Interactions of integrin GPIIb/IIIa-derived peptides with fibrinogen investigated by NMR spectroscopy

Letitia J. YAO* and Kevin H. MAYO†

Department of Biochemistry, Biomedical Engineering Center, University of Minnesota, 420 Delaware Street, S.E., Minneapolis, MN 55455, U.S.A.

Three peptides derived from platelet integrin receptor glycoprotein $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) have been identified recently as fibrinogen-binding sequences: GPIIb 300–314 and 656–667 and GPIIIa 211–223. NMR spectroscopy has been used here to investigate the interactions of these peptides with parent fibrinogen. Based on resonance broadening and chemical-shift changes of peptides in the presence and absence of fibrinogen, interactions in the fast ligand-exchange regime are apparent and interfacial residues can be proposed. Positively charged arginines and

histidines, along with several hydrophobic residues, are implicated as being crucial to the binding process. Transferred nuclear Overhauser effects and distance geometry calculations allow discussion of probable conformations in peptide-‘bound’ states. These identifications are consistent with other biological/chemical data and provide the basis for further studies aimed at understanding fibrinogen-mediated platelet aggregation on the molecular level.

INTRODUCTION

Platelet aggregation is the primary mechanism of haemostatic control and is dependent upon the binding of fibrinogen to the surface of activated platelets [1]. The platelet receptor mediating these adhesive interactions is a heterodimer complex of glycoprotein $\alpha_{IIb}\beta_3$ α -subunit (GP α IIb) and β -subunit (GPIIIa) [2,3], which are members of a superfamily of receptors that mediate such apparently diverse phenomena as platelet aggregation, platelet adhesion to collagen, fibroblast adhesion to fibronectin and vitronectin, and leucocyte binding to endothelial cells and matrix proteins [4,5]. Receptors within this integrin superfamily [4] are sequentially homologous heterodimers with α - and β -subunits. Because integrins mediate a broad spectrum of cell–cell or cell–substrate adhesive phenomena, knowledge of their functional domains is crucial to a molecular description of cellular interactions.

A common feature of many ligands that bind to GPIIb/IIIa is the presence of the Arg-Gly-Asp (RGD) amino acid sequence, originally identified as the cell-binding domain of fibronectin [6,7]. Since RGD-containing peptides in general inhibit the binding of adhesive proteins to GPIIb-IIIa, it appears that RGD mediates receptor binding. Other RGD-independent ligand-binding sequences have also been identified [8–11]. The identification of a variety of GPIIb and GPIIIa domains putatively involved in ligand recognition [12,13] indicates that the integrin’s receptor surface may be made up of regions from both subunits, which, although distantly located within the linear primary structure, are brought into juxtaposition by the spatial folding of the polypeptide chains [14]. Several groups have identified sequences in GPIIb/IIIa which may be ligand-binding sites on the receptor.

Charo et al. [15] showed that synthetic peptide GPIIIa 211–222, SVSRNRDAPEGG-NH₂, blocked the binding of fibronectin,

von Willebrand factor and vitronectin to purified GPIIb-IIIa and demonstrated direct binding of GPIIIa peptide 204–229 to fibrinogen and fibronectin by an enzyme-linked immunosorbent assay. Polyclonal antibodies to these peptides also blocked fibrinogen binding to purified GPIIb-IIIa as well as platelet aggregation. GPIIIa peptide 211–222 is highly conserved in the other β -subunits within the integrin superfamily {the fibronectin receptor family (β_1) [16] and the leucocyte-receptor family (β_2) [17]} and may represent a portion of the adhesive protein-binding domain of GPIIb-IIIa. This region of GPIIIa is positioned at the carboxyl side of GPIIIa sequences to which the RGD peptides cross-link [18,19].

GPIIb peptide 300–312 (GDGRHDLVGLGAPL) constitutes another specific but common recognition site on GPIIb/IIIa for both LGGAKQAGDV (fibrinogen γ -chain C-terminal peptide) and RGD-containing ligands. The fibrinogen γ -chain peptide cross-links selectively to residues 294–314 of GPIIb [20]. Taylor and Gartner [21] reported that GPIIb peptide 300–312 inhibits platelet aggregation and the adhesion of stimulated platelets to fibrinogen, fibronectin, vitronectin and von Willebrand factor and binds directly to fibrinogen and vitronectin. GPIIb peptide 300–312 inhibits ligand binding to GPIIb/IIIa, but not to GPIc-IIa, indicating selectivity in peptide bioactivity.

Calvete [12] showed that a synthetic peptide containing the polypeptide stretch GPIIb 656–667 binds to soluble fibrinogen. This interaction is Ca²⁺-independent and can be partially inhibited with synthetic fibrinogen γ -chain peptide 400–411, but not with GRGDS. In addition, peptide GPIIb 656–667 inhibits in a dose-dependent manner the aggregation of activated platelets (IC₅₀ = 170 μ M). The GPIIb 656–667 region may form part of the inducible fibrinogen-binding site and may not overlap with the integrin RGD-recognition domain. GPIIb peptide 656–667 contains the epitope for the platelet aggregation inhibitory monoclonal antibody M6, whose surface expression on GPIIb/

Abbreviations used: TOCSY, two-dimensional-NMR homonuclear Hartman–Hahn total correlated spectroscopy; NOE, nuclear Overhauser effect; TRNOE, transferred NOE; NOESY, two-dimensional-NMR NOE spectroscopy; GP, glycoprotein; IIb/IIIa, integrin receptor GP $\alpha_{IIb}\beta_3$; GPIIb, GP $\alpha_{IIb}\beta_3$ α -subunit; GPIIIa, GP $\alpha_{IIb}\beta_3$ β -subunit; DG, distance geometry; r.m.s.d., root mean squared deviation.

* Present address: Department of Pharmacology, c/o Professor Thomas James, University of California San Francisco, San Francisco, CA 94122, U.S.A.

† To whom correspondence should be addressed.

IIIa is platelet activation-dependent and shows a high hydrophobic complementarity to the fibrinogen γ 402–411 GPIIb/IIIa recognition sequence [22,23]. Both its ability to bind fibrinogen in solution and its selectivity for the γ -chain make GPIIb 656–667 unique among the peptides derived from GPIIb [21,24] or GPIIIa [15,25] reported to bind to fibrinogen.

In this study, NMR spectroscopy has been used to study the interaction of these three peptides with fibrinogen.

MATERIALS AND METHODS

Peptide synthesis

Peptides were synthesized on a Milligen Biosearch 9600 automated peptide synthesizer. The procedures used were based on Merrifield solid-phase synthesis utilizing fluoren-9-ylmethoxycarbonyl-BOP chemistry [26]. After the sequence had been obtained, the peptide support and side-chain protection groups were acid (trifluoroacetic acid and scavenger mixture) cleaved. Crude peptides were analysed for purity on a Hewlett-Packard 1090M analytical HPLC using a reverse-phase C₁₈ Vydac column. Peptides generally were about 90 % pure. Further purification was carried out on a preparative reverse-phase HPLC C₁₈ column using an elution gradient of 0–60 % acetonitrile with 0.1 % trifluoroacetic acid in water. Peptides were then analysed for amino acid composition on a Beckman 6300 amino acid analyser by total hydrolysis of samples using 6 M HCl at 110 °C for 18–20 h. N-terminal sequencing confirmed the purity of the peptide. Since GPIIb/IIIa peptides are derived from the central portion of native fibrinogen, the C-terminal carboxylates were synthesized in the amide form.

Soluble human fibrinogen was purchased from Sigma Chemical Co. and was dialysed three times against sodium citrate buffer. Since NMR spectra of fibrinogen recorded before and after dialysis were essentially the same, fibrinogen was used as is.

NMR measurements

Freeze-dried samples for NMR measurements were dissolved in H₂O/²H₂O (9:1) containing 10 mM sodium phosphate, 25 mM sodium citrate and 165 mM sodium chloride. Peptide concentration was 0.1–1.2 mM and fibrinogen concentration was 0.1 mM. Solution conditions were 10 mM sodium phosphate/25 mM sodium citrate/165 mM sodium chloride, pH 6.4. The desired pH was achieved by adding microlitre quantities of NaO²H or ²HCl to the protein/peptide sample and was not adjusted for isotope effects. The temperature was controlled at 288 K. All NMR spectra were acquired on a Bruker AMX-500 NMR spectrometer.

For sequential assignments, two-dimensional-NMR homonuclear Hartman–Hahn total correlated spectroscopy (TOCSY) (spin-locked with a MLEV-17 sequence and a mixing time of 60 ms) [27] and two-dimensional-NMR nuclear Overhauser effect (NOE) spectroscopy (NOESY) [28,29] experiments were performed. All spectra were acquired in the phase-sensitive mode (States-time proportional phase incrementation) [30,31]. The water resonance was suppressed by direct irradiation (1 s) at the water frequency during the relaxation delay between scans as well as during the mixing time in NOESY experiments.

The majority of the two-dimensional-NMR spectra were collected as 256 t_1 experiments, each with 1×10^3 complex data-points over a spectral width of 5 kHz in both dimensions with the carrier placed on the water resonance; 64 or 96 scans were generally time averaged per t_1 experiment. The data were processed directly on the Bruker AMX-500 X-32 or off-line on a Bruker Aspect-1 or SGI Indigo workstation with the Bruker

UXNMR program. Data sets were multiplied in both dimensions by a 30 to 60 degree shifted sine-bell or Lorentzian to Gaussian transformation function and generally zero-filled to 1×10^3 in the t_1 dimension before Fourier transformation.

Structural calculations

All structural calculations were done on a Silicon Graphics (SGI) Indigo Extreme workstation using an R4400 CPU running with 32 MB RAM. The correct L-amino acid peptide sequence was built using Insight II/Discover (Biosym Technologies, San Diego, CA, U.S.A.). To ensure that the starting peptide geometry was correct, initial structures were minimized without NOE-based restraints using the Discover program. A variable-target function distance geometry procedure (DGII in the Biosym suite of programs) was used [32,33]. Transferred NOE (TRNOE)-derived distance constraints were incorporated sequentially, starting with one pair at a time from sets of initial random structures. Final iterations incorporated all distance constraints simultaneously. During calculations, the conjugate gradient method was used to minimize the full matrix error function. Additional NOEs having low intensity or chemical-shift degeneracy were then identified from the analysis of interim structural models and were used iteratively in later calculations. At the final stage, *in vacuo* molecular dynamics (50 ps) and energy minimization were used to relieve structural irregularities, e.g. bad bond lengths and contacts, and to improve structures. Resulting structures were analysed and compared on an SGI Indigo Extreme workstation using Insight II. Most structures compared favourably with TRNOE-derived distances (less than 0.5 Å deviations) and with energetically favourable Φ, Ψ angles. Structures which did not meet these criteria were discarded.

RESULTS AND DISCUSSION

Figure 1 (lower spectral traces) shows the downfield NMR spectrum of fibrinogen (330 kDa) alone. Addition to the fibrinogen solution of any of the three GPIIb/IIIa-derived peptides at a 1:1 molar ratio shows little change in the spectra (Figure 1). Even peptide resonances, which in the absence of fibrinogen are generally well resolved (top trace in each panel), are highly broadened. As the peptide:fibrinogen ratio is increased, peptide resonances narrow and at the highest peptide:fibrinogen ratio correlate with chemical shifts of resonances in the free peptide. Resonances have been labelled according to the sequence-specific resonance assignments discussed below. In the presence of fibrinogen, resonances which are differentially shifted, shift towards free-state resonances as the peptide:fibrinogen ratio is increased. Observations of resonance narrowing and shifting towards free-peptide resonances with increasing peptide:fibrinogen ratio indicates that each of these peptides interacts with fibrinogen in the fast ligand-exchange regime [33–35]. This is supported by the observation that NOE build-up rates for the peptide are faster in the presence of fibrinogen. This will be discussed in more depth later. Furthermore, the observation that for GPIIb 656–667 (Figure 1) resonance narrowing is maximal at a peptide:fibrinogen ratio of about 12:1 while for GPIIb 300–314 (Figure 1) and GPIIIa 211–223 (Figure 1) resonance narrowing is maximal at a ratio of 5:1 or 6:1, follows the trend in equilibrium dissociation constants, K_d , for each peptide–fibrinogen complex: 2×10^{-5} M for GPIIb 656–667 [12], 30×10^{-5} M for GPIIb 300–314 [21] and 50×10^{-5} M for GPIIIa 211–223 [15]. The smaller the K_d (i.e. tighter binding), the larger the peptide:fibrinogen ratio needed to reduce linewidths. This occurs, since in the fast exchange regime,

Figure 1 NMR spectra of GPIIb/IIIa-derived peptides

Three panels of ^1H -NMR spectra (500 MHz) are shown for the GPIIb/IIIa-derived peptides investigated here. The peptide sequence numbers are given at the top of each panel which also shows a spectral tracing for the free peptide. At the bottom of each panel is shown a spectral tracing for fibrinogen alone. At the right-hand side of each panel is indicated the peptide:fibrinogen ratio associated with that spectral trace. Spectra were accumulated at 288 K as discussed in the text. Some resonances have been labelled as described in the text.

Figure 2 TOCSY spectra for GPIIb/IIIa-derived peptides $\pm$ fibrinogen

Three panels of TOCSY spectra are shown. Each panel contains two TOCSY contour plots. The left- and right-hand spectra of each panel are for free and 'interacting' peptides as labelled at the top of each panel. Solution conditions (288 K) are as described in the text.

resonance broadening and shifting are proportional to the population of peptide in the bound state. The terms 'free' and 'interacting' peptide will be used to refer to any of the peptides in the absence and presence of fibrinogen respectively.

TOCSY contour plots for free and fibrinogen-interacting peptides are compared in Figure 2. For spectra of peptide in the presence of fibrinogen, broader fibrinogen cross-peaks have been filtered out by the apodization used in processing two-dimensional-NMR data sets. Sequence-specific resonance assignments were made in combination with NOESY spectra by using standard assignment procedures [31]. Peptide resonance assignments in the free state are listed in Tables 1, 2 and 3, along with chemical shifts of peptide resonances in the presence of fibrinogen at the highest peptide:fibrinogen ratio indicated in Figure 1. It should be noted that peptide resonance chemical shifts in the presence of fibrinogen do not indicate fully bound-state chemical shifts but rather a weighted average of free and bound states. Although chemical shifts in the bound state can sometimes be measured by varying the peptide:fibrinogen ratio and back-extrapolating, most shift differences were too small to measure accurately at lower peptide:fibrinogen ratios where resonance broadening was maximal.

The remainder of the Results and Discussion section is organized into sub-sections, one for each peptide, in order to discuss more clearly individual cases in terms of identifying residues which better promote fibrinogen interactions and TRNOEs used to elucidate possible 'bound'-state conformations.

GPIIb peptide 656–667: GAHYMRALSNE

Peptide resonances most broadened in the presence of fibrinogen can be identified by comparing TOCSY spectra for interacting and free peptide (Figure 2). The more broadened cross-peaks are either weaker or absent in the interacting state relative to those in the free state. The C-terminal residues, SNVE, appear to be relatively unperturbed, while residues within the sequence, HYMRAL, have proton resonances which are broadened to varying degrees. The α H cross-peak of R661 and side-chain resonances of residues HYMR, and somewhat less so for A662 and L663, are significantly broadened. H658, Y659, M660, R661 and L663 also show the largest chemical-shift differences (see Table 1). From these observations, it may be suggested that the hexapeptide segment HYMRAL is crucial for interactions with fibrinogen.

Since GPIIb/IIIa and the vitronectin receptor share the same β -subunit but recognize different ligands [36,37], the distinct α -subunits of each receptor must impart ligand specificity. In this regard, it is noteworthy that the GPIIb 656–667 region has been poorly conserved within the primary structure of integrin α -chains. In particular, the GPIIb sequence (GAHYMRALSNE) corresponds to the α -subunit (α_v) sequence QADFIGVVRNNE of the vitronectin receptor [38]. The overall amino acid sequence identity between α_v and GPIIb is 60%. Moreover, although both vitronectin receptor and GPIIb/IIIa bind fibrinogen, only the former recognizes an epitope containing the RGD sequence at position 572–574 of the fibrinogen A α chain, whereas GPIIb/IIIa binds preferentially to the fibrinogen D-domain, which includes the γ -chain 400–411 binding site [24,39–41]. These observations suggest that the GPIIb 656–667 amino acid sequence contributes to the binding specificity of GPIIb/IIIa.

In general, NOEs for all three peptides are larger in the presence of fibrinogen. This is consistent with the presence of TRNOEs due to populations of fibrinogen-'bound' peptide [33–35]. Of these three peptides, however, GPIIb 656–667 exhibits

Table 1 Chemical shifts (p.p.m.) of free and bound peptide GAHYMRALS_NVE

Solution conditions for free peptide were: 1.2 mM peptide in 9:1 H₂O:²H₂O, 10 mM NH₂HPO₄, 25 mM sodium citrate and 165 mM NaCl, pH 6.4, at 288 K. Solution conditions for bound peptide were: 1.2 mM peptide + 0.1 mM fibrinogen in 9:1 H₂O:²H₂O, 10 mM NH₂HPO₄, pH 6.4, at 288 K. The ²H₂O peak was set to 4.86 ppm. ^a Peptide:fibrinogen ratio.

Residue	NH		α H		β H		others	
	Free	12:1 ^a	Free	12:1 ^a	Free	12:1 ^a	Free	12:1 ^a
G656	---	---	---	---	---	---	---	---
A657	---	---	4.212	4.214	1.216	1.218	---	---
H658	8.543	---	4.587	4.573	3.076 3.018	3.076 3.023	2H 8.234 4H 7.083	2H 8.247 4H 7.088
Y659	8.244	8.205	4.477	4.477	2.909	2.906	2,6H 6.996 3,5H 6.746	2,6H 7.041 3,5H 6.748
M660	8.267	8.272	4.341	4.346	1.944 1.864	1.938 1.862	γ CH ₂ 2.444 2.391 ϵ CH ₃ 1.944	γ CH ₂ 2.413 --- ϵ CH ₃ 1.938
R661	8.238	8.237	4.169	4.166	1.763 1.581	1.780 1.582	γ CH ₂ 1.697 δ CH ₂ 3.149 NH 7.199	γ CH ₂ 1.687 δ CH ₂ 3.143 NH ---
A662	8.384	8.391	4.238	4.241	1.338	1.341	---	---
L663	8.309	8.307	4.285	4.290	1.618	1.608	γ H 1.536 δ CH ₃ 0.815	γ H 1.532 δ CH ₃ 0.810
S664	8.267	8.272	4.386	4.391	3.830 3.794	3.833 3.795	---	---
N665	8.492	8.497	4.688	4.695	2.779 2.721	2.783 2.726	γ NH ₂ 7.614 6.901	γ NH ₂ 7.617 6.906
V666	8.085	8.089	4.089	4.062	2.051	2.053	γ CH ₃ 0.877	γ CH ₃ 0.875
E667	8.437	8.439	4.169	4.171	1.983 1.897	1.994 1.900	γ CH ₂ 2.221 NH ₂ 7.588 7.096	γ CH ₂ 2.224 NH ₂ 7.590 7.096

the smallest K_d value and therefore is the most tightly bound. This peptide also displays the largest number of TRNOEs. Figure 3 compares NOESY spectra for free peptide (300 ms mixing time) and peptide in the presence of fibrinogen (100 ms mixing time). Some NOEs, especially those in the α N region, are present in both spectra. For the free peptide, A662 and L663 NH- β H₂ (*i,i*) NOEs stand out, probably due to constrained internal motions of the Ala-Leu sequence. Free-peptide NOEs were negative or near zero. Free-peptide NOEs which were negative, were generally much smaller than those NOEs in the interacting state, which were all negative. At 100 ms, free-peptide NOEs were considerably attenuated and less numerous relative to those from the interacting-peptide state. Since most were not present in free-peptide NOESY spectra (except α N cross-peaks),

these NOEs were actually TRNOEs resulting from the dynamic equilibrium between free and fibrinogen-bound peptide.

NOESY spectra (50, 100, 200 and 400 ms) were analysed to identify TRNOEs and to check for possible spin diffusion. Most TRNOEs originate in the mid- to C-terminal sequence from R661 to E667. Figures 4 and 5 show that other TRNOEs are observed from H658 and Y659 side-chains and between NH-NH (*i,i*+1) positions respectively. In particular, Y659 and H658 ring protons show TRNOEs to A657 and M660 respectively. These TRNOEs are consistent with an extended N-terminal chain conformation.

TRNOE magnitudes and inter-proton distances were determined in two ways. Initially, TRNOE cross-peak intensities were identified as strong, medium or weak by analysing one-

Table 2 Chemical shifts (p.p.m.) of free and bound peptide GDGRHDLVGLPYM

Solution conditions for free peptide were: 0.6 mM peptide in 9:1 H₂O:²H₂O, 10 mM NH₂HPO₄, 25 mM sodium citrate and 165 mM NaCl, pH 6.4, at 288 K. Solution conditions for bound peptide were: 0.6 mM peptide + 0.1 mM fibrinogen in 9:1 H₂O:²H₂O, 10 mM NH₂HPO₄, pH 6.4, at 288 K. The ²H₂O peak was set to 4.86 ppm. ^a Peptide:fibrinogen ratio.

Residue	NH		α H		β H		others	
	Free	6:1 ^a	Free	6:1 ^a	Free	6:1 ^a	Free	6:1 ^a
G300	---	---	---	---	---	---	---	---
D301	---	---	4.632	4.626	2.697	2.685	---	---
G302	8.618	8.614	3.896	3.893	---	---	---	---
R303	8.236	8.231	4.212	4.203	1.716	1.709	γ CH ₂ 1.498 δ CH ₂ 3.128 NH 7.502	γ CH ₂ 1.497 δ CH ₂ 3.125 NH 7.501
H304	8.643	8.636	4.646	4.628	3.209 3.072	3.205 3.065	2H 8.329 4H 7.166	2H 8.325 4H 7.161
D305	8.396	8.394	4.521	4.508	2.545	2.540	---	---
L306	8.177	8.182	4.263	4.252	1.567	1.567	γ H 1.574 δ CH ₃ 0.831	γ H 1.567 δ CH ₃ 0.821
L307	8.250	8.247	4.327	4.315	1.629	1.618	γ H 1.528 δ CH ₃ 0.831	γ H 1.520 δ CH ₃ 0.825
V308	8.047	8.047	3.967	3.957	1.995	1.993	γ CH ₃ 0.888	γ CH ₃ 0.874
G309	8.445	8.443	3.877	3.876	---	---	---	---
A310	8.075	8.072	4.545	4.534	1.313	1.307	---	---
P311	---	---	4.330	4.320	2.195 1.696	2.193 1.694	γ CH ₂ 1.964 δ CH ₂ 3.752 3.577	γ CH ₂ 1.952 δ CH ₂ 3.749 3.579
L312	8.305	8.303	4.172	4.161	1.503	1.497	γ H 1.393 δ CH ₃ 0.822	γ H 1.397 δ CH ₃ 0.816
Y313	8.145	8.146	4.501	4.500	2.970	2.963	2,6H 7.087 3,5H 6.783	2,6H 7.072 3,5H 6.775
M314	8.205	8.208	4.343	4.331	2.349	2.344	γ CH ₂ 2.455 ϵ CH ₂ 1.839	γ CH ₂ 2.469 ϵ CH ₂ 1.829

dimensional F1 and F2 slices from selected NOESY spectral regions and by counting NOESY cross-peak contours [31]. Upper distance cut-offs were then assigned: 2.8 Å (strong), 3.5 Å (medium) and 4.5 Å (weak). The size of some broadened NOE cross-peaks were also taken into account and re-scaled up in magnitude if they were found to be broader than average. The second approach was more quantitative. TRNOE cross-peak volume integrals were determined and inter-proton distances were calibrated by using the tyrosine (Y659) 2,6 to 3,5 inter-proton distance of 2.48 Å; 0.5 Å was added to the upper limit for each inter-proton distance involving a side-chain. These calibrated distances are listed in Table 4. Both the qualitative and

quantitative approaches were used, given the potential inaccuracies in calibrating distances when a more internally mobile side-chain is used as the calibration factor. No good backbone inter-proton distance calibration, i.e. glycine or proline, was available. For the most part, calibrated distances fell within the error of the qualitative strong, medium, weak TRNOE classifications. Furthermore, when used as distance restraints in the distance geometry (DG) calculations discussed below, upper distances were relaxed by 0.2 Å to reduce the potential for over-constraining inter-proton distances.

Table 4 lists all TRNOEs for the interacting peptide, along with distance constraints calibrated with the Y659 2,6 to 3,5

Table 3 Chemical shifts (p.p.m.) of free and bound peptide SVSRNRDAPEGGF

Solution conditions for free peptide were: 0.5 mM peptide in 9:1 H₂O:²H₂O, 10 mM NH₂HPO₄, 25 mM sodium citrate and 165 mM NaCl, pH 6.4, at 288 K. Solution conditions for bound peptide were: 0.5 mM peptide + 0.1 mM fibrinogen in 9:1 H₂O:²H₂O, 10 mM NH₂HPO₄, pH 6.4, at 288 K. The ²H₂O peak was set to 4.86 ppm. ^a Peptide:fibrinogen ratio.

Residue	NH		α H		β H		others	
	Free	5:1 ^a	Free	5:1 ^a	Free	5:1 ^a	Free	5:1 ^a
S211	---	---	---	---	---	---	---	---
V212	8.701	8.704	4.176	4.178	2.032	2.030	γ CH ₃ 0.901	γ CH ₃ 0.898
S213	8.550	8.552	4.394	4.410	3.792	3.804	---	---
R214	8.522	8.523	4.304	4.305	1.809 1.698	1.813 1.707	γ CH ₂ 1.566 δ CH ₂ 3.137 NH 7.213	γ CH ₂ 1.564 δ CH ₂ 3.136 NH 7.203
N215	8.531	8.531	4.622	4.621	2.787 2.704	2.787 2.705	γ NH ₂ 7.658 6.954	γ NH ₂ 7.658 6.955
R216	8.459	8.460	4.233	4.234	1.830 1.723	1.835 1.734	γ CH ₂ 1.578 δ CH ₂ 3.150 NH 7.213	γ CH ₂ 1.576 δ CH ₂ 3.136 NH 7.203
D217	8.317	8.315	4.532	4.532	2.665 2.533	2.657 2.532	---	---
A218	8.045	8.044	4.514	4.515	1.260	1.261	---	---
P219	---	---	4.361	4.366	2.228 1.842	2.232 1.854	γ CH ₂ 1.921 δ CH ₂ 3.676 3.490	γ CH ₂ 1.922 δ CH ₂ 3.676 3.484
E220	8.701	8.704	4.166	4.171	1.995 1.929	2.002 1.927	γ CH ₂ 2.238	γ CH ₂ 2.245
G221	8.499	8.499	4.128 3.913	4.130 3.916	---	---	---	---
G222	8.177	8.181	3.819	3.807	---	---	---	---
F223	8.177	8.181	4.522	4.534	3.160 2.938	3.158 2.937	2,6H 7.246 3,5H 7.329 NH ₂ 7.650 7.142	2,6H 7.249 3,5H 7.327 NH ₂ 7.649 7.143

NOE. For peptide GPIIb 656–667 in the presence of fibrinogen, NOE cross-peak magnitudes reached near maximal intensity at 100–200 ms. Distance constraints therefore were derived using NOESY data with mixing times of 200 ms or less. Although most interacting-peptide TRNOEs were clean from free-peptide NOEs, some TRNOE magnitudes had to be corrected. Corrections were made by subtracting the integrated volume of the free-peptide NOE from that of the interacting-peptide NOE. With the exception of α N NOEs, free-peptide NOEs represented at most 20% of the NOE from the interacting peptide. For α N NOEs,

however, effects were larger and difficult to correct in this manner. In these cases, those distance restraints were not used in the DG calculations discussed below.

Based on these interproton distances (Table 4), a DG minimization protocol, described in the Materials and methods section, was used to determine the most probable bound-state ensemble of conformations for peptide GPIIb 656–667. The best calculated structures had favourable Φ, Ψ angles [42,43]. Of 20 structures calculated, 12 had backbone distance violations for the sequence Y659–L663 of less than 0.5 Å. The best seven structures (back-

Figure 3 NOESY spectra for free and interacting GPIIb peptide 656–667

NOESY spectra are shown for free (left-hand panel) and interacting (right-hand panel) GPIIb peptide 656–667. The mixing time was 300 ms for the free peptide and 100 ms for peptide in the presence of fibrinogen. The temperature was 288 K. All other conditions are as described in the text. Some α N cross-peaks have been traced sequentially.

Figure 5 NOESY spectrum for interacting GPIIb peptide 656–667

The NH–NH spectral region from a NOESY (100 ms) contour plot is shown for fibrinogen-interacting GPIIb peptide 656–667.

Figure 4 NOESY spectra for interacting GPIIb peptide 656–667

The aromatic- α H/up-field resonance region from a NOESY (100 ms) contour plot is shown. TRNOEs from H658 and Y659 ring-proton resonances to methylene and methyl groups are indicated.

Table 4 Distance constraints for bound GPIIb 656–667 derived from transferred NOE experiments

All distances were derived from the 200 ms NOESY spectrum of peptide GPIIb 656–667 in the presence of fibrinogen. The atom labels follow the convention adopted in the BIOSYM program.

	Upper Bounds (Å)
A657–A657:	HA–HB 3.4
H658–H658:	HB–HD2 3.7
Y659–Y659:	HA–HD 3.8; HB–HD 2.9
M660–M660:	HA–HG 3.6; HB–HN 3.8
R661–R661:	HA–HB 3.4; HB–HN 4.0
A662–A662:	HA–HB 2.6; HB–HN 3.3
L663–L663:	HA–HG 3.9; HB–HN 3.4; HD–HN 4.4; HG–HD 4.2
S664–S664:	HA–HB 3.0; HB–HN 3.4
N665–N665:	HB–H1 3.6; HB–HN 3.6
V666–V666:	HA–HB 3.5; HA–HG 3.7; HB–HG 3.2; HG–HN 3.7
E667–E667:	H1–HN 3.1; HA–HB 3.3; HA–HG 3.7; HB–HN 3.5; HG–HN 4.0
A657–H658:	HD–HB 5.5
Y659–M660:	HB–HN 3.3; HE–HN 5.0
M660–R661:	HA–HN 3.3; HB–HN 3.6; HG–HN 3.9
R661–A662:	HA–HB 4.2; HA–HN 3.0; HB–HN 3.5; HN–HN 3.1
A662–L663:	HA–HN 2.9; HB–HN 4.0; HN–HN 3.1
L663–S664:	HD–HN 5.2; HG–HN 4.1; HN–HN 3.1
S664–N665:	HA–HN 3.4; HB–HN 3.0; HN–HN 3.1
N665–V666:	H1–HG 5.9; HA–HN 3.4; HB–HN 4.2; HN–HN 3.1
V666–E667:	HA–HN 3.0; HB–HN 3.2; HG–HN 4.7; HN–HN 3.1
A657–Y659:	HB–HD 5.5; HB–HE 5.5
H658–M660:	HB–HN 4.0
A665–S664:	HB–HN 4.6
L663–N665:	HB–HN 5.7
Y659–L663:	HD–HD 5.5; HE–HB 5.5

bone root mean squared deviation range of 0.43–0.75 Å; average root mean squared deviation of 0.53 Å) have been superimposed in Figure 6 by using the backbone heavy atoms of residues Y659–L663. A657–R661 forms an extended β -structure with A662–S664 setting the stage for a C-terminal helix. It should be noted that although $i, i+1$ NH–NH TRNOEs were observed

Figure 6 TRNOE-based computer modelled structures for GPIIb peptide 656–667

Eight superimposed structures for fibrinogen-bound GPIIb peptide 656–667 are shown. Structures were generated as discussed in the Materials and methods section. Only those residues having relatively well-defined backbone positions, i.e. A657–S664, are indicated. Side-chains have been labelled.

from S664 to E667, other helix-constraining TRNOEs, e.g. $\alpha N(i, i+3)$, were not observed. In some of the DG structures, helix-like folds were identifiable within this C-terminal segment even though backbone Φ, Ψ angles deviated significantly from those of an α -helix. A helix within this segment is appealing since its amphipathicity would allow M660, A662, L663 and V666 to form a hydrophobic surface and R661, S664, N665 and E667 to form a hydrophilic, probably solvent-exposed, surface. This proposal is supported by line broadening and chemical-shift differences noted above. The sequence HYMR, as well as L663 and V666, is most affected. In fact, the best DG modelled structures (Figure 6) have H658, M660 and R661 side-chains extending out on one side of an extended chain segment. Y659 is sandwiched between A657, L663 and, in some structures, V666.

GPIIb 656–667 shows a high hydrophobic complementarity to fibrinogen γ -chain C-terminal peptide $\gamma 402$ –411 [22,23]. The fibrinogen $\gamma 402$ –411 peptide D410 and V411 carboxylate groups have been implicated as being important to fibrinogen γ -chain binding to GPIIb/IIIa [44,45]. The role of GPIIb residues H658 and R661 therefore may interact electrostatically with γ -chain D410 and/or V411 C-terminal carboxylates.

GPIIb peptide 300–314: GDGRHLLVGAPLYM

TOCSY spectral comparison (Figure 2) for GPIIb peptide 300–314 in the absence and presence of fibrinogen indicates that although most resonances in the bound state are broadened, those belonging to L307, L312, Y313 and M314 are most broadened. Chemical-shift differences are indicated in Table 2. H304, L306, L307, Y313 and M314 resonances are shifted most. Several others in the mid- and C-terminal segments, however, are

nearly equally shifted. In fact, the $\Delta\delta$ pattern suggests that most perturbations occur within the HDLL and PLYM sequences.

The amino acid sequence around HDLL has been implicated as being important for GPIIb/IIIa-fibrinogen binding [16,20,46,47], as has the GAPL sequence [21,48]. These sequences are present in the region of GPIIb (residues 294–314) found to be cross-linked by the fibrinogen γ -chain peptide [20]. GPIIb peptides 296–306 and 300–312 have similar potencies as inhibitors of the adhesion of stimulated platelets to fibrinogen [21], suggesting that the overlapping sequence (300–306) shared by these two peptides provides residues that are crucial for interactions with fibrinogen. However, the presence of this stretch alone cannot account for the inhibition by GPIIb 300–312, since a peptide lacking GAPL (300–308) is not as potent an inhibitor of platelet aggregation or platelet adhesion [49]. Therefore, it appears that the GAPL moiety contributes to function. This is consistent with the NMR results discussed above.

For GPIIb 300–314, most NOEs, although negative and generally larger in the interacting state, were also observed in the free state. Two exceptions, however, were apparent with $i, i+2$ TRNOEs between R303 and D305, i.e. NH–NH and βH_2 –NH (results not shown). These TRNOEs are suggestive of a turn centred at RH. The αH –NH $i, i+2$ TRNOE may be present, although overlap with some fibrinogen remnant cross-peaks makes this identification ambiguous. Owing to the limited number of clearly identifiable TRNOEs, DG calculations were not done. In any event, this possible turn may be similar to the β -turn type II found for GRGDSP hexapeptide free in solution [50]. Here, the turn residues are formed with GRGD. This sequence is highly conserved compared with GPIIb 300–314 which has GRHD. In GRGDSP, it was proposed that the arginine guanidino group forms a salt bridge with the aspartic acid carboxylate group. This GRGDSP sequence is known as a ligand-binding sequence which mediates interactions with various integrins [7]. As noted above, the sequence HDLL in GPIIb peptide 300–314 is probably crucial to fibrinogen binding.

GPIIIa peptide 211–223: SVSRNRDAPEGGF

For this peptide, the resonances most broadened in the presence of fibrinogen (see Figure 2) belong to S213, R214, N215 and R216. While backbone αH cross-peaks of S213, R214 and N215 are apparently most broadened, the side-chain resonances of R214, N215 and R216 are in fact the most broadened. S213, R214 and R216 are also among the most chemically shifted (see Table 3). This is particularly true for R216 for γH_2 and δH_2 resonances. Peptide GPIIIa 211–223 also shows larger NOEs in the presence of fibrinogen, consistent with peptide in the bound state, although no new conformationally constraining TRNOEs could be identified. However, for S213, R214 and N215, $\alpha N(i, i+1)$ NOEs are weaker in the interacting state. Unfortunately, only one NH–NH NOE near these residues can be observed between D217–A218. Other NH–NH NOEs within this sequence cannot be observed due to resonance overlap. The presence of weaker $\alpha N(i, i+1)$ NOEs may suggest some type of helix formation within this segment. With the C-terminal portion, the G221–G222 αN NOE is stronger in the bound state, and an NH–NH NOE is observed from E220 to G221. Since proline substitution alters activity, it may be that conformational restriction at P311 modulates function [25]. Proline is often found in position 2 of a β -turn [42]. In this respect, the E220–G221 NH–NH TRNOE noted above may suggest the presence of a turn centred at PE which is also chemically shifted in the bound state.

These results indicate that the sequence SRNR appears to

interact directly with fibrinogen. This is consistent with biological data which indicate that substitution of R214 with either tryptophan [51] or glutamine [52] substantially impairs the ability of mutant GPIIb/IIIa to bind fibrinogen. Steiner et al. [53] have shown that a shorter peptide, RNRDA, is even more active than peptide SVSRNRDAPEG. Cook et al. [25] have demonstrated, however, that substitution of the proline (Pro²¹⁹) to an alanine residue substantially reduces peptide binding to both fibrinogen and von Willebrand factor and displacement of parent GPIIb/IIIa bound to fibrinogen.

Conclusions

These data show the applicability of NMR to help identify sequences which may interact directly with fibrinogen and to derive bound-state conformations in GPIIb/IIIa peptide-fibrinogen interactions. For the three GPIIb/IIIa-derived peptides studied here, sequences apparently most involved in interacting with fibrinogen include RHDLL and PLYM from GPIIb 300–314 (GDGRHDLVLGAPLYM), HYMR from GPIIb 656–667 (GAHYMRALSNE) and SRNR from GPIIIa 211–223 (SVSRNRDAPEGGF). In all three peptides, arginines and hydrophobic residues are identified, and in two cases, histidines are present. Negatively charged residues appear to play a less significant role in GPIIb/IIIa for fibrinogen binding. In nature, arginines have been proposed to play a unique role in anion recognition [54–56]. In the context of Pearson's concept of soft acid, soft base interactions [57], arginine, a large diffuse cation (soft), is ideally suited to interact with large (soft) bio-anions, such as carboxylates. In this respect, both GPIIb-derived peptides have been proposed to interact with the fibrinogen γ -chain C-terminal sequence [20,22,23], whose terminal dodecapeptide contains two carboxylates (D410 and V411-COO⁻) which have been shown by Kloczewiak et al. [44,45] to be important for fibrinogen binding to GPIIb/IIIa.

This work was carried out by L.J.D. in partial fulfillment of the requirements towards a Ph.D. degree at the University of Minnesota. We are very grateful to Eric Eccleston and Dinesha Walek of the Department of Human Genetics Microchemical Facility, University of Minnesota, for the synthesis of peptides used in this study.

REFERENCES

- Davie, E. W., Fujikawa, K. and Kiesel, W. (1991) *Biochemistry* **30**, 10363–10370
- Marguerie, A., Plow, E. F. and Edgington, T. S. (1979) *J. Biol. Chem.* **254**, 5357–5363
- Bennett, J. S., Hoxie, J. A., Leitman, S. F., Vilaire, G. and Cines, D. B. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2417–2421.
- Hynes, R. O. (1987) *Cell* **48**, 549–554
- Phillips, D. R., Charo, I. F., Parise, L. V. and Fitzgerald, L. A. (1988) *Blood* **71**, 831–843
- Pierschbacher, M. D., Hayman, E. G. and Ruoslahti, E. (1981) *Cell* **26**, 259–267
- Pierschbacher, M. D. and Ruoslahti, E. (1984) *Nature (London)* **309**, 30–33
- Herbst, T. J., McCarthy, J. B., Tsilibary, E. C. and Furcht, L. T. (1988) *J. Cell Biol.* **106**, 1365–1373
- Gehlsen, K. R., Dillner, L., Engvall, E. and Roushathi, E. (1986) *Science* **241**, 1228–1229
- Chelberg, M. K., McCarthy, J. B., Skubitz, A. P. N., Furcht, L. T. and Tsilibary, E. C. (1990) *J. Cell Biol.* **111**, 261–270
- Mayo, K. H., Parra-Diaz, D., McCarthy, J. B. and Chelberg, M. (1991) *Biochemistry* **30**, 8251–8267
- Calvete, J. J. (1993) in *Cell Adhesion Molecules* (Mihich, E. and Hemler, M. E., eds.), pp. 63–91, Plenum, New York
- Plow, E. F., D'Souza, S. E. and Ginsberg, M. H. (1992) *Semin. Thromb. Hemostasis* **18**, 324–332
- Calvete, J. J., Mann, K., Alvarez, M. V., Lopez, M. M. and Gonzalez-Rodriguez, J. (1992) *Biochem. J.* **282**, 523–532
- Charo, I. F., Nannizzi, L., Phillips, D. R., Hsu, M. A. and Scarborough, R. (1991) *J. Biol. Chem.* **266**, 1415–1421
- Argraves, W. S., Suzuki, S., Arai, H., Thompson, K., Pierschbacher, M. D. and Ruoslahti, E. (1987) *J. Cell. Biol.* **105**, 1183–1190
- Kishimoto, T. K., O'Connor, K., Lee, A., Roberts, T. M. and Springer, T. A. (1987) *Cell* **48**, 681–690
- D'Souza, S. E., Ginsberg, M. H., Burke, T. A., Lam, S. C.-T. and Plow, E. F. (1988) *Science* **242**, 91–93
- Smith, J. W. and Cheresch, D. A. (1988) *J. Biol. Chem.* **263**, 18726–18731
- D'Souza, S. E., Ginsberg, M. H., Burke, T. A. and Plow, E. F. (1990) *J. Biol. Chem.* **265**, 3440–3446
- Taylor, D. B. and Gartner, T. K. (1992) *J. Biol. Chem.* **267**, 11729–11733
- Calvete, J. J., Arias, J., Alvarez, M. V., Lopez, M. M., Henschen, A. and Gonzalez-Rodriguez, J. (1991) *Biochem. J.* **273**, 523–532
- Calvete, J. J., Arias, J., Alvarez, M. V., Lopez, M. M., Henschen, A. and Gonzalez-Rodriguez, J. (1991) *Biochem. J.* **273**, 767–775
- Weisel, J. W., Nagaswami, C., Vilaire, G. and Bennett, J. S. (1992) *J. Biol. Chem.* **267**, 16637–16643
- Cook, J. J., Trybulek, M., Lasz, E. C., Khan, S. and Niewiarowski, S. (1992) *Biochim. Biophys. Acta* **1119**, 312–321
- Stewart, J. M. and Young, J. D. (1984) *Solid Phase Peptide Synthesis*, 2nd edn., pp. 135, Pierce Chemical Co., Rockford, IL
- Bax, A. and Davis, D. G. (1985) *J. Magn. Reson.* **65**, 355–360
- Jeener, J., Meier, B. H., Bachman, P. and Ernst, R. R. (1979) *J. Chem. Phys.* **71**, 4546–4553
- Wider, G., Macura, S., Anil-Kumar, Ernst, R. R. and Wuthrich, K. (1984) *J. Magn. Reson.* **56**, 207–234
- States, D. J., Haberkorn, R. A. and Ruben, D. J. (1982) *J. Magn. Reson.* **48**, 286–293
- Wuthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley-Interscience, New York
- Braun, W. and Go, N. (1985) *J. Mol. Biol.* **186**, 611–623
- Ni, F. (1994) *Prog. Nucl. Magn. Reson. Spectrosc.* **26**, 517–606
- Campbell, A. P. and Sykes, B. D. (1993) *Annu. Rev. Biophys. Biomol. Struct.* **22**, 99–122
- London, R. E., Perlman, M. E. and Davis, D. G. (1992) *J. Magn. Reson.* **97**, 79–98
- Hemler, M. E. (1990) *Annu. Rev. Immunol.* **8**, 365–400
- Hynes, R. O. (1992) *Cell* **69**, 11–26
- Fitzgerald, L. A., Poncz, M., Steiner, B., Rall, S. C., Jr., Bennett, J. S. and Phillips, D. R. (1987) *Biochemistry* **26**, 8158–8165
- Farrell, D. H., Thiagarajan, P., Chung, D. W. and Davie, E. W. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10729–10732
- Santoro, S. A. and Lawling, W. J., Jr. (1987) *Cell* **48**, 867–873
- Smith, J. W., Ruggeri, A. M., Kunicki, T. J. and Cheresch, D. A. (1990) *J. Biol. Chem.* **265**, 12267–12271
- Richardson, J. S. (1981) *Adv. Protein Chem.* **34**, 167–186
- Iijima, H., Dunbar, J. B., Jr. and Marshall, G. R. (1987) *Proteins* **2**, 330–338
- Kloczewiak, M., Timmons, S., Lukas, T. J. and Hawiger, J. (1984) *Biochemistry* **23**, 1767–1773
- Kloczewiak, M., Timmons, S., Bednarek, M. A., Sakon, M. and Hawiger, J. (1989) *Biochemistry* **28**, 2915–2919
- D'Souza, S. E., Ginsberg, M. H., Matsueda, G. R. and Plow, E. F. (1991) *Nature (London)* **350**, 66–68
- Poncz, M., Eisman, R., Heidenreich, R., Silver, S. M., Vilaire, G., Surrey, S., Schwartz, E. and Bennett, J. S. (1987) *J. Biol. Chem.* **262**, 8476–8482
- Gartner, T. K. and Taylor, D. B. (1990) *Thromb. Res.* **60**, 291–309
- Harker, L. A. (1990) in *Hematology* (Williams, J. W., Beutler, E., Erslev, A. J. and Lichtman, M. A., eds.), 4th edn., pp. 1559–1569, McGraw Hill, New York
- Johnson, W. C., Jr., Pagano, T. G., Basson, C. T., Madri, J. A., Gooley, P. and Armitage, I. M. (1993) *Biochemistry* **32**, 268–273
- Lanza, F., Stierle, A., Fournier, D., Morales, M., Andre, G., Nurden, A. T. and Cazenave, J.-P. (1992) *J. Clin. Invest.* **89**, 1995–2004
- Bajt, M. L., Ginsberg, M. H., Frelinger, A. L., III, Berndt, M. C. and Loftus, J. C. (1992) *J. Biol. Chem.* **267**, 3789–3794
- Steiner, B., Trzeciak, A., Pfenninger, G. and Kouns, W. C. (1993) *J. Biol. Chem.* **268**, 6870–6873
- Riordan, J. F., McElvany, K. D. and Borders, C. L., Jr. (1977) *Science* **195**, 884–886.
- Wallis, M. (1974) *Biochem. Biophys. Res. Commun.* **56**, 711–716
- Jukes, T. H. (1973) *Biochem. Biophys. Res. Commun.* **53**, 709–714
- Pearson, R. G. (1963) *J. Am. Chem. Soc.* **85**, 3533–3539