Investigation of Conformational Specificity at GPIIb/IIIa: Evaluation of Conformationally Constrained RGD Peptides†

Catherine E. Peishoff,*,‡ Fadia E. Ali,§ John W. Bean,‡ Raul Calvo,§ Cynthia A. D'Ambrosio,‡ Drake S. Eggleston,‡ Shing Mei Hwang,⊥ Tammy Page Kline,‡ Paul F. Koster,∥ Andrew Nichols,∥ David Powers,∥ Todd Romoff,§ James M. Samanen,§ Jeffrey Stadel,∥ Janice A. Vasko,∥ and Kenneth D. Kopple‡

Departments of Physical and Structural Chemistry, Peptidomimetic Research, Pharmacology, and Cellular Biochemistry, SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, King of Prussia, Pennsylvania 19406

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RGD-containing proteins and peptides are known to bind to the platelet GPIIb/IIIa receptor and inhibit platelet aggregation. That a conformational component to the specificity exists is suggested by significantly lower activity of linear RGD analogs relative to closely related cyclic peptides and small proteins containing the RGD sequence. Recently, conformations for a suite of RGD containing cyclic peptides have been defined by NMR-based methods and, for one molecule, by X-ray diffraction. We report here the NMR-based conformational analysis of an additional cyclic peptide, cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), and compare the conformational variations in the suite of peptides and related analogs. Biological activity data for these peptides shows a preference of the platelet GPIIb/IIIa receptor for one conformation of the RGD sequence, but suggests its ability to bind a second, distinct conformation.

Introduction

Peptides and small proteins containing the sequence Arg-Gly-Asp (RGD) antagonize binding of fibrinogen to its platelet GPIIb/IIIa receptor, thereby inhibiting platelet aggregation, an important step in thrombus formation.[1-3] The linear peptide acetyl-Arg-Gly-Asp-Ser-amide has an affinity for the receptor that is ~1000 times lower than that of fibrinogen, however,[4] suggesting that conformational stability provided by secondary and tertiary structure may be a factor in determining affinity.

Because only a few residues are necessary to express biological activity, RGD analogs are an ideal instance in which cyclic oligopeptides of variously restricted conformation may be designed to aid in identifying the biologically active (receptor-bound) conformations of a functional peptide sequence. For example, incorporation of RGD into a cyclic pentapeptide disulfide structure such as acetyl-Cys-(N^{α}-methyl)Arg-Gly-Asp-Pen-NH_2 cyclic disulfide, 1, can give analogs with high receptor affinity.[4-7] This cyclic peptide has a K_i of 0.175 ± 0.025 μM for the human GPIIb/IIIa receptor, comparable to that of fibrinogen, 0.28 ± 0.05 μM.[8] NMR studies of the probable solution conformation of this molecule have shown it to be similar to the solution conformation and crystal structure of a more constrained and more active analog, the semipeptide 2-mercaptobenzoyl-(N^{α}-methyl)Arg-Gly-Asp-2-mercaptoanilide cyclic disulfide, 2.[9,10]

In addition to cyclic disulfides, we have prepared and examined a number of RGD-containing homodetic cyclic peptides of limited conformational mobility. These include cyclo(Pro-Gly-Arg-Gly-Asp-D-Pro), 12, and cyclo(Pro-Arg-Gly-Asp-Gly-D-Pro), 9, for which NMR-based solution conformations have been reported.[11] Also included are cyclo(Pro-Arg-Gly-Asp-Gly-D-Phe), 11, a closely

† This paper is dedicated to Professor Ralph Hirschmann on the occasion on his 70th birthday.

‡ Department of Physical and Structural Chemistry.

§ Department of Peptidomimetic Research.

∥ Department of Pharmacology.

⊥ Department of Cellular Biochemistry.

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Table I. Activities of Conformationally Defined RGD-Containing Cyclic Peptides

no.	compound[e]	antiaggregatory[a] canine PRP/ADP IC_{50} (μM)	binding[b] human GPIIb/IIIa K_i (μM)	RGD conformational type/basis
1	Ac-Cys-(N^{α}-Me)**Arg-Gly-Asp**-Pen-NH_2	0.355 ± 0.035	0.175 ± 0.025	turn–extended–turn/NMR
2	Mba-(N^{α}-Me)**Arg-Gly-Asp**-Man	0.09 ± 0.02	0.004 ± 0.000	turn–extended–turn/NMR, X-ray
3	Ac-Cys-**Arg-Gly-Asp**-Pen-NH_2	4.12 ± 0.6		turn–extended–turn/analogy to 1
4	Ac-Cys-D-**Arg-Gly-Asp**-Pen-NH_2	4.1 ± 1.1	3.30 ± 0.15	turn–extended–turn/analogy to 1
5	Ac-Cys-D-(N^{α}-Me)**Arg-Gly-Asp**-Pen-NH_2	1.79 ± 0.23		turn–extended–turn/analogy to 4
6	Mba-**Arg-Gly-Asp**-Man	0.29 ± 0.09		turn–extended–turn/analogy to 2
7	Ac-Cys-**Arg-Gly-Asp**-D-Pen-NH_2	20.3 ± 2.2	3.13 ± 0.08	turn–extended–turn/analogy to 1
8	cyclo(Pro-**Arg-Gly-Asp**-D-Pro-Gly)	76.6 ± 16.0	>100	turn–extended–turn/NMR (this work)
9	cyclo(Pro-**Arg-Gly-Asp**-Gly-D-Pro)	5.26 ± 1.63	1.9 ± 0.4	Gly-Asp β turn/NMR
10	H-Gly-Pen-Gly-**Arg-Gly-Asp**-Ser-Pro-Cys-Ala-OH	10.1 ± 1.52		Gly-Asp β turn/NMR
11	cyclo(Pro-**Arg-Gly-Asp**-Gly-D-Phe)	17.7 ± 8.74	18.5 ± 1.0	Gly-Asp β turn/analogy to 9
12	cyclo(Pro-Gly-**Arg-Gly-Asp**-D-Pro)	>200		Arg-Gly β turn/NMR
13	cyclo(Pro-**Arg-Gly-Asp**-D-Phe)$_2$	>200		fully extended Arg-Gly-Asp/NMR,[c] crystal analogy[d]

[a] Inhibition of platelet aggregation in canine platelet-rich plasma induced by ADP, see ref 7. [b] Inhibition of binding of [^{3}H]Mba-(N^{α}-Me)Arg-Gly-Asp-Man to GPIIb/IIIa isolated from human platelets, reconstituted in liposomes, see ref 5. [c] Limited NMR only. [d] Analogy to gramicidin S urea complex. [e] Abbreviations used: Me, methyl; Pen, penicillamine; Mba, 2-mercaptobenzoic acid; Man, 2-mercaptoaniline.

Table II. Probable Solution Conformations of Cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), 8[a]

Cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), NMR in Methanol-d_3 at 213 K

			Pro		Arg		Gly		Asp		D-Pro		Gly	
class	E_{total}	E_{constr}	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ
I	10.93	0.08	−69	−23	−93	28	90	167	51	73	80	−61	−70	173
II	14.05	1.13	−69	−29	−94	27	81	180	55	71	40	45	−156	174

Cyclo(Ala-Ala-Gly-Ala-Gly-Gly), Crystal Structure[b]

Ala		Ala		Gly		Ala		Gly		Gly	
φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ
−62.0	−32.5	−95.4	13.7	105.7	179.1	53.8	37.7	90.9	−6.3	−119.0	−167.2

[a] Lowest energy conformations of each class returned by the constrained distance geometry searches. [b] See ref 13.

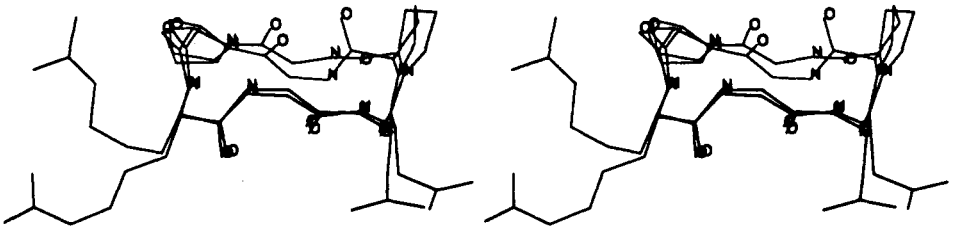

Figure 1. Stereoplots of the lowest energy conformers for the two backbone types of cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), 8, generated by the constrained distance geometry/minimization search procedure.

related analog, and cyclo(Pro-Arg-Gly-Asp-D-Phe)$_2$, 13, an RGD analog of the conformationally stable gramicidin S.

In this paper we report the solution conformation of cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), 8. Also, we discuss the activities of it and the above mentioned and related peptides in assays measuring inhibition of platelet aggregation in canine platelet-rich plasma and binding to purified human platelet GPIIb/IIIa receptor.[5] The preferred RGD conformations of the various cyclic peptides are classified, and conformation–activity correlations are proposed. Table I presents the compounds and data to be discussed.

Results

Conformation of Cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), 8. The conformation of cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), 8, was determined using the NMR-constrained distance geometry search methods described in the Experimental Section. It displays the common two β turn cyclic hexapeptide conformation. Two conformational classes were generated by the constrained search; they differ only in the rotation of the D-Pro^5-Gly^6 amide plane. Residues Gly^3-Asp-D-Pro-Gly^6 approximate a type I′ β turn and residues Gly^6-Pro-Arg-Gly^3 are contained in a type I β turn. Backbone dihedral angles for the lowest energy conformers of each set are listed in Table II, and an overlay representing the lowest energy conformers of the two variants in the D-Pro^5-Gly^6 region is shown in Figure 1. The uncertainty at D-Pro^5-Gly^6 may not be real. It arises from the inability, because of chemical shift degeneracies, to assign either a doubly bounded or anti distance constraint between the D-Pro^5 δ protons and the Gly^6 α and amide protons, between the D-Pro^5-Gly^6 α protons, or between the D-Pro^5 β protons and the Gly^6 amide proton. Only one doubly bounded constraint, Pro^5 H_α to Gly^6 H_N, could be applied between these residues. For the analogous Pro^1-Arg^2 region, however, the presence of a doubly bounded constraint between H_δ of Pro^1 and H_N of Arg^2 in addition to the constraint for Pro^1 H_α to Arg^2 H_N provided better definition. Of the three isomeric cyclic hexapeptide molecules, 8, 9, and 12, compound 8 is the best defined

Table III. Proton NMR Data for Cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), 8, in Methanol-d_3 at 213 K[a]

residue	H_N	H_α	H_β	H_γ	H_δ	$H_{N\epsilon}$	J H_N/H_α,[b] Hz	$\Delta\delta/\Delta T$[c]
Pro	-	4.32	2.38/1.94	2.04	3.68/3.60			
Arg	8.57	4.57	2.18/1.56	1.56/1.50	3.18	7.82	9.1	3.8
Gly	7.56	4.42/3.72					8.8/3.1	0.3
Asp	9.63	4.51	3.01/2.65				5.1	6.0
D-Pro	-	4.53	2.24/1.97	1.95	3.86/3.76			
Gly	7.47	4.62/3.83					8.9/2.3	1.4

[a] Chemical shifts reported as ppm relative to TMS. [b] Coupling constants obtained for Gly residues from P.E. COSY and for Arg and Asp from 1-D ^1H spectra. [c] Temperature coefficients for H_N reported as ppb/K.

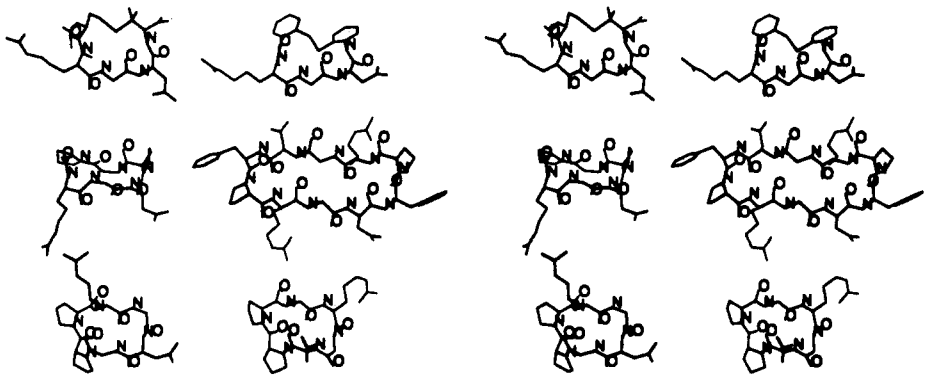

Figure 2. Low-energy conformations of compounds 1, 2, 8, 9, 12, and 13 shown in stereo: top row, 1,2; middle row, 8,13; bottom row, 9,12.

experimentally; 18 doubly bounded constraints were identified for this molecule, compared to 16 for compound 9 and 14 for compound 12. The H_N chemical shifts and their temperature coefficients are very clearly consistent with a dominant two β turn conformation in solution.[12] These coefficients are high for Arg and Asp and low for both Gly residues, indicating that the amide protons on both Gly residues are sequestered from solvent relative to those on Arg or Asp. (See Table III.) In addition, the dispersion in both Gly H–N–C–H coupling constants and in both Gly α proton chemical shifts strongly suggest a single highly favored conformer. The strong conformational preference may be due to an intrinsic stability of the type I,I' β turn combination and/or the absence of Gly from any turn position. Compound 8 is not the first example of a β I, β I' turn combination; crystallographic studies of cyclo(Ala-Ala-Gly-Ala-Gly-Gly)[13] indicate a type I β turn at Gly-Ala-Ala-Gly, a type I' β turn at Gly-Ala-Gly-Gly, and an overall backbone closely similar to that of 8, as shown in Table II.

Classification of RGD Conformations. The molecules of Table I can be classified into four distinct categories according to conformational preferences of the RGD backbone. Bases for the assignments and the preferred conformations are indicated in the table, and the categories are more specifically described below. Whether or not the backbone is directly involved in receptor binding, its conformation defines the separation and relative orientations of the C_α–C_β vectors of the Arg and Asp residues. Differences in the positioning of these vectors result in different limits on the positioning of the Arg and Asp side chains. Because analogs lacking either all or part of the RGD sequence display significantly reduced affinity for GPIIb/IIIa,[7] it may be presumed that the guanidine and carboxylic acid groups are critical binding elements that maintain a specific three-dimensional relationship at the receptor.

To illustrate the differences among the categories, Figure 2 contains stereoplots of preferred backbone conformations for compounds 1, 2, 8, 9, 12, and 13.

Turn–Extended–Turn RGD Conformation: Compounds 1–8. The RGD region of molecules in this category can be described as preferring a Gly residue with ϕ, ψ values such that the $C_{\alpha_{Arg}}$–$C_{\alpha_{Asp}}$ distance is 6.8 Å or greater, while the Arg and Asp residues each adopts a conformation(s) that produce a reversal of chain direction through the RGD sequence. (See 1, 2, and 8, Figure 2.) In terms of the angles between consecutive Cα–Cα vectors, this general form can be described as maintaining ∠Xxx-Arg-Gly < 110°, ∠Arg-Gly-Asp > 130°, and ∠Gly-Asp-Xxx < 110°, hence the designation turn–extended–turn. In this group is 2, the most active compound listed in Table I, for which both X-ray and NMR studies show the turn–extended–turn RGD arrangement to be favorable. NMR studies indicate an analogous conformation for 1.[9]

Within the turn–extended–turn category, a range of local conformations at Arg and Asp are still possible. For the Asp residues, a C_{7eq} (−80°, 80°) conformation is preferred in the dominant solution conformations of 1 and 2[9] (See Figure 2.) In the crystal structure of 2, and in a minor component of 2 identified in solution at −70 °C, Asp takes a more nearly right-handed helical conformation (ϕ, ψ = −60°, −30°), corresponding also to the i + 1 position of a type I β turn. In 8 (this paper), Asp is in the i + 1 position of a type I' β turn, i.e., left-handed helical (ϕ, ψ = 60°, 30°).

The Arg residue in 8 takes the conformation corresponding to the i + 2 position of a type I β turn (ϕ, ψ = −90°, 0°) (Figure 1). The most probable conformation of (N^α-methyl)Arg in 1 and 2, ca. ϕ, ψ = −120°, 60°, departs somewhat from the canonical values but would also fit the i + 2 position.

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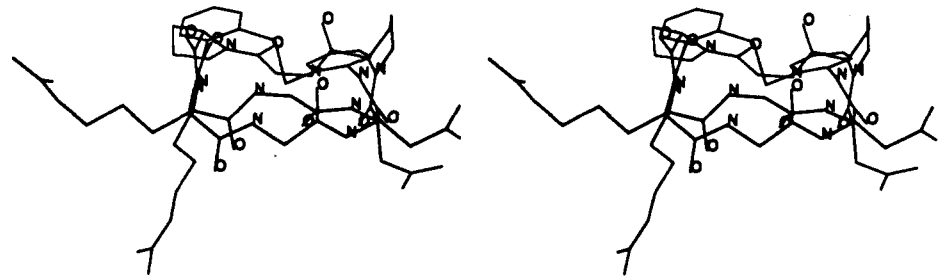

Figure 3. Overlay of compounds 2, cyclic disulfide Mba-(N^{α}-Me)Arg-Gly-Asp-Man, and 8, cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), shown in stereo. The overlay optimizes the overlap of the amide bonds preceding Arg, the amide bonds following Asp and the α carbons of Arg and Asp.

Analogs of 1 lacking the N^{α}-methyl substituent (e.g. 3 and 4) may be assigned to this group, although experimental data show them to be less well defined conformationally. Turn–extended–turn conformations are likely components of the conformational distribution; however, we have found that quantitative nuclear Overhauser data obtained for 4 are inconsistent with any single RGD backbone conformation,[14] and similar observations have been reported for Ac-Cys-Arg-Gly-Asp-Cys-NH_2 cyclic disulfide.[15]

Gly-Asp β Turn Conformation: Compounds 9–11. The cyclic hexapeptide 9 and the 10-residue disulfide 10 have been found to prefer conformations in which Gly and Asp occupy the $i+1$ and $i+2$ positions, respectively, of a β turn. NMR data for compound 9 are accommodated at low energy by ring conformations including β turns of type I, II, II′, and III, with no clear preference for any one type, although the lowest energy conformers have type I Gly-Asp turns, as shown in Figure 2.[11] The conformation of Gly-Asp in 10 has been reported to correspond to a type I turn on the basis of NOE, coupling constant, and variable temperature data.[16] The backbone of compound 11, equating its D-Phe-Pro sequence with the stable D-Phe-Pro type II′ β turns seen in gramicidin S[17,18] and with the D-Pro-L-Pro sequence of compound 9, should also contain a Gly-Asp β turn. In such arrangements, the distance between the Arg and Asp α carbons is 6 Å or less.

Arg-Gly β Turn Conformation: Compound 12. The RGD region of the cyclic hexapeptide 12 adopts predominantly a type II β turn arrangement, with Arg and Gly in the $i+1$ and $i+2$ positions, according to NMR studies.[11] Its backbone is illustrated in Figure 2. The separation of the Arg and Asp α carbons is similar to that of the Gly-Asp turn conformations, 6 Å or less.

Fully-Extended RGD: Compound 13. NMR evidence, the pattern and dispersion of the H_N chemical shifts, their temperature and solvent dependences, and the $HNC^{\alpha}H$ coupling constants, confirms that compound 13 shares with gramicidin S,[19–21] the molecule on which it was based, a stable conformation comprised of two D-Phe-Pro type II′ β turns spaced by antiparallel three-residue β-like strands containing the RGD sequences. From a report of gramicidin S crystal structure coordinates,[18] the α carbon separations for appropriately placed Arg and Asp residues would be 6.6 Å. In the crystal, the residues corresponding to the Arg, Gly, and Asp residues of 13, Val-Orn-Leu, have all ϕ and ψ greater in magnitude than 110°, except for one of the ψ_{Leu} values at 96°. The replacement of Val-Orn-Leu with Arg-Gly-Asp to construct compound 13 replaces two side-chain-bearing residues with Gly residues. A model of 13 was built starting from the backbone and β carbon coordinates of gramicidin S, and was energy minimized as described in the Experimental Section. This conformer, shown in Figure 2, retains the extended RGD sequences.

Structure/Activity Relationships. Turn–Extended–Turn RGD Conformation. Both the highly active heterodetic cyclic pentapeptide 2 and the much less active homodetic cyclic hexapeptide 8 adopt the turn–extended–turn conformation that maintains a ~7 Å separation between the α carbons of Arg and Asp. Because 8 does not contain (N^{α}-methyl)Arg, which has an advantageous effect on potency, other things being equal, its activity might be expected to be comparable to that for compounds 3 or 6 rather than 1 or 2. However, 8 is still much less active than 3 or 6. To indicate conformational differences that might account for this, Figure 3 compares the lower energy conformer of compound 8 and the major component conformer from the low-temperature NMR study of compound 2.[9] The two are overlaid to superimpose the α carbons of Arg and Asp and to minimize graphically the difference in the backbone regions flanking the RGD sequence. Several differences are apparent. First, the Arg and Asp side chains take a more axial orientation relative to the overall ring plane in the hexapeptide 8. Second, the distance between the Arg and Asp β carbon atoms is shorter (~7.8 Å) than the corresponding distance in the cyclic disulfide 2 (~9.8 Å). Third, the orientation of the Gly-Asp amide plane in compound 8, which maintains the Asp H_N syn to the Asp H_{α} ($\phi_{Asp} \approx -80°$), is opposite that in compound 2, in which the corresponding protons are anti ($\phi_{Asp} \approx +60°$). (The Asp H_N to Asp H_{α}

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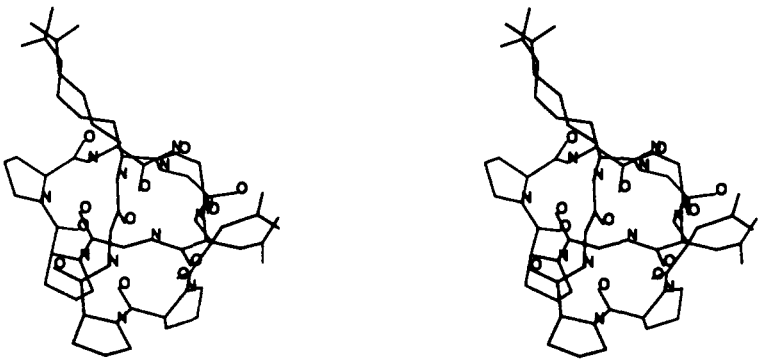

Figure 4. Stereoplot of compounds 9, cyclo(Pro-Arg-Gly-Asp-Gly-D-Pro), and 12, cyclo(Pro-Gly-Arg-Gly-Asp-D-Pro), overlaid to minimize the difference in the C_α–C_β vectors of Arg and Asp and the backbone between them.

crosspeak is the strongest nongeminal crosspeak in the NOE spectrum of 8.) Any of these differences in conformation could influence the interactions of 8 with receptor, particularly if backbone atoms are involved in receptor binding. The overlay of 8 on 2 in Figure 3 further suggests that there is a portion of 8, the D-Pro ring, that extends significantly outside the molecular volume of 2 and could interact unfavorably with the receptor.

In compounds 3 and 4, antiaggregatory activity is independent of the stereochemical configuration at Arg. Upon N^α-methyl substitution at Arg, the L-Arg isomer increases 12-fold in activity (3 → 1), but that of the D-Arg isomer only doubles (4 → 5). NMR-based conformational analysis of 1 indicates that the most probable arrangement at (N^α-methyl)Arg has the N^α-methyl group anti to the Arg α proton,[9] and the relationship also holds in the solution and crystal structures of 2. However, as indicated earlier, the analogs lacking the N^α-methyl substituent seem to be less well defined conformationally. The suggestion, therefore, is that 3 and 4, in spite of the difference in configuration at Arg, can more readily adopt similar receptor-bound arrangements of important binding groups than can 1 and 5, which are conformationally restricted to a certain extent by the N^α-methyl group.

Gly-Asp β Turn Conformation. As a class, compounds 9–11 are active, but less active, than compounds which display the turn–extended–turn conformation. It is conceivable that they could adopt the turn–extended–turn conformation on binding to the receptor. However, if this is to be so, the cost in energy to adopt the new conformation cannot be high, because compound 9 is already about as active as 3, and only 20-fold less active (the equivalent of ~2 kcal) than 6. Compounds 3 and 6 are the N^α-methyl free analogs of the cyclic disulfide turn–extended-turn class to which 9, which also bears unsubstituted Arg, should be compared. However, examination of the 500 energy-minimized structures generated in the constrained distance geometry search for conformations of 9,[11] without regard to the extent of NMR constraint violations, showed that the lowest energy set of conformations that approximate a turn–extended–turn conformation ($|\phi|_{Gly}$ and $|\psi|_{Gly} \geq 120°$) are found more than 11 kcal (in steric and strain energy) above the lowest energy Gly-Asp β turn conformation. Although the constrained distance geometry search is not exhaustive, this result seems a strong indication that for cyclic peptide 9, at least, the turn–extended–turn conformation is not sufficiently accessible to explain the observed activity.

Arg-Gly β Turn Conformation. Although the 6-Å spacing of the Arg and Asp α carbons is approximately the same in the cyclic hexapeptide 12 as in its isomer 9 just discussed, the activity of 12 is not detected in our platelet aggregation assay. Figure 4 shows the two compounds superimposed to minimize the difference in the Arg and Asp C_α–C_β vectors and the backbone between them. If maintaining the guanidine and carboxylic acid relationship is critical, this overlay may suggest reasons for the diminished activity. In addition to differences in the local conformations about the Arg and Asp residues, there are large differences in the volume distribution of the non-RGD moieties relative to the RGD sequences. Unfavorable steric interactions of these non-RGD sections with the receptor may be absent for 9 but significant for 12.

Fully Extended RGD Conformation. The gramicidin S analog 13 was found to be inactive in the platelet assay. In its expected conformation, the Arg C_α to Asp C_α distances are similar to the corresponding distances in active molecules of the turn–extended-turn type, but the turns at Arg and Asp are, of course, absent. This could result in suboptimal positioning of backbone elements important to binding. In respect to the orientation of the Arg and Asp C_α–C_β vectors, compound 13 is similar to compound 8, which has only modest activity in the platelet assay. (The C_β–C_β distance in the energy minimized model of 13 is 8.1 Å.) In Figure 5, these two compounds are overlaid to optimize overlap of their C_α–C_β vectors and to position the backbone of 8 as much as possible within the backbone volume of 13. The D-Phe-Pro residues flanking the RGD sequences of 13 are seen to add volume not present in analogous regions of other RGD analogs. It is likely that a combination of the extra volume and positional changes in the RGD backbone atoms is responsible for the inactivity of compound 13.

To summarize, analogs representing the turn–extended–turn RGD conformation display high affinity and efficacy, analogs representing the Gly-Asp β turn conformation display moderate affinity and efficacy, while analogs representing the Arg-Gly β turn and the fully-extended RGD conformations display significantly reduced efficacy.

Discussion

It has been assumed in the foregoing that the local conformation of the RGD motif in these cyclic peptides is a critical recognition factor for the receptor, i.e., that the activity of the differently constrained cyclic peptides can be rationalized in terms of relative orientations of the Arg and Asp side chains and backbone elements of the RGD sequences, plus the distribution of non-RGD molecular volume. Other constitutional factors not examined here, the nature of residues flanking the RGD sequence

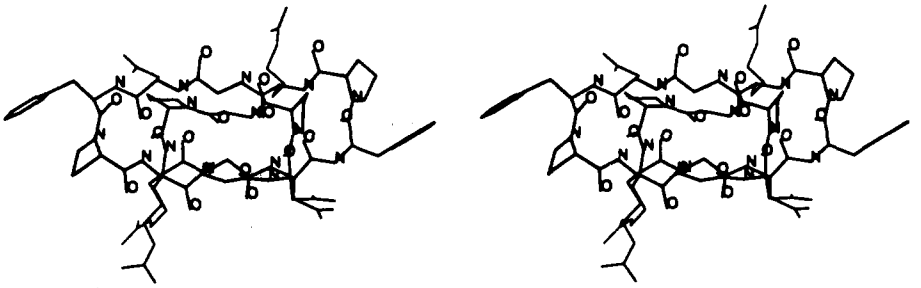

Figure 5. Stereoplot of compounds 8, cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), and 13, cyclo(D-Phe-Pro-Arg-Gly-Asp)$_2$, overlaid to optimize the overlap of the Arg-Gly-Asp regions while keeping the backbone of 8 within the backbone volume of 13.

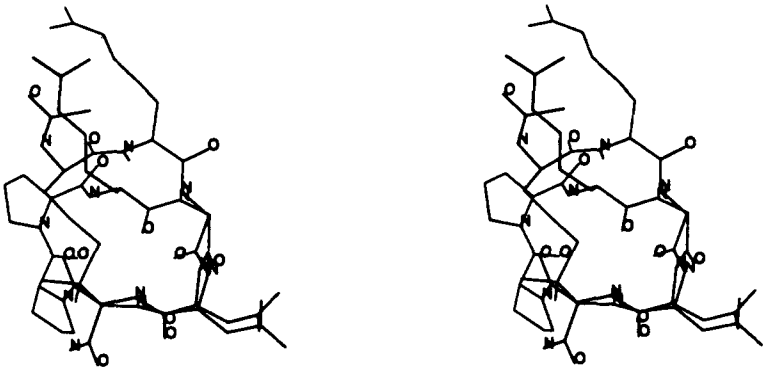

Figure 6. Stereoplot of an overlay of compounds 1, Ac-Cys-(N^{α}-Me)Arg-Gly-Asp-Pen-NH_2 cyclic disulfide, and 9, cyclo(Pro-Arg-Gly-Asp-Gly-D-Pro), made to optimize the overlap of the Gly-Asp regions.

for example, undoubtedly influence receptor affinity. Therefore, before the fully-extended RGD and Arg-Gly β turn conformations are rigorously excluded as receptor-bound conformations, additional analogs which address some of the constitutional differences between the low- and high-affinity analogs should be shown to display similarly low activities.

It does appear, however, that two conformational classes, the turn–extended–turn and the Gly-Asp β turn groups, include molecules with considerable affinity for the GPIIb/IIIa receptor. These conformations also require further experimental testing if they are to be proposed as receptor-bound conformations. Some aspects of the turn–extended–turn model have been tested, and one such test is reported in a separate paper in this issue.[22]

We have indicated that conformations of compound 9 with an extended Gly residue are likely to be too high in energy to account for its receptor affinity on the basis of a receptor-bound turn–extended–turn conformation. Similarly, a review of the distance geometry search outputs obtained for 1 and 2[9] suggests that they can only approach conformations with Gly-Asp β turns at high energy (ca. 10 kcal steric and strain energy above the turn–extended–turn structures). While a yet unknown third conformation that is the true receptor-bound conformation may be readily accessible to the cyclic pentapeptide disulfides on the one hand and 9 on the other, we have not found a common low energy conformation among the set of conformations derived for these analogs. The hypothesis that there are two conformations recognizable by the GPIIb/IIIa receptor is worth exploration.

In fact, comparison of likely solution conformations of compounds 1 and 9 reveals strong similarity in that part of the backbone region between the Gly α-carbon and the α-carbon of the residue following Asp. This is illustrated in an overlay of the conformations of these compounds in which the differences in the Gly-Asp region are minimized, Figure 6. The main differences between these conformations appear in the relative separation of the Arg and Asp side chains, and in the local conformation about Arg.

Given suggestions in the literature that there may be two sites for fibrinogen fragments on GPIIb/IIIa (a γ-chain site on GPIIb,[23,24] and an α-chain site on GPIIIa[25]), it is tempting to speculate that these two conformational types may relate to these two binding sites. At present, there is no data to support this hypothesis. A second hypothesis may be suggested by the fact that GPIIb/IIIa occurs in two states on the platelet: a fibrinogen competent state, one capable of binding fibrinogen, in the activated platelet, and a fibrinogen-incompetent state in the resting platelet.[26] In contrast to fibrinogen, the smaller snake venom RGD containing peptides, which bind to the same number of sites on resting or activated platelets, display a 10 fold difference or less in K_i.[27,28] However, current evidence

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Table IV. Distance Constraints (Å) Derived from NOE Measurements of Cyclo(Pro[1]-Arg[2]-Gly[3]-Asp[4]-D-Pro[5]-Gly[6]), 8, in Methanol-d_3 at 213 K

A. Doubly Bounded Constraints

atom 1	atom 2	lower bound	upper bound	atom 1	atom 2	lower bound	upper bound
$2H_N$	$1H_A$	2.75	3.52	$4H_N$	$4H_A$	1.98	2.53
$2H_N$	$1H_{D1}$	2.63	3.36	$4H_N$	$5H_{D1}$	2.46	3.14
$2H_N$	$2H_A$	2.43	3.10	$4H_A$	$5H_{D1}$	1.98	2.53
$2H_N$	$3H_N$	2.09	2.67	$4H_A$	$5H_{D2}$	1.93	2.47
$2H_N$	$6H_{A2}$	2.87	3.67	$6H_N$	$5H_A$	2.55	3.26
$3H_N$	$2H_A$	2.32	2.96	$6H_N$	$6H_{A1}$	2.31	2.96
$3H_N$	$3H_{A2}$	2.12	2.71	$6H_N$	$6H_{A2}$	2.09	2.68
$4H_N$	$3H_{A1}$	2.08	2.66	$6H_{A1}$	$1H_{D1}$	2.30	2.94
$4H_N$	$3H_{A2}$	2.05	2.62	$6H_{A1}$	$1H_{D2}$	2.15	2.75

B. Omitted Constraints

atom 1	atom 2	atom 1	atom 2	atom 1	atom 2	atom 1	atom 2
[a]							
$1H_{D1}$	$6H_{A2}$	$1H_{D2}$	$6H_{A2}$	$5H_{D2}$	$6H_N$		
[b]							
$1H_A$	$3H_{A1}$	$1H_{D1}$	$5H_{D1}$	$3H_N$	$6H_N$	$5H_A$	$6H_{A1}$
$1H_A$	$3H_{A2}$	$1H_{D1}$	$5H_{D2}$	$3H_{A1}$	$4H_A$	$5H_A$	$6H_{A2}$
$1H_A$	$5H_{B2}$	$1H_{D2}$	$2H_A$	$3H_{A1}$	$5H_{D2}$	$5H_{B2}$	$6H_N$
$1H_{B1}$	$5H_{B1}$	$1H_{D2}$	$3H_{A2}$	$3H_{A2}$	$5H_{D1}$	$5H_{D1}$	$6H_N$
$1H_{B2}$	$5H_A$	$1H_{D2}$	$5H_{D1}$	$3H_{A2}$	$5H_{D2}$	$5H_{D1}$	$6H_{A1}$
$1H_{B2}$	$5H_{B1}$	$2H_A$	$5H_A$	$3H_{A2}$	$6H_{A2}$	$5H_{D1}$	$6H_{A2}$
$1H_{B2}$	$5H_{B2}$	$2H_A$	$5H_{D2}$	$4H_N$	$5H_A$	$5H_{D2}$	$6H_N$
$1H_{B2}$	$5H_{D1}$	$2H_A$	$6H_N$	$4H_N$	$5H_{D2}$	$5H_{D2}$	$6H_{A1}$
$1H_{B2}$	$6H_{D2}$	$2H_A$	$6H_{A1}$	$4H_A$	$5H_A$	$5H_{D2}$	$6H_{A2}$
$1H_{B2}$	$6H_N$	$2H_A$	$6H_{A2}$	$4H_A$	$5H_{B1}$		
$1H_{D1}$	$3H_{A1}$	$2H_{B1}$	$5H_{B1}$	$4H_A$	$6H_{A1}$		
$1H_{D1}$	$3H_{A2}$	$2H_{B2}$	$3H_N$	$4H_A$	$6H_{A2}$		

[a] NOE is observed, but could not be quantified. [b] Unable to determine if NOE occurs due to overlap with diagonal or another crosspeak.

suggests that the sequence around and including RGD in the snake venom peptides is conformationally mobile[29] and therefore adaptable to the receptor environment. It is conceivable that the active site-configuration of GPIIb/IIIa in resting and activated platelets is sufficiently different that the relatively constrained molecules, the protein fibrinogen and the small cyclic peptides, can detect the differences by their inability to adapt conformationally.

Experimental Section

Synthesis. Syntheses of the heterodetic cyclic peptides **1**, **3**, and **4** were reported by Samanen et al.[7] Syntheses of the heterodetic cyclic peptides **2** and **5–7** will be reported by Ali et al.[30] Syntheses of the homodetic cyclic peptides **8**, **9**, and **11–13** were reported by Ali and Samanen.[31] The decapeptide **10**[32] was obtained from Telios Pharmaceuticals, Inc. Analytical data for peptides **8**, **9**, and **11–13** are reported in Table V.

In Vitro Inhibition of Aggregation of Canine Platelet-Rich Plasma. The procedure used has been reported in Samanen et al.[7]

In Vitro Inhibition of [^{3}H]SK&F 107260 Binding to Purified, Reconstituted Human GPIIb/IIIa. The procedure has been reported in Stadel et al.[5]

^{1}H NMR Spectra of Cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), 8. Proton NMR spectra were obtained using a Bruker AMX 500 spectrometer. A 3.8 mM solution of **8** in methanol-d_3 was used. Data were collected at 213 K, where the viscosity of methanol (~2.7 cp[33]) was sufficient to yield negative NOE's detectable for distances up to 3.2 Å. At lower temperatures, general line broadening occurs. The data were processed using the program package FELIX (Hare Research, Woodinville, WA). Assignments were made from P.E.COSY,[34] TOCSY,[35,36] and NOESY[37,38] spectra. The NOESY spectra were measured at mixing times of 50, 80, 110, and 140 ms. For the P.E.COSY spectrum, a 25° sine bell apodization function was applied along both t_1 and t_2. Both domains were zero-filled to 2048 real data points. The NOESY spectra were apodized with a 90° sine bell squared function in both dimensions and zero-filled to 1024 × 1024 matrices. Standard procedures were used for making proton chemical shift assignments. Coupling constants were obtained from the P.E.-COSY spectrum for the Gly residues and from a 1-D ^{1}H spectrum for the Asp and Arg residues. Chemical shift and coupling constant data are listed in Table III.

Generation of Distance Constraints from ^{1}H NMR Data. For compound **8**, NOESY build-up rates were obtained by least-squares fitting of the four measured points. Build-up rates for which the linear fit gave correlation coefficients of 0.95 or greater were used to calculate distance constraints. In this system, the NOE build-ups for geminal proton pairs on Pro or Gly proton pairs were nonlinear. For this reason, the strongest three-bond interaction, Asp H_N–H_α, was used as the reference, corresponding to a distance of 2.2 Å, which is the geometric lower limit. [Using this reference, the geminal proton distances (using the 50-, 80-, and 110-ms data points) are calculated to be longer than the standard 1.75 Å: Pro[1] H_β–H_β, 1.95 Å; Gly[3] H_α–H_α, 1.87 Å; Gly[6] H_α–H_α, 1.81 Å]. Diastereotopic protons were not chirally assigned

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Table V. Peptide Analytical Data

compound	TLC sys[a]	TLC R_f	HPLC CH_3CN[b]	HPLC K'	MS $(M+H)^+$	amino acid analysis (molar ratio)
Mba-(N$^{\alpha}$-Me)Arg-Gly-Asp-Man, **2**	1	0.75	10–50 g	3.4	602.3	Asp (0.93), Gly (1.00)
	2	0.77	21 i	3.0		
Ac-Cys-D-(N$^{\alpha}$-Me)Arg-Gly-Asp-Pen-NH_2, **5**	1	0.61	1–50 g	6.4	624.3	Asp(1.01), Gly (1.00), Cys/Pen (+)[c]
	2	0.54	6 i	5.8		
Mba-Arg-Gly-Asp-Man, **6**	1	0.68	10–50 g	6.0	588	Asp (0.81), Gly (1.28), Arg (1.00)
	2	0.70	20 i	3.6		
Ac-Cys-Arg-Gly-Asp-D-Pen-NH_2, **7**	1	0.42	0–50 g	3.3	620.2	Asp (1.00), Gly (1.03), Arg (0.88), Cys/Pen (+)
	3	0.11	5 i	2.7		
cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly), **8**	1	0.44	0–50 g	6.87	580.3	Asp (1.00), Gly (1.94), Pro (2.05), Arg (1.02)
	2	0.45	5 i	3.98		
cyclo(Pro-Arg-Gly-Asp-Gly-D-Pro), **9**	1	0.35	1–50 g	6.7	580.5	Asp (1.00), Gly (2.00), Pro (2.00), Arg (1.05)
	2	0.5	5.5 i	4.8		
cyclo(Pro-Arg-Gly-Asp-Gly-D-Phe), **11**	1	0.64	1–50 g	8.5	630.3	Asp (1.00), Gly (1.98), Pro (1.04), Phe (0.97), Arg (1.05)
	2	0.64	13 i	5.98		
cyclo(Pro-Gly-Arg-Gly-Asp-D-Pro), **12**	1	0.3	0–50 g	9.44	580.3	Asp (1.06), Gly (2.00), Pro (1.81), Arg (1.29)
	2	0.42	5 i	6.3		
cyclo(D-Phe-Pro-Arg-Gly-Asp)$_2$, **13**	1	0.55	0–50 g	12.9	1145.5	Asp (2.00), Gly (2.08), Pro (2.71), Phe (2.02), Arg (1.74)
	2	0.58	25 i	6.09		

[a] TLC system: 1 = nBuOH/AcOH/H_2O/EtOAc, 1:1:1:1; 2 = nBuOH/AcOH/H_2O/pyridine, 15:5:10:10; 3 = nBuOH/H_2O/iPrOH/$CHCl_3$, 6.5:2:1.5:0.3. [b] Analytical HPLC run in either gradient mode (g) or isocratic mode (i) with the percentage CH_3CN employed in mixture with 0.1% TFA. [c] Quantitative determination of the presence of Cys and Pen indicated by "+" due to difficult quantitation.

and thus are labeled using numbers, e.g. H_{A1}, to distinguish them without implying configuration.

Constraints were of two types: doubly bounded constraints, which had both upper and lower bounds defined as −10% and +15% of the calculated distance (Table IV), and anti distance constraints, which were assigned a lower bound of 3.0 Å and an upper bound of 999 Å. Only data for backbone protons, i.e., HN, H_α, Pro H_β, and Pro H_δ were used to define doubly bounded and anti distance constraints. Anti distance constraints[11] were applied to only those proton pairs which showed no measurable crosspeak in the NOESY spectrum taken with 140-ms mixing time. A total of 18 doubly bounded constraints and 177 anti distance constraints were used. Pairwise interactions which were omitted due to chemical shift degeneracy or overlap with the diagonal are listed in Table IV, Section B.

Conformation Generation and Evaluation for Cyclo(Pro-Arg-Gly-Asp-D-Pro-Gly). A total of 500 conformations[11,39] were generated using the distance geometry program DGEOM (QCPE, Department of Chemistry, Indiana University, Bloomington, IN 47405). Torsion angle sampling was applied during the run and distance correlation was turned off. All bonds were considered fully rotatable with the exception of amide bonds which could be either cis or trans. Chiral volumes were applied only to actual stereocenters, not to atoms carrying prochiral protons. Eighteen doubly bounded distance constraints derived from NMR NOE data, Table IV, were applied along with the 177 anti distance constraints. The conformations were evaluated energetically using the molecular mechanics program AMBER 3.0[40,41] modified to accept distance bounds. Each was minimized for 10 000 iterations or until the norm of the gradient of the energy reached 0.01 kcal mol^{-1} Å^{-1}. All partial charges were set to zero to eliminate electrostatic energy terms. The distance constraints were applied using a flat-bottomed well potential with harmonic sides such that no penalty was assessed for distances falling within the bounds. A force constant of 50 kcal mol^{-1} Å^{-2} was used on each constraint. The conformers were ordered according to constraint violation energy and the set below 1.5 kcal mol^{-1} was saved. These conformers were reordered on total energy and the set within 5 kcal mol^{-1} of the lowest energy conformer was saved. This left 30 of the original 500 structures. Increasing the total energy window to 10 kcal mol^{-1} increases this number to 68, but does not change the number of conformational sets found. Representative structures were checked for conformational stability by minimizing with both partial charges and distance constraints for an additional 75 iterations followed by 75 iterations with partial charges, but without distance constraints. All conformers remained in their original set.

Model Building of (D-Phe-Pro-Arg-Gly-Asp)$_2$, 13. A conformational model was built for compound **13** using the coordinates reported for gramicidin S crystallized as the urea complex.[18] Only backbone and β carbon atoms were included. The remaining atoms were added and minimization performed (1000 iterations, no electrostatics) using AMBER 3.0.[40,41]

Acknowledgment. Support for T.P.K. was provided under a grant from the National Institutes of Health, GM-39526.

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