this includes the second largest GPIbα-thrombin interface in each of the two crystals forms [14]. Notably, the GPIbα 1-thrombin 2 interface in 1P8V crystals is smaller than a thrombin-thrombin interface found in 1OOK crystals.

Thrombin 2 in 1OOK crystals simultaneously contacts both GPIbα and thrombin 1 bound to the same GPIα protein; in combination, the two interfaces add up to 2,636 Å². This means that the binding of one thrombin molecule to GPIα creates an extensive binding site for another thrombin molecule, and supports a sequential binding hypothesis proposed by the authors.

BINDING TO FUNCTIONAL REGIONS OF THROMBIN

Substrate specificity in thrombin is determined by its catalytic site and two positively charged areas on the same face of the molecule, termed exosite I and II [16,17]. Exosite I binds ligands such as thrombomodulin and substrates such as fibrinogen, while exosite II binds heparin (facilitating inhibition by antithrombin); factor V and factor VIII bind to both exosites [18].

None of the observed binding modes of thrombin to GPIbα obstructs the catalytic site of thrombin. Thrombin 1 in 1P8V binds to the concave region towards the C-terminal end of the LRR domain, using exosite II. The involvement of this exosite has been implicated through site-directed mutagenesis [19,20] and is consistent with induction of platelet adhesion [5,13]. Thrombin 3 in 1OOK binds in a similar area of GPIbα, although the binding does not involve any of the exosites (Fig. 3). Thrombin 1 in 1OOK binds to the C-terminal end of the LRR domain, using exosite I. Thrombin 2 in 1P8V binds to a similar region of GPIbα, also using exosite I, although the two thrombin molecules are rotated for about 180° relative to one another. Thrombin 2 in 1OOK crystals simultaneously interacts with the C-terminal end of GPIbα and thrombin 1, using exosite II.

The structure of the complex between the N-terminal domain of GPIbα and the A1 domain of von Willebrand factor [21] shows that thrombin 1 in 1OOK and thrombin 2 in 1P8V, but not any other thrombin molecules bound to GPIbα in these crystals, would obstruct the A1 domain binding to GPIbα.

INVOLVEMENT OF THE C-TERMINAL REGION OF GPIbα CONTAINING SULFATED TYROSINE RESIDUES

An anionic sequence found in the C-terminal region of the LRR domain of GPIbα has been found to play an important role in the GPIbα-thrombin interaction [6]. This sequence contains three tyrosine residues that undergo post-translational sulfation. In both crystal structures, the primary GPIbα-thrombin interface involves this anionic sequence of the GPIbα molecule; however, the anionic region has swung into different positions (Fig. 1), and therefore thrombin is bound in a different juxtaposition relative to the bulk of GPIbα. In 1P8V, the anionic sequence (residues 268-284) contributes ~1,200 Å² to the interface, and several positively charged thrombin residues from exosite II compensate the negatively charged sulfate groups. In 1OOK, the anionic sequence similarly contributes a substantial area (1,000 Å²) to the interface. However, although the sulfates form favourable contacts with thrombin residues, there does not appear to be the same degree of charge complementarity in terms of basic residues from thrombin interacting with GPIbα. In 1P8V, the residue Asp277 and the sulfated tyrosines Tyr276 and Tyr279 form six contacts at <3.5 Å with polar atoms of the side chains of positively charged thrombin residues (including Arg98, Arg123, Lys248 and Lys252). Sulfated Tyr278 (not present in the model; see next section) could also conceivably make favourable interactions with positively charged residues in this structure. In 1OOK by contrast, the acidic region makes most favourable contacts with the thrombin 2 molecule (that is not the thrombin molecule exhibiting the largest interface), comprising three contacts at <3.5 Å with polar atoms of side chains of positively charged thrombin residues (Asp277 and sulfated Tyr276 with thrombin Arg98 and Arg123). The K248E mutation in thrombin has been found to reduce the affinity for GPIbα 25-fold, and the dependence of the interaction on ionic strength suggested the involvement of about four ionic interactions [19]; the structure 1P8V therefore appears to be more consistent with these observations [5].

Comparison of various structures of GPIbα alone and in complex with different ligands sheds light on the structural attributes of the C-terminal anionic sequence containing the sulfated residues. In most free structures of the N-terminal fragment of GPIbα (1GWB [22], 1P9A [1], and 1M0Z [21]), the C-terminal region beyond residue 266 is not visible in the electron density map and is presumed disordered; this region is only present in the model of one of the molecules in the

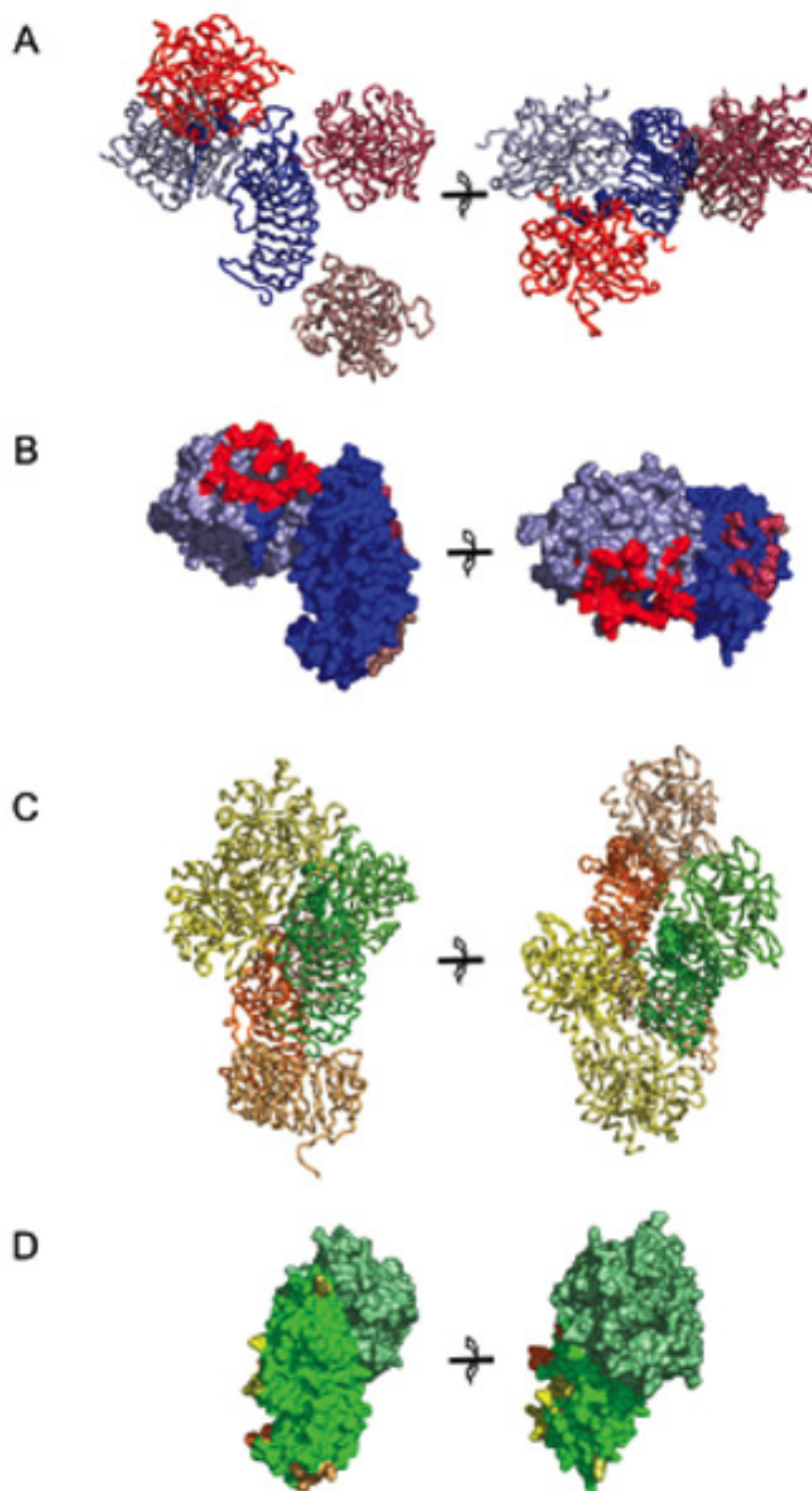


Fig. (3). All protein-protein interactions in 100K and 1P8V crystals. (A) All molecules contacting GpIb α 1 and thrombin 1 in the 100K crystals. GpIb α 1, blue; thrombin 1, light blue; thrombin 2, red; thrombin 3, raspberry; thrombin 4, dark salmon. The two views are related by a 90° rotation around the x-axis. The left orientation is analogous to Fig. 1. The molecules are shown in ribbon representation. The side chains of the sulfated Tyr276 and Tyr279 are shown in sphere representation. (B) Surface representation of the GpIb α 1 (blue)-thrombin 1 (light blue) complex in 100K crystals. The GpIb α 1 and thrombin 1 residues interacting with other molecules in the crystals are coloured as in A: thrombin 2, red; thrombin 3, raspberry; thrombin 4, dark salmon. (C) All molecules contacting GpIb α 1 and thrombin 1 in the 1P8V crystals. GpIb α 1, green; GpIb α 2, orange; GpIb α 3, light orange; thrombin 1, light green; thrombin 2, yellow; thrombin 3, pale yellow; thrombin 4, wheat. The orientation is the same as in A after superposition of GpIb α residues 1-265. (D) Surface representation of the GpIb α 1 (green)-thrombin 1 (light green) complex in 1P8V crystals. The GpIb α 1 and thrombin 1 residues interacting with other molecules in the crystals are coloured as in C: GpIb α 2, orange; GpIb α 3, light orange; thrombin 2, yellow; thrombin 3, pale yellow; thrombin 4, wheat.

asymmetric unit in 1GWB, where the last modelled residue corresponds to residue 279. In this case, the anionic sequence is found in a similar juxtaposition with the rest of GpIb α as in 1P8V; however, the detailed conformations and the contacts between the anionic sequence and the bulk of the LRR domain of GpIb α differ. Uff *et al.* [22] suggested that the observed structure of the anionic sequence is in equilibrium with a more extended structure in solution, taking advantage of the flexibility of a hinge comprising residues 269-272. The anionic sequence is disordered also in the structure of GpIb α in complex with the A1 domain of vWF [21]. In conclusion, the available GpIb α structures suggest that the C-terminal region can sample several conformations, including a disordered conformation making no contacts with the rest of the molecule, and thrombin binding does not appear to stabilize one specific conformation either.

RECOMBINANT PROTEINS USED FOR STRUCTURAL STUDIES

One possible explanation for the discrepancies of the 100K and 1P8V structures could be a difference in the recombinant protein fragments used in the study. Human α -thrombin was obtained in both cases from the same commercial source. The catalytic site of the protease was blocked by different inhibitors (PPACK in 100K [1] or DFP in P8V [2]); however, the inhibitor and the catalytic site do not contribute to GpIb α binding in any of the interfaces found in the crystals. The GpIb α fragments were expressed in *Drosophila melanogaster* cells for 100K study [1], or CHO cells as a fusion protein with human IgG1 for 1P8V study [2], and contained residues 1-290 and 1-288 of mature human GpIb α , respectively. The protein in 100K crystals additionally contained a C65A substitution intended to eliminate an unpaired cysteine (this mutation was reported not to have an effect on the function), while the protein in 1P8V crystals contained the mutations N21D and N159D that removed two glycosylation sites (these residues are remote from thrombin binding sites). Dumas *et al.* [2] report that they only used the protein containing three sulfated residues for crystallization, and that the protein containing only two sulfated residues was removed by purification. The deposited coordinates of both 100K and 1P8V models contain sulfate groups on residues 276 and 279, but not 278. Although there are differences in protein reagents used in the two studies, none of the differences appear significant enough to explain the different binding modes.

CRYSTALLIZATION CONDITIONS

Another possible reason for the difference in the two crystal structures could be the physical environment resulting from different crystallization conditions. In the case of 100K, the crystallization conditions involved a protein solution at the concentration of 25 mg/ml, the reservoir solution contained 12% PEG 6000 and 100 mM ammonium phosphate (pH 7), and the temperature was 4 °C. The respective conditions for 1P8V were 10 mg/ml, 14% PEG 400, 100 mM MES (pH 6), and 18 °C. Again, while the two environments clearly differ, they are not drastically dissimilar and do not clearly point to any variations that could be responsible for the different binding modes.

ELECTROSTATIC DIFFERENCES BETWEEN THE STRUCTURES

We conclude that electrostatic phenomena appear to be the most likely reason for the differences between the 100K and 1P8V structures. The effect of the pH of the crystallization solution was therefore analysed in more detail. The electrostatic energies for all crystal interfaces were calculated over the pH range 4.5 to 9.0, using the Adaptive Poisson-Boltzmann Solver package [23]. This software finds a numerical solution of the Poisson-Boltzmann equation (PBE), one of the most popular models for describing electrostatic interactions between molecules in ionic aqueous solutions.

The total electrostatic energies of all interfaces in the 100K crystals revealed a sharp increase in electrostatic energy below and above pH 6, indicating that the structure is most stable at this pH (Fig. 4A). By contrast, the 1P8V crystals showed the lowest energies at pH values 5–5.5 (Fig. 4B). These results correlate with crystallization conditions; the 100K crystals were obtained at pH 7, while 1P8V crystals were obtained at a lower pH (pH 6). Unfortunately, the stability of the crystals does not directly relate to interactions occurring in a biologically relevant situation. The crystal structure 100K [1] was produced under pH conditions closer to the physiological blood pH level (pH 7.4); however, we need to analyze individual interfaces to shed light on the biological relevance of individual interactions. The electrostatic energy curves as a function of pH for the two largest GpIb α -thrombin interfaces in each of the crystal (Fig. 4C, 4D) show similar trends as the all-crystal-interface curves. Although the calculated absolute electrostatic energy values should be viewed only as rough approximations, they do support a better complementarity of the major interface in 1P8V, when compared to 100K.

To shed further light on the electrostatic complementarities in different interfaces, we also calculated the electrostatic energies of binding using the program FastContact [24]. This software estimates the electrostatic and desolvation component of the free energy based on a distance-dependent dielectric and an empirical contact potential for the desolvation contribution, developed using a database of crystal structures. The most favourable energies were observed for the two largest GpIb α -thrombin interfaces in either crystal structure (Table 1). Interestingly, both calculated energy values are of very similar size. However, the sulfated tyrosines make a significant contribution only to the energy of the largest interface in 1P8V, but not in 100K; the calculated electrostatic energies in absence of sulfates are -46.06 and -28.38 kcal/mol for 100K and 1P8V, respectively. This observation agrees with the conclusion above that the structure 1P8V is more consistent with the mutational analysis of this region of the protein.

CONCLUSIONS

The discrepancies of the two crystal structures reported for the GpIb α -thrombin complex is particularly surprising because very similar fragments of proteins were used in the studies, and the crystallization conditions do not differ drastically. Both structures appear to have been determined at a satisfactory technical standard. For these reasons, the dis-

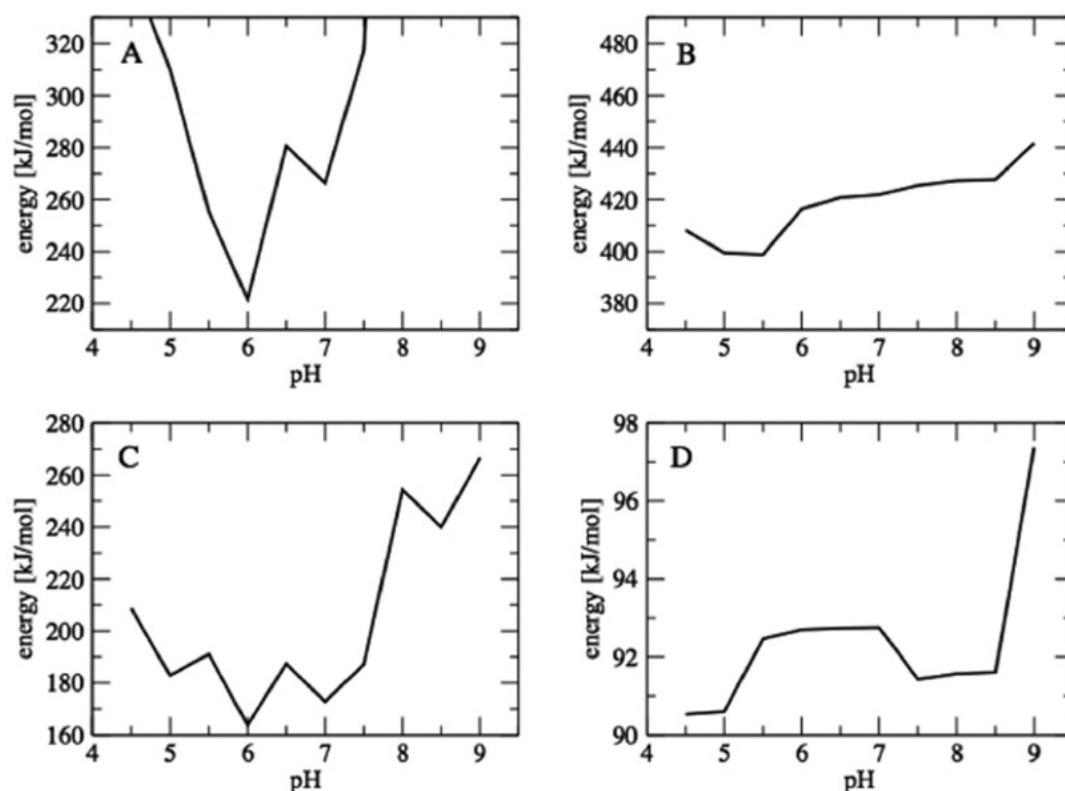


Fig. (4). Electrostatic energy of binding as a function of pH, calculated by numerically solving the Poisson-Boltzmann equation. Electrostatic energies were first calculated for each of the interfaces that occur in the crystals. The results were then added for all interfaces to calculate the total electrostatic energy of binding. The Protein Data Bank files were transformed into pqr format using the pdb2pqr program [29]. A titration over the pH range of 4.5 to 9.0 at 0.5 pH increments was performed using the program propKa [30]. Hydrogens were added in this conversion step and the Amber force-field was implemented to determine charges and radii. The transformation did not take account for sulfated tyrosines 276 and 279, and these were added separately. The partial charges and radii of SO₄ were assigned according to [31]: sulfur: +1.712; oxygen attached to sidechain (R-O-S): -0.747; remaining oxygens: -0.753. Because the pK_a of SO₄ is 2, charges on this group were not changed over the titrated range. The electrostatic calculations were completed by numerically solving the Poisson-Boltzmann equation using the Adaptive Poisson-Boltzmann solver package (APBS) [23]. The parameters for the calculation were not altered from those automatically determined by the pdb2pqr program. The dielectric constants for the solvent and protein were 78.54 and 2.0, respectively. The calculations were performed at 298.15 K with a 0.15 M concentration of monovalent ions. (A) Total electrostatic energy of all interfaces in 100K. (B) Total electrostatic energy of all interfaces in 1P8V. (C) Electrostatic energy of the GPIb α 1-thrombin 1 interface in 100K. (D) Electrostatic energy of the GPIb α 1-thrombin 1 interface in 1P8V.

crepancies suggest a fascinating plasticity in binding between the two proteins that may be biologically significant.

The main conclusions resulting from our analyses are as follows. The 1:1 GpIb α -thrombin complex detected for the crystallized fragments in solution is conceivably the only strong interaction. The structures suggest that the anionic sequence is likely the first point of interaction, and the interaction with the rest of LRR domain of GpIb α occurs subsequently and may alternate between two or more different binding modes. The primary GpIb α -thrombin interface in 1P8V crystals appears most consistent with functional studies and performs best according to the structural analyses we present in this paper, compared to all other observed interfaces. The interactions could be studied further by site-directed mutagenesis, and the effects examined by binding assays and cellular studies. For example, GpIb α residue Arg123 and thrombin heavy chain residues Arg68, Glu124 and Lys145 form charged interactions only in one GpIb α -thrombin 1 interface, but not in any other GpIb α -thrombin 1

or GpIb α -thrombin 2 interfaces in 100K and 1P8V crystals (Fig. 2); mutations to alanine or reversing the charge could shed light on the relative importance of the different interactions observed in the crystals.

While some functional evidence suggests that the binding of the second molecule of thrombin may be biologically relevant [1], the structures do not clearly identify the most likely binding site for the second molecule. This does not eliminate the possibility that interactions of GpIb α molecule with more than one thrombin molecule may play a role in the biological context; analogous to the primary binding mode, the binding of the second thrombin molecule may adopt a number of alternative modes.

The analysis suggests that the interaction between GpIb α and thrombin is highly pH-dependent and a small change in pH is likely to contribute to the formation of alternate binding modes. The crystallization conditions used for 100K resemble the physiological pH of 7.4 slightly better than the

conditions used for 1P8V, and the ionic strengths are similar to the physiological conditions in both cases.

The consequences of the interaction of thrombin with GpIb α in the initiation and propagation of coagulation are complex, leading to both prothrombotic and antithrombotic effects. Prothrombotic effects can be caused by (i) the release of factor Va; (ii) the masking of exosite II, resulting in a reduction of the accessibility to heparin-antithrombin complexes; and (iii) the masking exosite I, resulting in a reduction of the accessibility to thrombomodulin and activation of the protein C pathway [18]. Antithrombotic effects can be caused by (i) the masking of exosite I, resulting in reduction of thrombin binding to fibrinogen and the reduction of fibrin formation; and (ii) the masking of both exosites, resulting in a reduction of the activation of factor V, factor VIII and factor XI [18]. Clearly, the complex biological effects of thrombin-GpIb α binding will depend on the concentrations of various components including GpIb α , fibrinogen, factor V, factor VIII and factor XI and their relative affinities for thrombin. One of the important steps in understanding this complex system is to study systematically the molecular recognition of specific pairs of interacting molecules, such as the interaction between thrombin and GpIb α studied by Celikel *et al.* [1] and Dumas *et al.* [2]. The high versatility in this biological system may reflect the requirement to operate efficiently under a number of different physiological conditions, to fulfil its essential role in haemostasis. It appears we may only be at a very early stage of understanding the complex interplay of the recognition events in the process of platelet adhesion.

The case of the divergent structures of the GpIb α -thrombin complex suggests that further examples of divergent structures will undoubtedly arise in the future, especially as more complex biological systems and transient protein-protein complexes are studied. It would be interesting to systematically identify all structures currently available in the Protein Data Bank that exhibit interesting discrepancies. Such an analysis would require the identification of all structures containing identical or similar protein chains, extraction of the biological units of the structures in the case of oligomeric or complex structures, superposition of these structures (for example using the combinatorial extension approach [25]), and identification of the structures that exhibit discrepancies. Such structures will undoubtedly uncover novel concepts in the biology of protein-protein interactions.

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LIST OF ABBREVIATIONS

- 1OOK = Protein Data Bank ID for the GpIb α -thrombin crystal structure by Celikel *et al.* [1]
 1P8V = Protein Data Bank ID for the GpIb α -thrombin structure by Dumas *et al.* [2]
 GpIb α = Platelet glycoprotein Ib α

- LRR = Leucine-rich repeat
 Rmsd = Root mean square distance
 vWF = Von Willebrand factor

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