Probing the β-Chain Hole of Fibrinogen with Synthetic Peptides That Differ at Their Amino Termini[†,‡]

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ABSTRACT: In a recent report, we showed that alanine can replace glycine at the amino terminus of synthetic B-knobs that bind to human fibrin(ogen). We now report a survey of 13 synthetic peptides with the general sequence XHRPYam, all tested with regard to their ability to delay fibrinolysis in an *in vitro* system activated by t-PA, the results being used as measures of binding affinity to the βC hole. Unexpectedly, some large and bulky amino acids, including methionine and arginine, are effective binders. Amino acids that branch at the β carbon (valine, isoleucine, and threonine) do not bind effectively. Crystal structures were determined for two of the peptides (GHRPYam and MHRPYam) complexed with fibrin fragment D-dimer; the modeling of various other side chains showed clashing in the cases of β-carbon substituents. The two crystal structures also showed that the enhanced binding observed with pentapeptides with carboxyl-terminal tyrosine, compared with that of their tetrapeptide equivalents, is attributable to an interaction between the tyrosine side chain and a guanidino group of a nearby arginine (β406). The equivalent position in γ-chains of human fibrin(ogen) is occupied by a lysine (γ338), but in chicken and lamprey fibrin(ogen), it is an arginine, just as occurs in β chains. Accordingly, the peptides GPRPam and GPRPYam, which are surrogate A-knobs, were tested for their influence on fibrin polymerization with fibrinogen from lamprey and humans. In lampreys, GPRPYam is a significantly better inhibitor, but in humans, it is less effective than GPRPam, indicating that in the lamprey system the same tyrosine−arginine interaction can also occur in the γ-chain setting.

In vertebrates, fibrin formation involves two homologous knob−hole interactions. In one case, the thrombin-catalyzed removal of the fibrinopeptide A exposes the sequence Gly-Pro-Arg, a knob-like structure that binds to a hole on the γC domain of neighboring molecules and initiates the polymerization event. In the second, removal of fibrinopeptide B exposes the sequence Gly-His-Arg-, a knob that eventually binds to a hole on the βC domain. Under native conditions, the latter process occurs secondarily, and its importance for the structural stability of the clot is questionable (*1*−*3*). Instead, it has been proposed that its physiological function may have to do with the initiation of fibrinolysis (*4*), a conclusion drawn from the observation that synthetic B-knobs enhance the turbidity of fibrin clots and delay their subsequent fibrinolytic destruction. It was also found that adding a tyrosine to the carboxyl terminus of the most often used synthetic B-knob, GHRPam, increases the turbidity effect and greatly exaggerates the delay in fibrinolysis (*4*).

The finding that chicken fibrin B-knobs have alanine instead of glycine (*5*) led to the realization that the interaction between the B-knob and the βC hole is less specific than the interaction between the A-knob (Gly-Pro-Arg) and the γC hole. Not only does chicken fibrinogen bind GHRPam in its βC hole (*6*), but human fibrinogen and its fragments bind AHRPam in theirs also (*7*). In no case was it found that an amino-terminal alanine could be accommodated by the γC hole. The basis of the difference in binding specificity for the two homologous sites was found to reside in the reduced flexibility of a single key aspartyl side chain deep in the γ-chain hole (*7*). It is significant that in the absence of A-knobs (GPRPam[1]), GHRPam also binds to the γC hole, but the same is not true of knobs with amino-terminal alanine (*7*, *8*).

The question arose, is the βC hole promiscuity unlimited when it comes to the amino-terminal residue? To answer this question, we have examined 11 additional peptides with regard to their ability to enhance clot turbidity and delay fibrinolysis. In line with the finding that the addition of a carboxyl-terminal tyrosine to B-knobs greatly increases their effectiveness (*4*, *7*), the synthetic peptides had the general sequence XHRPYam, where X was any of 13 amino acids, including glycine and alanine.

The structural basis of how tyrosine increases binding was established from crystal structures of fragment D-dimer

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[‡] The atomic coordinates and structure factors for the new structures described in this article have been deposited in the Protein Data Bank (DD-MHRPY = entry 2Q9I; DD-GHRPY = 2Z4E).

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[1] Abbreviations: GHRPYam, Gly-His-Arg-Pro-Tyr-amide or XHRPYam, where X is M, A, S, R, L, K, E, F, T, N, I, V, or acetyl; GPRPam, Gly-Pro-Arg-Pro-amide; GHRPam, Gly-His-Arg-Pro-amide; PEG, polyethylene glycol; MPD, methylpentanediol; t-PA, tissue plasminogen activator; CNS, crystallographic and NMR system.

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complexed with GHRPYam in one instance and MHRPYam in another. As it happens, cocrystallization of the D-dimer with the pentapeptides was never successful, and these structures were obtained by soaking the tyrosine-ending peptides into particularly sturdy crystals that had initially been formed in the presence of the tetrapeptide, GHRPam (*9*).

As predicted (*4*), a nearby arginine side chain was found to interact with the tyrosine side chain. In mammals, the equivalent position in the γ chain is occupied by a lysine, but in chicken and lamprey, it is an arginine, just as occurs in the βC situation, presenting an opportunity for comparing the effect of these two basic residues. Accordingly, we tested the synthetic A-knobs GPRPam and GPRPYam on fibrin formation in humans and lampreys, with the expectation that the tyrosine-ending pentapeptide would be a better binder in the lamprey case but not necessarily so in the human system.

MATERIALS AND METHODS

The synthetic peptides MHRPYam, AHRPYam, RHRPYam, SHRPYam, LHRPYam, KHRPYam, EHRPYam, FHRPYam, THRPYam, NHRPYam, IHRPYam, and VHRPYam were purchased from Sigma-Genosys. The peptides Gly-Pro-Arg-Pro-amide, Gly-His-Arg-Pro-amide, and Gly-His-Arg-Pro-Tyr-amide were those described in earlier publications from this laboratory (*4*, *8*, *9*, *10*) and had been synthesized by the BOC-procedure (*11*). The concentrations (concns) of tyrosine-containing peptides were determined spectrophotometrically at $\lambda = 274.5$ using a molar extinction coefficient of 1340 M^{-1} cm^{-1} (*12*). Acetylated peptides were prepared from stock solutions of peptides as follows: aliquots were freeze-dried and then redissolved in 50 μL glacial acetic acid, warmed to 100 °C in glass-stoppered tubes, and treated with 25 μL acetic anhydride for 2 min before being quenched with 500 mL of H_2O. Preparations were freeze-dried, redissolved in water, and aliquots shown to be ninhydrin-negative.

Fibrinogen was prepared from human blood plasma as described previously (*10*); protocols for the purification of fragments D and D-dimer have also been fully described in previous publications (*10*). Human thrombin was purchased from Enzyme Research Corp.; t-PA was obtained from Sigma-Genosys. The details of the assay involving thrombin-fibrinogen and thrombin-tPA-fibrinogen have been described in a recent publication (*4*). Briefly put, human fibrinogen, thrombin, t-PA, and appropriate peptide additives were mixed in disposable cuvettes and the turbidity followed at 350 nm.

Half-Lysis-Time-Ratios. The half-lysis time was defined as the point where the clot turbidity dropped to one-half its maximum. The half-lysis-time-ratio was the ratio of the half-lysis time in a system with peptide additive compared with that of the control with no peptide.

Fibrin Polymerization Assays. The effect of the peptides GPRPam and GPRPYam on thrombin-catalyzed fibrin formation in human and lamprey systems was evaluated by monitoring the turbidity of appropriately mixed thrombin and fibrinogens. Lamprey thrombin and fibrinogen were prepared as described previously (*13*). In both the lamprey and human systems, the fibrinogen concn was 3 μM; thrombin concns were determined empirically to give approximately the same clotting times in the two systems.

Crystal Preparation. Numerous attempts were made to obtain crystals of the tyrosine-ending pentapeptides (XHRPYam) with fragments D or D-dimer from human fibrin(ogen) (>1000 drops set with GHRPYam, MHRPYam, AHRPYam, SHRPYam, and RHRPYam over the course of a year). Because none were successful, recourse was made to soaking peptides into the most reliable crystals in our experience, those formed in the presence of the tetrapeptide GHRPam (*9*). These were grown at room temperature from sitting drops containing equal volumes of a 9 mg/mL D-dimer in 0.05 M Tris at pH 7.0, 5 mM $CaCl_2$, containing 4 mM GHRPam, and well solution composed of 0.05 M Tris at pH 8.0, containing 9−12% PEG, 2.5−10 mM $CaCl_2$, and 2 mM sodium azide. In the case of soaking with GHRPYam, the DD-GHRPam crystals were simply transferred to new drops containing 1 mg/mL D-dimer, 0.3 mM GHRPYam, 4.5% PEG, 5 mM $CaCl_2$, and 1 mM sodium azide. In the case of soaking with MHRPYam, the mother liquor was removed from the D-dimer-GHRPam drops in a stepwise manner and replaced with an equivalent solution containing 0.6 mM MHRPYam, 2 mg/mL D-dimer, 4.5% PEG, 5 mM $CaCl_2$, and 1 mM sodium azide. In both cases, soaking was conducted overnight, after which MPD was added as a cryoprotectant to a final concn of 15% and the crystals frozen with liquid nitrogen. If the soaked crystals were not frozen within 20 h, they deteriorated beyond use.

X-ray diffraction data for D-dimer complexed with MHRPYam were collected at the UCSD X-ray Crystallography Facility; data for D-dimer complexed with GHRPYam were collected at the Advanced Light Source, Lawrence Berkeley National Laboratory (Berkeley, CA). Data were processed with Denzo and Scalepack (*14*). In both cases, it was possible to refine structures directly after rigid body placement of the model DDGH (PDB ID 1FZF) with CNS (*15*). Ligands were modeled in electron density with the modeling program package O (*16*). Illustrations were prepared with the aid of PyMol (*17*).

RESULTS

Effectiveness of Various XHRPYam Peptides in Delaying Fibrinolysis. The nature of the amino-terminal residue in XHRPYam peptides greatly influences both the degree of turbidity enhancement during fibrin formation and the delay in fibrin-stimulated, t-PA-activated fibrinolysis. Although glycine remains the most effective terminal residue, methionine is almost as good, followed by alanine and serine (Figure 1A). Under the conditions of the assay, all four of these pentapeptides have a significant effect at concentrations as low as 20 μM. In contrast, valine and isoleucine have virtually no effect even at 2400 μM (Figure 1B; Figure 2). Seven other amino acids fell in between these extremes (Figure 1C; Figure 2). The one clear trend was that substitution at the β-carbon greatly diminishes the effectiveness of peptides. Thus, serine was much more effective than threonine and leucine considerably more so than isoleucine or valine (Figure 2). Other effects were more idiosyncratic, as reflected by arginine being better than lysine. Glutamic acid (EHRPYam) was less effective than asparagine (NHRPYam) or threonine (THRPYam) (Figure 2).

Acetylated Peptides Are Inactive. Acetylation of the peptide GHRPYam completely abolished its ability to

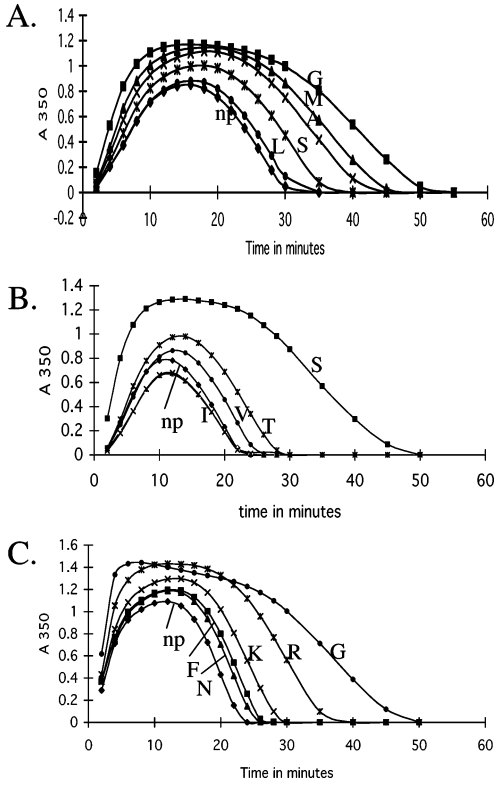

FIGURE 1: Relative effectiveness of synthetic B-knobs in delaying the fibrin-stimulated tPA-activation of fibrinolysis. The concentration of human fibrinogen at time-zero was 3.3 μM. A mix of thrombin and t-PA was added such that their final concentrations were 0.08 μg/mL and 0.29 μg/mL, respectively. (A) Final peptide concn = 20 μM; GHRPYam (G); MHRPYam (M); AHRPY (A), SHRPY (S), LHRPYam (L); control = no peptide (np). (B) Final peptide concns of THRPYam (T), VHRPYam (V), and IHRPYam (I) were all 1200 μM; SHRPYam (S) = 200 μM; control = no peptide (np). (C) Peptide concn for NHRPYam (N) and FHRPYam (F) = 400 μM; peptide concn for GHRPYam (G), RHRPYam (R), and KHRPYam (K) = 200 μM; control = no peptide (np).

enhance turbidity or delay fibrinolysis, even when the final concn was 1000 μM. Similar results were obtained with acetylated LHRPYam and also *N*-acetyl-HRPYam, which is the equivalent of deaminated GHRPYam (data not shown).

Attempts to Cocrystallize XHRPYam Peptides with Fragments D or D-Dimer. In spite of more than 1000 attempts, it was not possible to obtain diffraction-grade crystals of human (or lamprey) fragments D or D-dimer by cocrystallization with the tightly binding pentapeptides (GHRPYam, MHRPYam, AHRPYam, SHRPYam, RHRPYam, or LHRPYam). In the case of D-dimer, the tightly binding peptides invariably gave showers of microcrystals or needles within hours of being set, even in the absence of a precipitant (Figure 3A). The peptides VHRPYam and IHRPYam did not produce such showers, presumably because these peptides do not bind at all.

Numerous remedies were attempted, including the addition of osmolytes, denaturants, and organic solvents as well as varying peptide, protein, and calcium concns, ionic strengths, and pH. Although needle showers could be prevented by adding the A-knob GPRPam, suitable crystals were never obtained in such mixtures. Accordingly, an alternative strategy was undertaken in which peptides were soaked into D-dimer crystals that had been prepared with the tetrapeptide GHRPam (Figure 3B).

Crystal Structure of Fragment D-Dimer Complexed with GHRPYam. The structure of fragment D-dimer from human fibrinogen complexed with the synthetic peptide GHRPYam was determined at 2.7 Å (Table 1). All four holes (B and E chains have βC holes; C and F chains have γC holes) were found to contain electron density. However, the electron density in the β-chain holes clearly corresponded to GHRPYam (Figure 4A), whereas that in the γ-chain holes was wholly attributable to GHRPam bound in the initial crystallization. In the latter case, there was no sign of a fifth residue (i.e., tyrosine). In the βC holes, the tyrosine side chain of the ligand is juxtaposed to the guanidino group of arginine β406. As for the other interactions between the ligand and host, they appear to be identical to those observed with GHRPam (*7*, *9*).

Crystal Structure of Fragment D-Dimer Complexed with MHRPYam. As anticipated, the ligand MHRPYam is restricted to the β-chain holes, although some lingering density corresponding to residual GHRPam was observed in the γC hole of the C chain. The latter density was not attributable to MHRPYam on two counts: first, there was no evidence of a side chain of any sort at position-1, nor was there any density at all beyond proline-4.

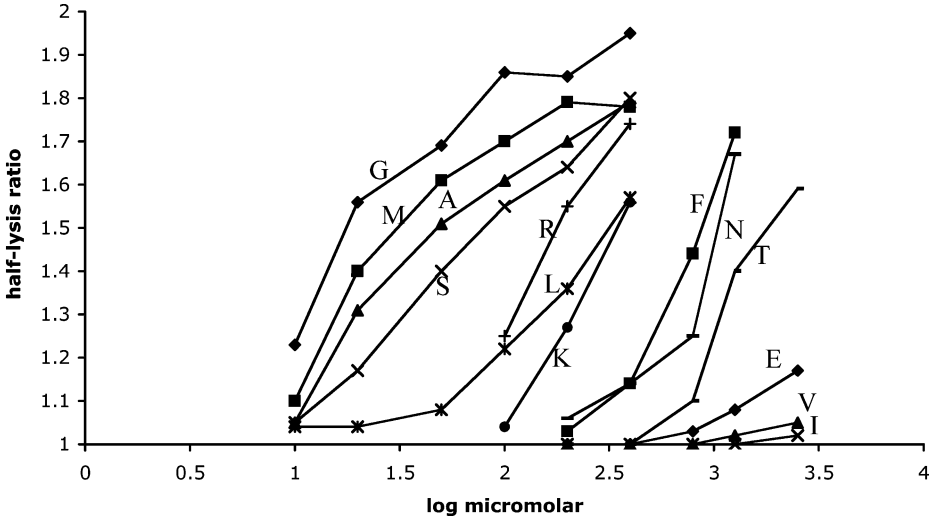

FIGURE 2: Plot of half-lysis-time-ratios (see Materials and Methods) vs log peptide concn (taken from data that include those shown in Figure 1 and many more).

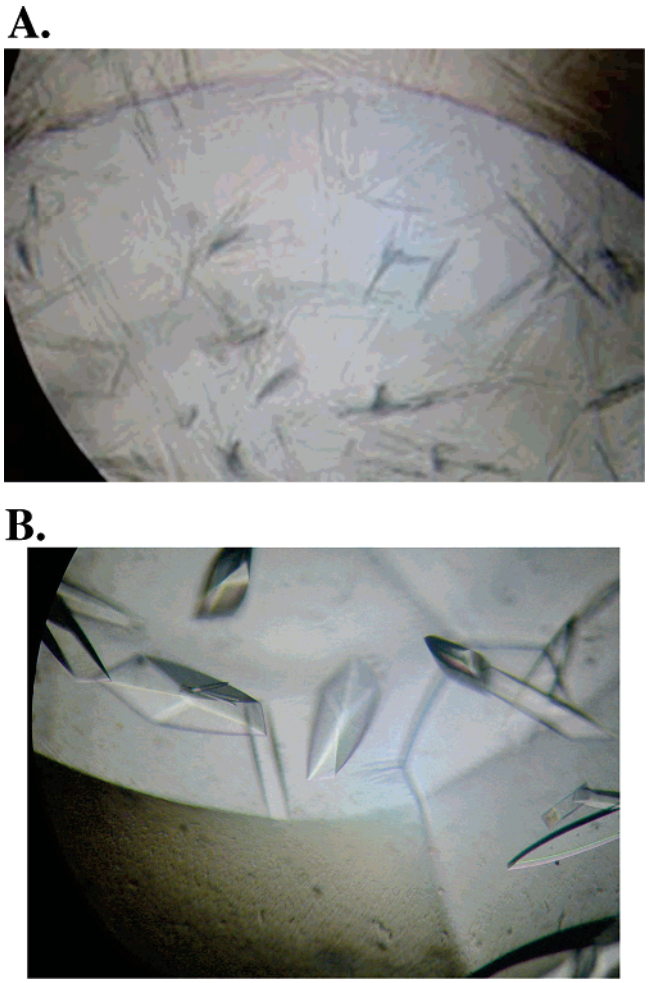

FIGURE 3: Differences in crystal forms of D-dimer brought about by having carboxyl-terminal tyrosine added to synthetic B knobs. (A) Needle clusters and thin plates formed with D-dimer in the presence of 500 μM GHRPYam and no precipitant. (B) Diffraction-grade crystals generated in the presence of 2 mM GHRPam and 10% PEG.

Table 1: Data Collection and Refinement Statistics for DD-GHRPY and DD-MHRPY

		DD-GHRPY	DD−MHRPY
space group		$P2_12_12_1$	$P2_12_12_1$
unit cell	*a* (Å)	54.0	54.4
	b (Å)	147.9	147.2
	c (Å)	231.4	231.5
highest resolution		2.7 (2.7−2.8)[a]	2.8 (2.8−2.9)[a]
no. of observations		541,496	153,213
no. unique reflections		55,743 (5468)[a]	44,180 (3794)[a]
completeness (%)		99.9 (99.6)[a]	95.4 (83.5)[a]
redundancy		9.7 (7.9)[a]	3.5 (2.5)[a]
R_{sym} (I)[b]		0.07 (0.22)[a]	0.09 (0.57)[a]
mosaicity (deg)		0.4	0.4
refinement resolution range (Å)		30−2.7	30−2.8
no. of residues in protein		1478	1478
no. of residues in model		1297	1297
R-factor[c]		0.216	0.219
R_{free}[d]		0.275	0.276
rmsd for ideal bond lengths (Å)		0.007	0.007
rmsd for bond angles (deg)		1.33	1.33

[a] The values in parentheses describe the highest resolution shell. [b] $R_{sym} = (\Sigma|I - \langle I\rangle|)/(\Sigma|I|)$. [c] Crystallographic *R*-factor = $[(\Sigma||Fo| - |Fc||)/\Sigma|Fo|]$ with 95% of the native data. [d] R_{free} is the *R*-factor based on 5% of the native data withheld from the refinement.

In contrast, an *Fo* − *Fc* omit map clearly showed both the methionine and tyrosine side chains in the βC hole (Figure 4B). The tyrosine orientation is the same as that

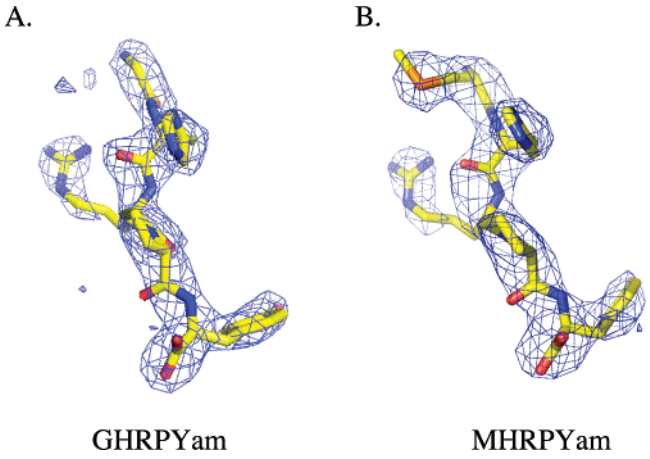

FIGURE 4: (A) Electron density corresponding to GHRPYam in the βC hole of the fragment D-dimer (from human fibrinogen) as observed in an *Fo* − *Fc* map calculated at 2.7 Å resolution and contoured at 3.5 σ. The peptide was not included in the calculation. (B) Electron density corresponding to MHRPYam in the βC hole of the fragment D-dimer as observed in an *Fo* − *Fc* map calculated at 2.8 Å resolution and contoured at 3.0 σ. The peptide was not included in the calculation.

observed in the GHRPYam-D dimer crystal structure (Figure 4A). As for the methionine side chain, the CB and CG atoms thread their way between the functional groups of Aspβ432 and Serβ443, and van der Waals distances are observed between the CG atom and the CB atoms of Serβ443 and Aspβ432, in one instance, and between the terminal CE atom and the CE2 and CZ2 atoms of Trpβ444, in another (Figures 5 and 6).

Modeling Other Side Chains. The side chains of those amino acids that were ineffective as terminal residues were modeled into the MHRPYam-D-dimer structure in an effort to gauge what clashes might be occurring in those situations. In the cases of isoleucine and valine, which are totally devoid of activity in the clot−lysis assay, the CG atoms clash severely with the carboxyl group of Aspβ432 and the hydroxyl of Serβ443. A similar result was observed when the substitution was threonine. In contrast, the ineffectiveness of EHRPYam appears not to be so much from clashing as it does from the side chain carboxylate interacting with the guanidino group of arginine-3 of the peptide itself, compromising the interaction of that group with host residues βGlu397 and βAsp398. The effect may also be attributable to repulsion by the generally electronegative nature of the hole environment.

Influence of GPRPYam (A-Knob) on Fibrin Formation. The observation that enhancement of binding for pentapeptide B-knobs was attributable to an interaction between the tyrosine side chain and a guanidino group of a nearby arginine prompted an examination of the situation with A-knobs and homologous γC domains. In the case of human fibrinogen, there is a lysine at the position corresponding to Arg β406, but chicken and lamprey fibrinogens have arginine, just as occurs in the βC domain. Consistent with these observations, GPRPYam is a better inhibitor of fibrin formation than GPRPam when lamprey fibrinogen is used, but the tetrapeptide GPRPam is better than the tyrosine-ending pentapeptide when human fibrinogen is used (Figure 7).

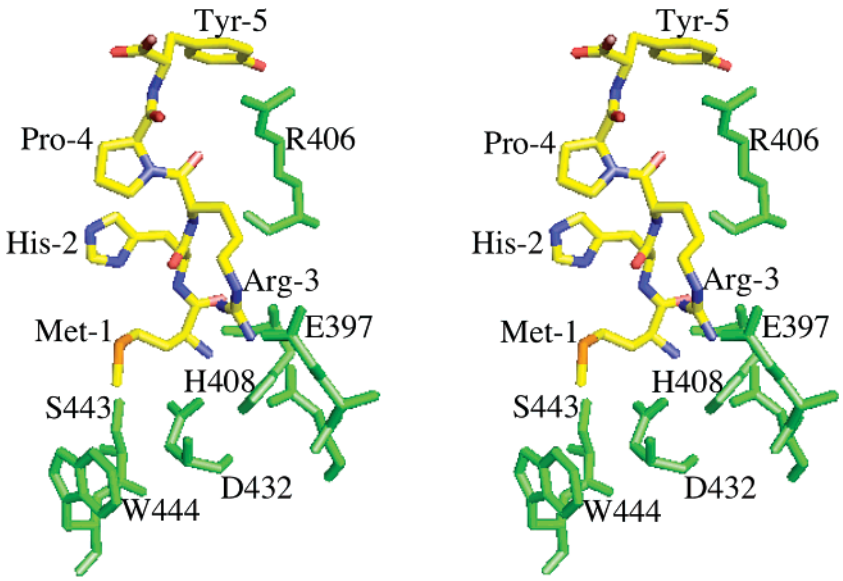

FIGURE 5: Stereodiagram of MHRPYam bound to the βC hole showing positions of methionine (Met-1) and nearby β-chain side chains (H408, D432, S433, W444, and E397) as well as the juxtaposition of the terminal tyrosine (Tyr-5) with βArg406.

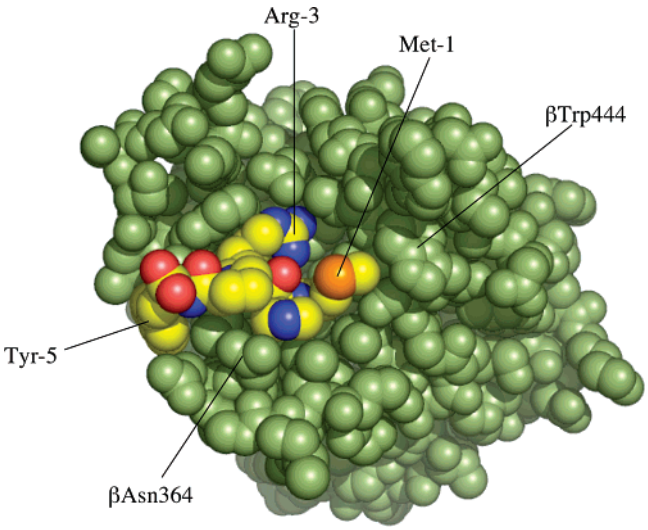

FIGURE 6: Space-filling model of βC domain (human fibrinogen) with bound MHRPYam. Some key residues are labeled on the peptide (Met-1, Arg-3, and Tyr-5) and host protein (βAsn364 and βTrp444).

DISCUSSION

Although it has been recognized that the βC hole is a less specific binder than the γC hole (*7*), the extent of the promiscuity was not fully appreciated. That large bulky amino acids like methionine, leucine, or arginine can substitute for glycine was quite unexpected, even allowing for the open nature of the βC pocket. There are limits, however, and those amino acids that branch at the β carbon, especially valine and isoleucine, are ineffective.

At first glance, it seemed surprising that MHRPYam was as good or even better a binder than AHRPYam. Comparison with the FD-AHRP structure (PDB ID 2H43) revealed that the CB atoms of the alanine and methionine are disposed similarly in the two structures, both in a more or less energy-neutral or mildly unfavorable situation proximal to the carboxyl group of βAsp432. In the case of MHRPYam, however, stabilizing interactions occur between the CG and CE atoms and several host methylene groups (Figure 5).

The fact that chickens have alanine at the amino termini of their fibrin β chains (*5*) does not necessarily imply that it will be likely that other non-glycine amino acids will turn up at this position in other species, if only because there is the hurdle of needing thrombin to cleave the adjacent bond in fibrinogen.

Tyrosine Contribution to Binding. In humans, the amino-terminal sequence of the fibrin β chain begins with GH-RPL−, but in bovine the corresponding sequence begins with

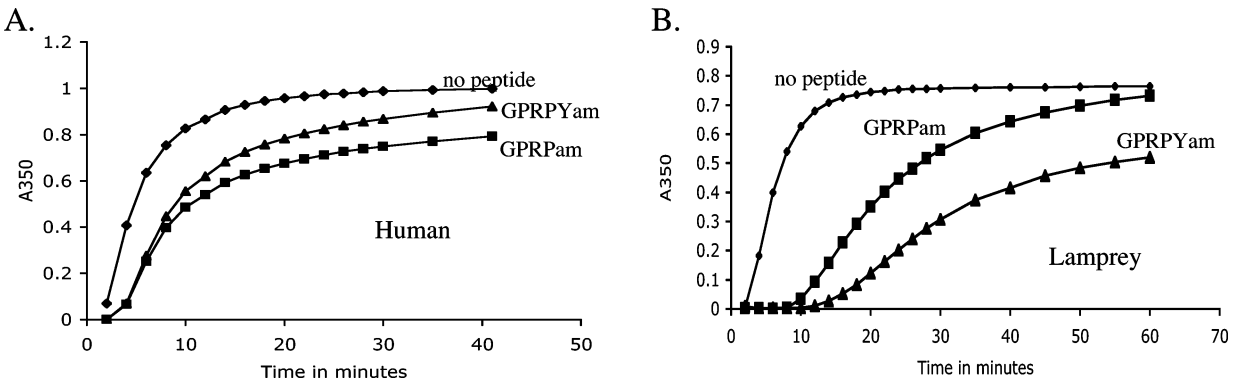

FIGURE 7: Effect of adding a carboxyl-terminal tyrosine residue to synthetic A-knob on thrombin-catalyzed polymerization of fibrin. In both cases, the GHRPam and GHRPYam concns were 100 μM. (A) Human thrombin and fibrinogen. (B) Lamprey thrombin and fibrinogen. The two fibrinogens differ in that human fibrinogen has a lysine residue at γ338 and lamprey fibrinogen has an arginine at the equivalent position.

GHRPY−. Nonetheless, the synthetic peptide GHRPYam is a considerably more effective inhibitor of tPA-activated fibrinolysis than is GHRPLam (*4*).

The interaction of the tyrosyl side chain with the guanidino group of Arg β406 was anticipated (*4*). Although a survey of amino-aromatic interactions was reported many years ago (*18*), they tend not to be well appreciated and are often overlooked. Validation of the importance in this case was obtained from an experiment with the homologous situation involving the A-knobs GPRPam and GPRPYam and their impact on fibrin formation with human and lamprey fibrinogens. The opposite effects of the added tyrosine in the two situations appears entirely attributable to human fibrinogen having a lysine at γ-chain residue 338 and lampreys having arginine. This is also consistent with GHRPYam being found only in the βC holes in the crystal DD-GHRPY.

Do the Tyrosine-Ending Peptides Induce a Further Conformational Change? The question arises, why were we not able to crystallize fragment D or D-dimer in the presence of tightly binding pentapeptides such as GHRPYam or MHRPYam, when the same preparations of D or D-dimer crystallize so easily and well with the tetrapeptide GHRPam? Certainly, the causes of enhanced turbidity of fibrin and the spontaneous formation of needle showers or microcrystals must be the same. The simplest explanation is that the tightly bound tyrosine-ending peptides cause a conformational change that leads to associations that preclude good crystal growth (Figure 3A). Although it was possible to determine *how* the peptide is bound to the β-chain hole in the soaked crystals, the larger scale conformational consequences of such binding had to be bypassed for the moment. Instead, the observed crystal packing was that dictated by the binding of GHRPam. It is significant that these crystals slowly deteriorate in the presence of GHRPYam.

It must be emphasized that the anticipated conformational change is over and beyond the side-chain excursion of residues βGlu397 and βAsp398 that occurs upon binding of the tetrapeptide GHRPam (*9*) and may involve a shift of the βC domain away from the coiled coil (*19*). As a result, αLys157, long implicated in t-PA activation (*20*), could become exposed. X-ray structures have shown the site to be inaccessible in both fragments D and D-dimer (*10*).

In summary, we have shown that the βC hole of human fibrin(ogen) can bind an assortment of peptides with amino-terminal residues other than glycine. In contrast to GHRPam, non-glycine peptides bind only to the βC hole and not to the homologous γC hole. Beyond that, the tight binding to the βC hole observed for peptides with carboxyl-terminal tyrosine is mainly attributable to an interaction of the tyrosine side chain with the guanidino group of a nearby arginine. This observation was reinforced by a parallel case of tight binding by GPRPYam to a fibrinogen with arginine in the equivalent position in the γC hole.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on August 10, 2007, with an incorrect labeling of the abscissa in Figure 2. The correct version was published on August 28, 2007.

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