A β-Turn Is Present in the 392–411 Segment of the Human Fibrinogen γ-Chain. Effects of Structural Changes in This Segment on Affinity to Antibody 4A5†

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ABSTRACT: The interaction between fibrinogen γ-peptide 392–411, LTIGEGQQHHLGGAKQAGDV, and monoclonal antibody 4A5, an antibody with a high affinity for both for the peptide and native fibrinogen, is being studied as a model for peptide–antibody interaction. Two-dimensional NMR studies of the free peptide at pH 5.2 indicated the presence of a significant population, about 60%, of type II β-turn, spanning residues Gln^{407}–Asp^{410}. At pH 2.7, little, if any, turn structure is present. The D-Ala^{409} analog, which, for steric reasons, would be expected to preserve the β-turn, and the L-Ala^{409} analog, which would not be expected to have this conformational feature, were synthesized, and NMR studies confirmed the respective structural predictions. The affinity of the D-Ala analog for antibody 4A5 is even greater than that displayed by native γ392–411, while the affinity of the L-Ala analog is less than one-tenth that of the native peptide. Both conformational and steric effects involving residues 407–410 may be important in recognition by antibody 4A5. Since γ392–411 includes a platelet receptor binding locus of fibrinogen, and this and related peptides are inhibitors of platelet aggregation, the D-Ala^{409} and L-Ala^{409} analogs were tested for platelet binding. Neither of the analogs displays any measurable platelet binding, indicating that the recognition requirements for the platelet receptor differ considerably from those for antibody 4A5.

Recognition of antigens by antibodies is of fundamental importance in the body's defense against pathogens. Since much of the immune response is directed against proteins, significant effort has been expended in studying protein–antibody interaction (Benjamin et al., 1984; Lerner, 1984; Davies et al., 1988). Protein epitopes are often discontinuous, consisting of amino acid segments which are removed in sequence from one another, but are brought into spatial proximity by the three-dimensional structure of the protein (Arnon et al., 1971). However, many continuous, or linear, epitopes exist (Lerner et al., 1984). Due to the antigenic and immunogenic properties of linear sequences, it has been possible to prepare antibodies of "predetermined specificity" (Lerner et al., 1984): peptides corresponding to a particular sequence of a protein are used to generate antibodies which will cross-react with the native protein containing this sequence. We are employing a fibrinogen-derived peptide, and an antibody raised against this peptide, to study the conformational features involved in the peptide–antibody interation.

Fibrinogen, a key protein in blood clotting, is a soluble homodimer of molecular weight 340 000, each monomer containing an Aα-, Bβ-, and γ-chain (Doolittle, 1984). It undergoes a remarkable transformation when thrombin is activated to cleave fibrinopeptides A and B. The resulting fibrin monomers are soluble, but spontaneously aggregate to form a fibrin gel. Under the action of thrombin-activated factor XIII, this matrix is simultaneously stabilized by the formation of ε(γ-Gln)Lys cross-links between adjacent fibrin molecules. One part of the fibrin molecule that seems to play an important role in the aggregation and cross-linking steps is the carboxy-terminus portion of the fibrin γ-chain that is present in the globular fragment D (residues Aα105–206, Bβ134–461, and γ63–411) of fibrin (Budzynski, 1986), with cross-links formed between Gln^{398} and Lys^{406}. Confirmatory immunochemical evidence has been reported by Matsueda and Bernatowicz (1988), who find that monoclonal antibody 4A5 (see below), which binds γ392–411, blocks factor XIII mediated cross-linking and decreases clot strength. The C-terminal region of the γ-chain is also one of the fibrinogen regions involved in platelet aggregation. The portion of the γ-chain which interacts with the platelet Gp-IIb/IIIa receptor, a member of the integrin receptor family, has been localized to residues 400–411. Peptides corresponding to this, and even shorter sequences, inhibit platelet aggregation (Kloczewiak et al., 1984). This same fibrinogen region is also involved in the staphylococcal clumping reaction (Strong et al., 1982).

A monoclonal antibody has been generated which binds tightly to the fibrinogen γ-chain C-terminal region. Antibody 4A5 was raised against a peptide consisting of residues 395–401 and 402–411, cross-linked, as in fibrin, via the side chains of Gln^{398} and Lys^{406} (Bernatowicz et al., 1988). The affinity of 4A5 is 1.1×10^{-9} M for fibrinogen and 8.3×10^{-9} M for the 20-residue C-terminal peptide γ392–411, LTIGEGQQHHLGGAKQAGDV. The antibody interacts strongly with a peptide corresponding to γ402–411, but there is virtually no interaction with a peptide related to γ395–402, indicating that most, if not all, of the epitope lies in the 10 C-terminal amino acids (Matsueda & Bernatowicz, 1988).

NMR spectroscopy is being utilized to study fibrinogen γ392–411, both free in solution and when bound to antibody 4A5, with the aim of finding if the peptide possesses secondary or tertiary structure which is important in recognition by molecules which interact with fibrinogen, as well as using the peptide–antibody system as a model to study immune

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recognition. Most short linear peptides exist in solution as a mixture of rapidly interconverting conformations, but several linear peptides have been found to have substantial populations of discrete conformations (Dyson et al., 1988; Wright et al., 1988). It has been suggested that antigenic peptides will often possess a high degree of structure and that this favored solution conformation is in fact which is recognized by the antibody (Dyson et al., 1985, 1988, 1992). Initial results on γ392–411 in solution, presented below, indicate that the C-terminal region of the peptide contains a significant population of β-turn structure at pH 5.2 but much less turn structure at pH 2.7. By synthesizing peptide analogs and determining their affinity for 4A5, we show that changes in the conformational and steric properties of this region have significant effects on antibody binding.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized with an Applied Biosystems 430A synthesizer utilizing *t*-Boc chemistry. The peptides were cleaved from the resin and deprotected using the low–high HF method (Tam et al., 1983) and purified by HPLC with a reverse-phase C_{18} column using a gradient of 0–45% acetonitrile in 0.1% trifluoroacetic acid. Purity of peptides was verified by analytical HPLC, by amino acid analysis, and, in some cases, by sequencing.

NMR Spectroscopy. NMR spectra were acquired at 400 MHz on a JEOL GX-400 spectrometer. Samples were generally 10 mM peptide in 90% H_2O/10% D_2O, 0.1 M NaCl, 1 mM EDTA, and 1 mM sodium azide. Double quantum filtered correlated spectroscopy (COSY)[1] (Aue et al., 1976), NOESY (Jeener et al., 1979), ROESY (Bothner-By et al., 1984), and TOCSY (Bax & Davis, 1985) experiments were run in the phase-sensitive mode, using the method of States et al. (1982). Relaxation delays of 1 s were used for COSY and TOCSY, and 2 s for NOESY and ROESY. A mixing time of 400 ms was utilized with NOESY and ROESY, and a spin lock time of 70 ms was employed for TOCSY. The data matrix size, after zero-filling in t_1, was 1K × 1K. To estimate *J* values from COSY spectra, additional zero-filling in t_2 yielded a 4K × 1K matrix. One-dimensional HMQC experiments (Muller, 1979; Bax et al., 1983) on [^{15}N]Ala peptides were run with 32K points. Processing was carried out on a uVAX II employing the FTNMR software from Hare Research. Unshifted, or slightly shifted (30° or less), sine windows were used for COSY, exponential (LB = 1) and 90°-shifted sine windows for NOESY, and skewed 45°-shifted sine windows for ROESY and TOCSY.

Except for variable-temperature experiments, spectra were recorded at either 3 °C or 10 °C. The low temperature was chosen to (1) maximize the chance to detect a stable structure in solution and (2) shift the water peak downfield of where most α-CH resonances occur. The pH was generally 5.2. At this pH, the carboxyl groups would be expected to be in their physiological, ionized state, while this pH would also allow water suppression via presaturation, a technique not applicable at much higher pH values due to rapid exchange of amide resonances with water, leading to broadening and disappearance of the crucial amide resonances. Experiments were also run at pH 2.7, where the carboxyl groups would be largely protonated, to gauge whether the ionization state of these groups had an effect on the peptide's conformation.

Preparation of Antibody 4A5. The synthesis of the cross-linked peptide immunogen has been previously described (Bernatowicz et al., 1988). MAb-4A5 was produced in ascites fluid and purified by DEAE Affigel-Blue–Sepharose (Calbiochem). The antibody has been previously characterized (Matsueda & Bernatowicz, 1988).

Antibody Binding by Peptides. The effects of amino acid substitution on antibody recognition were determined with a competitive inhibition ELISA. Antigen, 25 μL of 10 μg/mL human fibrinogen in 0.01 M Tris, 0.15 M NaCl, and 0.02% NaN_3, pH 7.4 (TBSA), was placed in wells of microtiter plates (Falcon) for 1 h. The antigen solution was removed and replaced with 200 μL of 10% horse serum in TBSA, to minimize nonspecific antibody binding, for 2 h. The test antibody solutions (100 μL) were then added for 1 h. The test solutions were prepared in advance by mixing an inhibitor solution with an equal volume of antibody 4A5 solution which had been diluted to give 75% of maximal binding when mixed with an equal volume of TBSA. Inhibitor solutions were prepared by dissolving each peptide at 2 mg/mL and submitting aliquots for amino acid analysis to determine exact molar concentrations. Prior to being mixed with antibody 4A5, each inhibitor solution was diluted serially by 1/0.316 to give a half-log dilution series. After incubation of the test solutions with the antigen-coated wells, unbound 4A5 was removed by repetitive washing steps. The specifically bound 4A5 was then detected by using a peroxidase-conjugated second antibody employing the ABTS substrate as described in the kit from Kirkegaard and Perry. Results are presented as percent inhibition = 100[1 – (binding in the presence of peptide)/(binding in the absence of peptide)].

Platelet binding to peptides was determined by the inhibition of binding of ^{125}I-fibrinogen (Timmons et al., 1984). The platelets were prepared as previously described (Timmons et al., 1989).

RESULTS

Conformation of Peptide γ392–411. NMR spectroscopy was employed to determine the conformation of γ392–411 in aqueous solution. The general strategy was to assign all resonances of the ^{1}H NMR spectrum of γ392–411 and to then use a variety of NMR experiments to ascertain whether the peptide displayed a preferred conformation. Resonances were assigned using standard two-dimensional (2D) NMR methods (Wuthrich, 1986). This involved COSY (Aue et al., 1976) and TOCSY (Bax & Davis, 1985) to group the resonances into individual amino acids, and NOESY (Jeener et al., 1979) and ROESY (Bothner-By et al., 1984) to allow sequential, and thereby specific, assignment. Due to the correlation time of the peptide, ROESY data were in general of higher quality than NOESY data; all intraresidue and sequential $\alpha_{CH\text{-}NH}$ cross-peaks were observed. Resonance assignments for γ392–411 (Table I) are in complete agreement with those published for γ385–411 (Mayo et al., 1990), though the tabulated values for the NH shifts differ from those of 385–411 due to different temperatures: 10 °C for γ392–411 and 30 °C for γ385–411 (Mayo et al., 1990). The NH chemical shift for Ile^{394} in γ392–411 differs significantly from the corresponding shift in γ385–411, probably due to additional structure in the N-terminal region of the latter peptide (see below).

The observed NOEs (NOESY or ROESY) were either intraresidue or between sequential residues, implying that the

[1] Abbreviations: COSY, correlated spectroscopy; TOCSY, total correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy; HMQC, heteronuclear multiple quantum coherence spectroscopy; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate).

Table I: Proton Chemical Shifts at pH 5.2, 10 °C, Shift Differences of Amide NH Protons between pH 5.2 and 2.7, and Temperature Dependence of Amide Chemical Shifts for Fibrinogen γ-Chain Peptide 392–411 in 90% H_2O/10% D_2O

	chemical shift (ppm)				NH shift difference,	temp coefficient of NH shift ($\times 10^{-3}$ ppm/°C)	
residue	NH	$C_\alpha H$	$C_\beta H$	other	pH 5.2 vs pH 2.7	pH 5.2	pH 2.7
Leu^{392}		4.15	1.74	γH 1.64, δH 0.94			
Thr^{393}	8.86	4.46	4.16	γH 1.23	0.10[a]	7.3	6.1
Ile^{394}	8.59	4.15	1.86	γH 0.93, 1.24, 1.51; δH 0.88	0.02	9.5	9.7
Gly^{395}	8.66	3.97			−0.01	6.7	7.5
Glu^{396}	8.36	4.29	2.31	γH 2.01, 2.07	−0.01	5.2	5.8
Gly^{397}	8.66	3.97			−0.01	7.9	8.3
Gln^{398}	8.28	4.29	1.98 2.11	γH 2.35; $CONH_2$ 6.96, 7.64	−0.04	5.5	5.8
Gln^{399}	8.47	4.23	1.88 1.96	γH 2.33; $CONH_2$ 6.96, 7.58	−0.02	6.5	6.5
His^{400}	8.59	4.66	3.13 3.24	4H 7.29; 2H 8.60	−0.03	7.7	7.0
His^{401}	8.70	4.70	3.20 3.27	4H 7.31; 2H 8.61	−0.01	7.4	6.4
Leu^{402}	8.62	4.38	1.68	γH 1.70; δH 0.90, 0.94	0.00	8.6	8.0
Gly^{403}	8.63	3.98			0.00	8.6	8.2
Gly^{404}	8.38	3.98			0.00	6.4	6.1
Ala^{405}	8.33	4.31	1.40		−0.02	7.2	7.2
Lys^{406}	8.47	4.29	1.71 1.82	γH 1.47; δH 1.69; εH 3.01; ϵNH_2 7.59	−0.01	8.5	8.2
Gln^{407}	8.63	4.35	1.99 2.14	γH 2.39; $CONH_2$ 6.96, 7.64	0.19	8.7	7.5
Ala^{408}	8.58	4.27	1.42		0.06	9.5	8.9
Gly^{409}	8.53	3.96 4.01			0.02	8.6	7.8
Asp^{410}	8.26	4.68	2.67 2.79		−0.10	2.9	5.1
Val^{411}	7.81	4.05	2.11	γH 0.91	−0.37	7.5	8.2

[a] Positive numbers indicate downfield shift at higher pH.

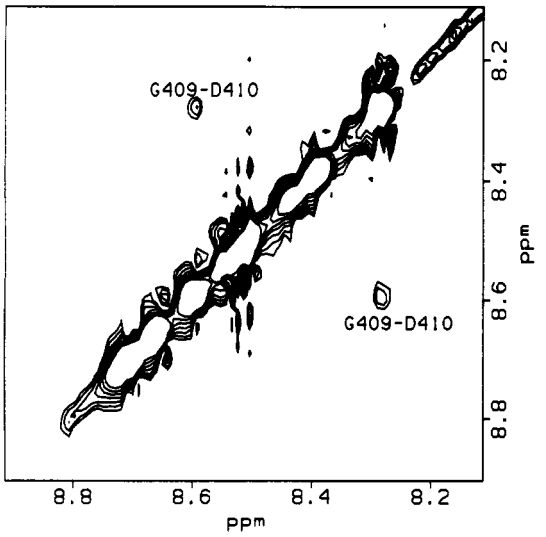

FIGURE 1: Amide NH–NH region of the 400-ms ROESY spectrum of 10 mM γ392–411 in 90% H_2O/10% D_2O, pH 5.2, 3 °C. The slight difference in chemical shifts from those listed in Table I is due to the lower temperature of the spectrum displayed in the figure.

peptide is largely unstructured (Wuthrich, 1986). The presence of sequential NH–NH cross-peaks indicated that some stable structure might be present (Wright et al., 1988). In particular, at 3 °C the ROESY spectrum showed a Gly^{409}–Asp^{410} NH–NH cross-peak of moderate intensity (Figure 1). Additional experiments were performed to see if there was corroborating evidence for a preferred conformation.

A series of COSY experiments were performed at temperatures from 4 to 40 °C to measure the temperature dependence of the chemical shift of the NH protons (Table I). This temperature dependence reflects the solvent exposure of the NH proton involved, with fully exposed NH protons having temperature coefficients in aqueous solution of (6–10) $\times 10^{-3}$ ppm/°C and NH protons which are shielded from solvent having much lower temperature coefficients (Kopple et al., 1969). A strong indication of nonrandom structure in γ392–411 is the low value for the temperature dependence of the NH of Asp^{410}, signifying that this proton is shielded from solvent and may participate in a hydrogen bond.

A family of structures occurring frequently in peptides and proteins is the β-turn, in which the carbonyl oxygen of residue "*i*" is hydrogen-bonded to the backbone amide proton of residue "*i*+3" (Smith & Pease, 1980). Among the NMR parameters characterizing a β-turn are a moderate NH_{i+2}–NH_{i+3} NOE and a low chemical shift temperature dependence for the NH of residue "*i*+3". Both the Gly^{409}–Asp^{410} NH–NH cross-peak and the low-temperature dependence of the NH of Asp^{410} are consistent with the presence of a β-turn from Gln^{407} to Asp^{410}. Another spectral parameter characterizing such a turn is a low (about 4 Hz) αCH–NH coupling constant for residue *i*+1 of the turn, in this case Ala^{408}. The usual *J* value for a non-Gly residue in an unstructured region is from 6 to 8 Hz. Measurement of *J* values directly from the COSY spectra indicated that Ala^{408} had a *J* value of about 5-Hz, Ala^{405} had a *J* value of about 6 Hz, and *J* values for all other non-Gly residues were 6.5–8.5 Hz. Because determination of small *J* values from COSY spectra can be somewhat inaccurate, the Ala values were measured from [^{15}N]Ala peptides via the HMQC (Muller, 1979; Bax et al., 1983) experiment. This experiment gives spectra in which only protons bonded to the enriched nucleus are observed. Since HMQC was performed as a one-dimensional experiment, it could be run under conditions of very high spectral resolution and sensitivity, and therefore yielded very precise *J* values, which are given in Table II. If about 60% of the peptide is in a turn at pH 5.2 [based on Dyson et al. (1988); see below], about 30% is in a turn at pH 2.7, and the *J* value for Ala not in a turn is 5.5–6.0 Hz (the value observed for Ala^{405}), then

Table II: Coupling Constant Values ($J_{\alpha CH\text{-}NH}$ in Hertz) of Alanine Residues of γ392–411, Derived from One-Dimensional HMQC Experiments[a]

pH	Ala[408]	Ala[405]
5.2	4.8	5.6
2.7	5.4	5.7

[a] Estimated accuracy for all J values is ±0.1 Hz.

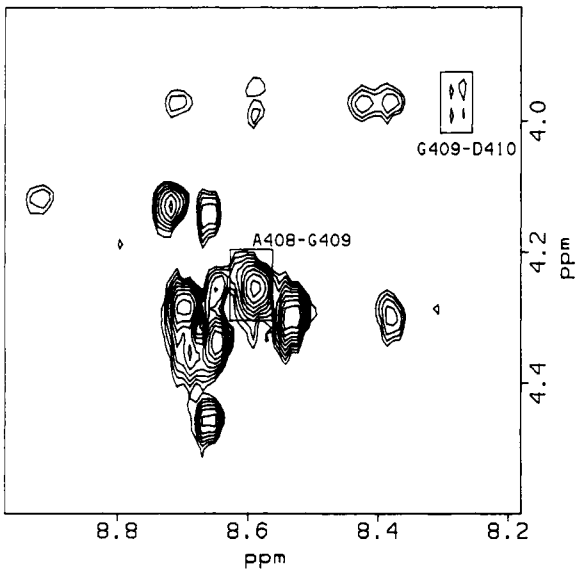

FIGURE 2: Portion of the αCH–NH region of the same ROESY spectrum as Figure 1. Cross-peaks are from the αCH of the first residue to the NH of the second residue.

the J value for the Ala[408] residue in the turn would be 4–4.5 Hz, in good agreement with the observed value.

Several types of β-turns exist, characterized by particular ranges of dihedral angles in residues i+1 and i+2. The most common turns are called type I and type II. When the turn population is very high, type I and type II turns can be differentiated by the intensity of the αCH_{i+1}–NH_{i+2} NOESY cross-peak (Dyson et al., 1985; Wuthrich, 1986; Stradley et al., 1990), in this case between Ala[408] and Gly[409]. In a type I turn, the αCH_{i+1}–NH_{i+2} internuclear distance will be 3.5 Å, and the cross-peak will be weak to moderate, while in a type II turn, the distance is 2.1 Å, and the cross-peak will be very strong. As shown in Figure 2, the Ala[408]–Gly[409] cross-peak is very intense, indicating that the β-turn is mostly type II (see Discussion). The relatively weak αCH_{i+2}–NH_{i+3} cross-peak between Gly[409] and Asp[410] is also characteristic of a β-turn. Thus, NMR criteria involving amide shift coefficients, J values, and NOEs all point to a β-turn from residues 407 to 410[2] (Figure 3).

Experiments were repeated at pH 2.7, where the carboxyl groups of Asp[410] and Val[411] would be largely protonated. A portion of the NH–CH of the COSY spectrum of γ392–411 at pH 5.2 and 2.7 is compared in Figure 4, and the shift differences of corresponding amide protons between the two pH values are listed in Table I. The 0.2 ppm shift of the Gln[407] backbone NH is of particular note, since this proton is three residues removed from the nearest carboxyl group,

[2] There was some evidence for a weak Ala[408]–Asp[410] CH–NH NOE cross-peak, which corresponds to the i+1 to i+3 cross-peak expected in a β-turn, but the observation of this interaction was not totally reproducible. In subsequent studies with peptide γ402–411, employing higher concentrations than those used with 392–411, this cross-peak could be reproducibly observed. A ROESY spectrum of 402–411 displaying this weak cross-peak is shown in Figure 3.

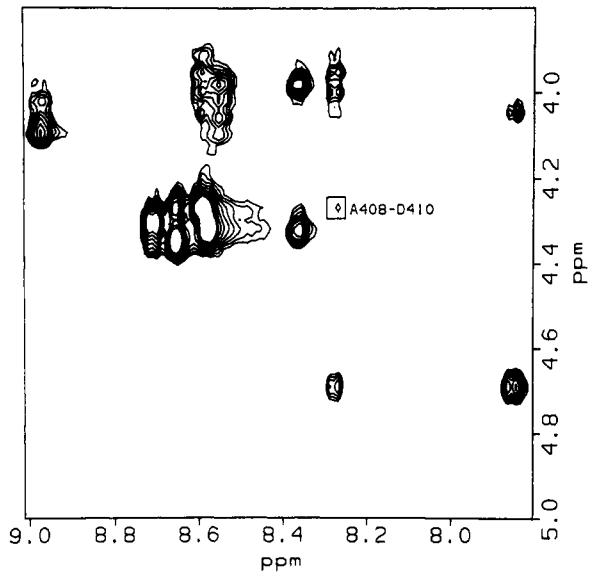

FIGURE 3: Portion of the ROESY spectrum of γ402–411. Peptide concentration, 50 mM; all other conditions the same as for Figure 1.

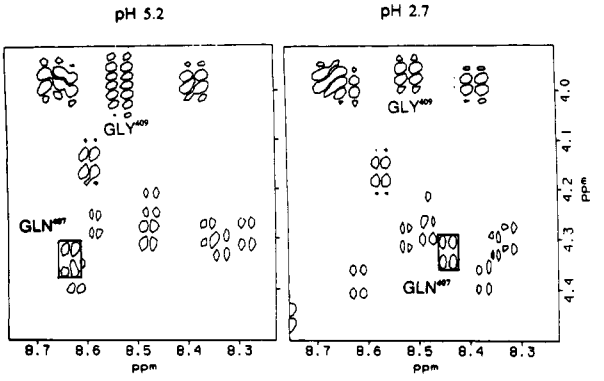

FIGURE 4: Portion of the αCH–NH region of COSY spectra at 10 °C of γ392–411 at pH 5.2 (left) and pH 2.7 (right).

and the electrostatic effect caused by ionization of the carboxyl groups would not be expected to induce such a large effect. Whether this shift is due to direct interaction with a carboxyl group, brought into proximity to the Gln NH as part of a folded structure, or by a conformational change which occurs on ionization, it is further confirmation that a highly populated, defined structure exists at the C-terminal end of the peptide. The increased temperature dependence of the NH of Asp[410] at low pH (Table I) and the increase in the J value of Ala[408] at low pH (Table II) are both consistent with a reduced population of turn structure at pH 2.7. As expected, the J value for Ala[405] is pH-independent, since this residue is not affected by the pH-dependent conformational change.

Conformation of Ala[409] and D-*Ala[409] Analogs.* In order to verify the β-turn hypothesis, the peptides in which Gly[409] was replaced by D-Ala or L-Ala were synthesized. A type II β-turn favors either Gly or a D-amino acid in the i+2 position of the turn. The presence of an L-amino acid is energetically unfavorable due to steric interference between the carbonyl oxygen of the second residue and the β-hydrogens of the third residue (Richardson, 1981). One can therefore predict that the D-Ala[409] substitution should lead to a retention of the conformation observed in γ392–411 while the L-Ala substitution would disrupt the observed structure.

The chemical shifts, temperature dependences of backbone NH resonances, and the pH dependence of these parameters for residues 392–406 were virtually identical in the two analogs

Table III: Conformationally Significant Spectral Parameters of γ392–411 and D-Ala^{409} and L-Ala^{409} Analogs

	pH	γ392–411	D-Ala^{409} analog	L-Ala^{409} analog
chemical shift of backbone NH of Gln^{407} (ppm)	5.2	8.63	8.64	8.53
	2.7	8.44	8.41	8.45
temp coefficient of Asp^{410} NH chemical shift (×10^{-3} ppm/°C)	5.2	2.9	3.0	5.9
	2.7	5.1	5.0	7.3

and in native γ392–411 (data not shown). However, with respect to the temperature coefficient of the NH shift of Asp^{410}, the chemical shift of the backbone NH of Gln^{407}, and the pH dependence of these parameters, the D-Ala^{409} peptide has spectral properties very similar to native γ392–411, while the L-Ala analog differed significantly (Table III). Fibrinogen γ392–411 and the D-Ala analog appear to undergo a transition from a structured form at pH 5.2 to a less structured form at pH 2.7, while the L-analog is always less structured. The results with the D-Ala^{409} and L-Ala^{409} analogs strongly support the hypothesis that a type II β-turn exists from residues 407 to 410 in peptide γ392–411.

Dyson and co-workers have done extensive studies on linear peptides in aqueous solution, including substantial work on peptides having the structure Tyr-Pro-X-Asp-Val (Dyson et al., 1988). They find that the peptide in which X is Gly, and thus contains the same Gly-Asp-Val carboxyl-terminal tripeptide as does γ392–411, exists substantially in a β-turn conformation. Their chemical shift temperature coefficient of the Asp NH, 3.0 × 10^{-3} ppm/°C, is virtually identical to that observed in γ392–411 and the D-Ala analog at pH 5.2. The Asp NH temperature coefficient which they find for the unstructured peptide, YPADV, 7.2 × 10^{-3} ppm/°C, is very similar to the value for L-Ala^{409}(γ392–411) at pH 2.7. Dyson et al. quantitate the amount of turn present by assuming an NH resonance in a 100% populated turn would have a coefficient of zero, 7.2 × 10^{-3} ppm/°C represents the coefficient where no turn is present, and intermediate coefficients are linearly dependent on the percentage population of the turn. A value of 3 × 10^{-3} ppm/°C, which is observed for both γ392–411 and the D-Ala^{409} analog at pH 5.2, would indicate a turn population of about 60%, a coefficient of (5–6) × 10^{-3} ppm/°C, seen with γ392–411 and the D-Ala analog at pH 2.7, as well as the L-Ala analog at pH 5.2, would indicate a turn population of 20–35%, and the L-Ala analog at pH 2.7 would be completely unstructured.

Peptide Binding Studies to Antibody 4A5 and to the Platelet Receptors. The relative affinities of native γ392–411, the L-Ala^{409} analog, and the D-Ala^{409} analog for 4A5 are shown below (Figure 5). Though the L-Ala analog has an affinity which is less than one-tenth that of the native peptide, the D-analog binds with almost 10-fold greater affinity than the native peptide.

The inhibition of fibrinogen binding to platelets by γ392–411 and by the D- and L-Ala analogs was determined (Figure 6). The inhibition of binding of ^{125}I-fibrinogen by γ392–411 is similar to that of related fibrinogen peptides (Kloczewiak et al., 1984), but neither analog displays any interaction with the platelet receptor.

DISCUSSION

While NOEs observed with γ392–411 at pH 5.2 gave some indication of a peptide containing ordered structure, measurement of amide NH temperature coefficients and *J* values, experiments at pH 2.7, and, above all, work with Ala^{409} and

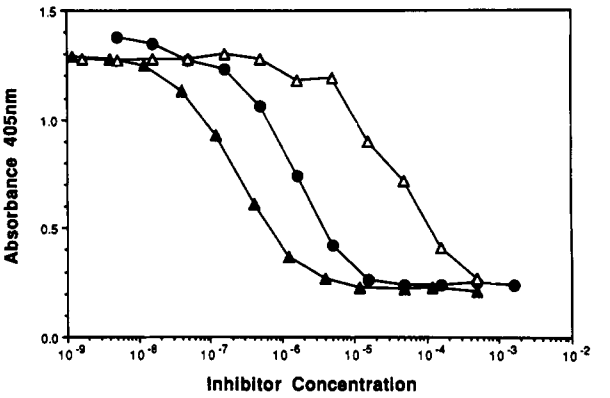

FIGURE 5: Inhibition of fibrinogen binding to antibody 4A5 by γ392–411 (filled circles), the D-Ala^{409} analog (filled triangles), and the L-Ala^{409} analog (open triangles) as determined by competitive ELISA (see Materials and Methods).

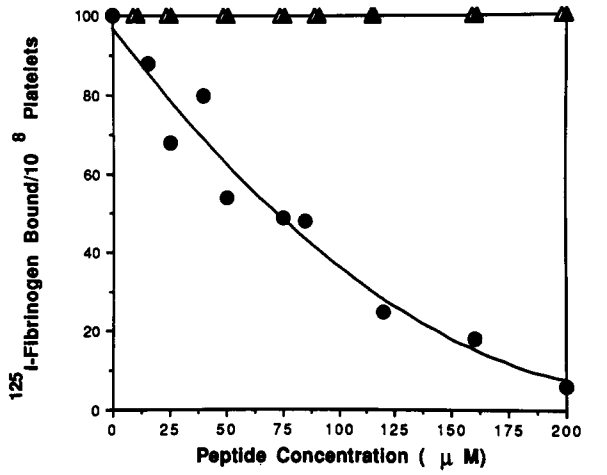

FIGURE 6: Inhibition of fibrinogen binding to platelets by γ392–411 and analogs (symbols same as for Figure 5).

D-Ala^{409} analogs led to a firm conclusion of a β-turn involving residues 407–410, Gln-Ala-Gly-Asp. There is some ambiguity with regard to whether the type of turn present is I, II, or a mixture of both. The observed strong Ala^{408}CH–Gly^{409}NH cross-peak is expected from a type II β-turn, but a strong cross-peak would also arise from the 40% of peptide molecules which are unfolded. The presence of a high percentage of type I turn would diminish the intensity of this cross-peak, but even though the Ala^{408}–Gly^{409} cross-peak is as intense as any in the spectrum, the presence of a type I turn cannot be ruled out. We see no evidence for the NH$_{i+1}$–NH$_{i+2}$ cross-peak, in this case Ala^{408}Gly^{409}, which would be indicative of a type I β-turn, but this cross-peak, were it present, would be too close to the diagonal to allow its observation. The best evidence for the type II structure in γ392–411 is the higher turn population in the native peptide (Gly^{409}) and the D-Ala^{409} analog, and the lower turn population in the L-Ala^{409} analog. It is only a type II β-turn which is sterically unfavorable in the latter peptide. If a type I turn were present in the native peptide, the turn would not be expected to be more highly populated in the D-Ala analog than in the L-Ala analog.

It is somewhat surprising that a significant population of γ392–411 exists in a β-turn conformation at pH 5.2. On the basis of qualitative Chou–Fasman rules (Chou & Fasman, 1978), the QAGD sequence would not be predicted to be in a turn conformation. Dyson et al. (1988) found high β-turn populations in peptides which included related sequences but had the turn-favoring proline residue in the *i*+1 position of the turn. As with 392–411, these related peptides (Dyson et

al., 1988) had more highly populated turns when the carboxyl group of the Asp residue in position $i+3$ was ionized. Molecular dynamics simulations indicated that turns in these peptides are stabilized by a bifurcated hydrogen bond, which in 392–411 would connect the carbonyl of Gln^{407} and the amide NH protons of Asp^{410} and Val^{411} (Wright et al., 1990). No NMR evidence for this bifurcated hydrogen bond was detected by Wright et al. (1990), nor was such evidence seen in the present studies.

The most intriguing of our results is that the D-Ala^{409} analog, which, like native γ392–411, has a high population of β-turn spanning residues 407–410, binds to antibody 4A5 with 2 orders of magnitude higher affinity than the L-Ala^{409} analog, which has a much lower turn population. The choice of analogs was guided solely by the NMR results on the native peptide and the expectation, which was confirmed, concerning the relative amounts of β-turn structure which they would possess. Nevertheless, because the analogs possess different steric as well as conformational properties, we cannot unequivocally assess the significance of the β-turn structure for recognition by 4A5.

One interpretation is that the β-turn from residues 407 to 410 is recognized by the antibody and that the peptide binds in the β-turn conformation. This would be consistent with the widely held belief (Benjamin et al., 1984) that antibodies to flexible molecules recognize specific conformers which are present in solution and bind to those distinct conformers. The high population of turn conformation present in solution also bolsters the hypothesis that antigenic regions of peptides often contain a population of ordered structure which is high enough to be detected by NMR (Dyson et al., 1985, 1988, 1992).

The stronger antibody binding of the D-Ala analog relative even to the native peptide likely indicates a favorable hydrophobic interaction between the D-Ala methyl group and the antibody. Recently, Robinson and co-workers (Hinds et al., 1991) have synthesized antigens which bound to an antibody with much higher affinity than the original peptide immunogen, a peptide which had been shown to possess a β-turn structure (Dyson et al., 1988). They concluded that the increased affinity was due to a stabilization of the turn structure, with a contribution from favorable antigen–antibody contacts. The L-Ala^{409}(γ392–411) analog may have about 20% structured conformation, as opposed to about 60% for the native peptide, yet the latter binds to the antibody with 10–20 times higher affinity than the former. We have no spectral evidence as to the type of turn present in the L-Ala analog. On the basis of the known unfavorable interactions of an L-amino acid in the third position of a type II β-turn, the small degree of structure observed in the L-Ala^{409} analog is likely either a type I β-turn or a nonturn structure, and the type II turn which may be necessary for recognition by the antibody is not only not present in solution but also cannot easily be formed even when the peptide binds to the antibody. Since the conformational studies presented above all involve studies of the free peptides, the possibility does exist that the β-turn is not involved in binding and that the relative binding of γ392–411 and the two analogs is due solely to steric considerations, with the L-Ala group going into a sterically unfavored position and the D-Ala group fitting fortuitously into a cavity where it can favorably interact with the antibody. Antibody conformational change has been shown to occur in the course of peptide–antibody interaction (Rini et al., 1992), and the conformation of the antibody-bound peptide may also differ significantly from its unbound conformation. Work is presently under way in these laboratories to elucidate the antibody bound conformation for γ392–411, as well as for the D-Ala and L-Ala analogs.

While the substitution of D-Ala for Gly^{409} leads to increased affinity to 4A5, the D-Ala analog, as well as the L-Ala analog, is inactive in binding to the platelet receptor. This receptor, a membrane of the integrin family, recognizes γ392–411 as well as RGD-containing peptides (Kloczewiak et al., 1984; Plow et al., 1985; Timmons et al., 1989). Thus, the platelet receptor, unlike the antibody, may not recognize the β-turn structure which is present in solution. However, the high receptor affinity of cyclic analogs such as AcHHLGGAcyclo-(S,S)CRGDPen-NH_2 (Samanen et al., 1991) indicates that some kind of folded structure may be involved in receptor binding. The receptor may recognize the β-turn structure which is present in γ392–411, but cannot accommodate any side chain, whether it be D or L, in position 409. Recent studies on naturally occurring platelet binding peptides such as echistatin (Saudek et al., 1991; Chen et al., 1991) and kistrin (Adler et al., 1991) have not seemed to indicate any universally recognized conformational features for the RGD sequence which is crucial for this binding.

The conclusions presented here on the solution conformation of γ392–411 differ from those reported for peptide γ385–411 (Mayo et al., 1990). In the latter study, a series of loops and turns was postulated for residues 385–401, with little or no ordered structure in residues 402–411; also, no clear structure was seen in peptide γ400–411. The sequential assignments for γ392–411 agree with those for γ385–411, and the NOE data are very similar, so these are not sources of disagreement on structure. Our results with γ392–411 are consistent with a low population of ordered structure in residues 392–399, as evidenced by the pH-dependent shift of the NH of Thr^{393}, amide shift temperature coefficients of between 5 and 6 × 10^{-3} ppm/°C for Glu^{396} and Gln^{398}, and the presence of weak sequential NH–NH cross-peaks (not displayed in Figure 1, but visible at lower contour plotting) between residues in this region. In the longer γ385–411, this structure may be further stabilized.

The most important difference between the investigation with γ385–411 and those with γ392–411 is the pH of the experiments. It is not surprising that there was little evidence of conformational preference in residues 401–411 of peptide γ385–411, since the peptide was studied only at pH 3. Little structure was detected in γ392–411 at this pH, and it was only at pH 5.2, when the carboxyl groups of Asp^{410} and Val^{411} were ionized, that unambiguous evidence of a significant population of β-burn conformation was obtained. This points out the potential danger in the rather common practice in peptide NMR of doing NMR experiments at pH values where the carboxyl groups are protonated, rather than being in their usual ionized state. Especially in short peptides, which have very few stabilizing secondary or tertiary interactions, the change in charge may significantly affect the structure of the peptide.

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