Thrombin Receptor Activating Peptides: Importance of the N-Terminal Serine and Its Ionization State As Judged by pH Dependence, Nuclear Magnetic Resonance Spectroscopy, and Cleavage by Aminopeptidase M†

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ABSTRACT: Peptides derived from the recently identified thrombin receptor were tested for their ability to induce platelet aggregation in platelet-rich plasma. The 14 amino acid peptide identified as the new N-terminus after thrombin cleavage (T-14) and an 11 amino acid peptide (T-11) lacking the 3 C-terminal amino acids of T-14 were studied. Both induced platelet aggregation at micromolar concentrations, with T-11 about twice as potent as T-14. Induction of platelet aggregation by these two peptides showed an unusual pH dependence, being more potent at pH 7.2 than at pH 8.1; thrombin-induced aggregation showed a reverse pH dependence. Proton NMR studies of T-11 demonstrated that the chemical shift of the C-α proton of the N-terminal serine had a pH dependence that mirrored the aggregation potency. Acetylating the N-terminus of T-11 resulted in loss of aggregating activity, and this peptide did not show the pH-dependence change in chemical shift. The T-14 and T-11 peptides lost aggregating activity when incubated in plasma due to cleavage of the N-terminal serine by an enzyme identified as aminopeptidase M based on its pattern of inhibition and the ability of purified aminopeptidase M (EC 3.4.11.2) to cleave the T-11 peptide. Endothelial cell aminopeptidase M was also able to cleave T-11. Inhibiting aminopeptidase M with amastatin enhanced aggregation induced by T-11 but not thrombin. These studies suggest that ionization of the N-terminus of the T-11 and T-14 peptides may be important in initiating platelet aggregation. In addition, aminopeptidase M in plasma and on endothelial cells can inactivate the peptides by cleavage of the N-terminal serine. This observation needs to be considered in designing and interpreting experiments using these peptides. Plasma aminopeptidase M does not, however, appear to inactivate the tethered ligand produced by thrombin's cleavage of the receptor on platelets ex vivo, but this does not exclude a potential effect on platelets or endothelial cells in vivo at low thrombin concentrations.

Thrombin is a potent platelet agonist in vitro, producing shape change, aggregation, and release of granule contents (Zucker & Borelli, 1955; Grette, 1962; Davey & Luscher, 1968; Charo et al., 1977). Patients with coagulation abnormalities that decrease thrombin production, such as hemophilia (Forbes & Madhok, 1991), and patients treated with anticoagulants that inhibit thrombin generation or thrombin's enzymatic activity (Levine & Hirsh, 1986) are prone to excessive hemorrhage. Moreover, studies in experimental animal models and in humans indicate that thrombin plays a central role in platelet thrombus formation (Chesebro & Fuster, 1991). Thus, it is likely that thrombin contributes to both hemostasis and thrombosis in humans. Thrombin has also been shown to affect a variety of other cells, producing phosphoinositide metabolism, prostacyclin synthesis, chemotaxis, and mitogenesis (Hung et al., 1992), but the physiological and pathological consequences of these activities are less clear. Thrombin's importance has stimulated research on the mechanism by which it activates platelets. Although multiple thrombin binding proteins and substrates have been identified on and in platelets, including glycoprotein Ib (Okumura et al., 1978; Ganguly & Gould, 1979; Hagen et al., 1981; Takamatsu et al., 1986), thrombospondin (Baenziger et al., 1971; Detwiler et al., 1992), glycoprotein V (Phillips & Agin, 1977), protease nexin (Gronke et al., 1987), factor XIII (Hagen et al., 1981), platelet factor 4 (Hagen et al., 1981), and fibrinogen (Hagen et al., 1981), it has been difficult to ascribe unequivocally a transducing receptor function to any of these proteins.

Recently, Vu et al. (1991a) used expression cloning in *Xenopus laevis* oocytes to identify a functional thrombin receptor from Dami cell mRNA; a similar or identical mRNA is also present in platelets and endothelial cells. The cDNA contains an open reading frame of 425 amino acids and a hydropathy plot suggests the presence of seven transmembrane domains. A putative thrombin cleavage site was identified between R41 and S42. Moreover, a 14 amino acid peptide mimicking the new amino terminus produced by thrombin (SFLLRNPNDKYEPF, S42–F55; T-14) (Table I) was able to aggregate gel-filtered platelets directly, whereas a mutant form of the receptor that could not be cleaved by thrombin at this site failed to respond to thrombin. The authors concluded that thrombin induces platelet activation, at least in part, by cleaving this receptor, which in turn, permits the

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Table I: Peptides	
SFLLRNPNDKYEPF-NH_2	T-14
FSLLRNPNDKYEPF-NH_2	T-14FS
SFLLRNPNDKY-NH_2	T-11
Ac-SFLLRNPNDKY-NH_2	Ac-T-11
LLRNPNDKYEPF-NH_2	T-12

new amino terminus to function as a tethered ligand, interacting with another region of the receptor to induce the activation signal(s). An adjacent region of 11–13 amino acids was found to be homologous to a portion of the C-terminal tail of hirudin, the high-affinity thrombin-binding protein obtained from the *Hirudo medicinalis* leech. In a subsequent study, these authors demonstrated that the hirudin homology region contributes significantly to thrombin's ability to activate oocytes containing the receptor and to cleave peptides containing the R41–S42 cleavage site (Vu et al., 1991b).

Similar thrombin receptors have been cloned from a hamster cell line cDNA (Rasmussen et al., 1991) and a murine source (Vu et al., 1991b). Each of these receptors also has a thrombin cleavage site, and the first six amino acids after the cleavage site are identical to the human sequence except for a substitution of phenylalanine for the first leucine (SFFLRN) (Vu et al., 1991b; Rasmussen et al., 1991). Huang et al. (1991) studied platelet phosphoinositol metabolism induced by thrombin and the T-14 peptide described by Vu et al. and concluded that most, if not all, of thrombin's activation of platelets could be accounted for by cleavage of the thrombin receptor. Brass and Woolkalis (1992) found that the T-14 peptide could activate phospholipase C and phosphatidylinositol 3-kinase in platelets, and Brass (1992) demonstrated that the peptide could cause homologous desensitization of the HEL cell thrombin receptor. The T-14 peptide was also shown by Ngaiza and Jaffe (1991) to increase endothelial cell intracellular calcium and stimulate prostacyclin production in a manner similar to that of thrombin. While the present studies were in progress, Vassallo et al. (1992) reported on the structure–function relationships of peptides from the thrombin receptor. They found the activity in the first six amino acids and stressed the importance of the phenylalanine and arginine residues, as well as the presence of the N-terminal amino acid. Similar results were obtained with peptides from the hamster receptor by Vouret-Craviari et al. (1992).

In the present study we have analyzed the effects of several peptides derived from the thrombin receptor described by Vu et al. (1991a) on the aggregation of platelets in platelet-rich plasma (PRP). We studied the pH dependence of platelet aggregation induced by thrombin and the peptides because previous studies by one of us of the pH dependence of ristocetin-induced platelet aggregation (Coller et al., 1976) provided important information on potential electrostatic interactions (Coller, 1978). Our current studies demonstrate that aggregation induced by several of the thrombin peptides has an unusual pH dependence, different from that of thrombin-induced aggregation. Studies of the 600-MHz proton NMR of one of the active peptides demonstrated that the C-α proton associated with the N-terminal serine (α_{ser}) underwent a pH-dependent change in chemical shift over the same pH range. Both the platelet-aggregating activity and the pH dependence of the chemical shift were abolished by acetylating the N-terminus. Moreover, we discovered that the peptides are rapidly cleaved in plasma and by cultured endothelial cells by an aminopeptidase activity identified as aminopeptidase M. The loss of just the N-terminal serine by this enzyme results in loss of platelet-aggregating activity.

MATERIALS AND METHODS

Enzymes and Antibodies. Thrombin (human) was obtained from Sigma (St. Louis, MO) in semipurified form and as a gift from Dr. Jolyon Jesty, State University of New York at Stony Brook, in purified form (3500 units/mg) (Neuenschwander & Jesty, 1988). Antibody 10E5, directed against GPIIb/IIIa, has previously been described (Coller et al., 1983). Purified human aminopeptidase M was provided by Dr. S. Mizutani (Nagoya University School of Medicine, Nagoya, Japan) (Kurauchi et al., 1986).

Platelet Aggregation. PRP was obtained from blood anticoagulated with sodium citrate (0.01 volume of 40% trisodium citrate) or acid–citrate–dextrose solution A (ACD-A; 8.5 mL of blood:1.5 mL of ACD-A) by centrifugation at 700*g* for 3.5 min at 22 °C. Platelet-poor plasma (PPP) was obtained by centrifuging the PRP at 2000*g* for 8 min at 22 °C. The pH of the citrated PRP after preparation was ~7.7 whereas the pH of the ACD-A PRP was ~7.0. The pH of the PRP was adjusted, when necessary, with 1 M NaOH or 1 M HCl. Platelet aggregation was performed in a dual-channel aggregometer (Chronolog, Havertown, PA) using matched stir bars.

Peptides. Peptides (Table I) were prepared on an automated peptide synthesizer (Applied Biosystems 430A; Foster City, CA) using *t*-Boc chemistry, 4-methylbenzhydrylamine resins, and *N*-methylpyrrolidone as the coupling solvent as previously described (Beer et al., 1992). The protecting groups were β-benzyl ester for aspartic acid, benzyl for serine, 2-bromobenzyloxycarbonyl for tyrosine, 2-chlorobenzyloxycarbonyl for lysine, and tosyl for arginine. Arginine, asparagine, tyrosine, and selected proline and phenylalanine residues were double-coupled. Dimethylsulfide and anisole were included in all of the hydrogen fluoride cleavage solutions. Peptides were assessed by reverse-phase HPLC and selected peptides were purified by this technique. Acetylation of the N-terminus of T-11 to form Ac-T-11 was accomplished with acetic anhydride prior to cleavage from the resin. The mass of selected peptides was determined by fast atom bombardment mass spectrometry as previously described (Beer et al., 1992).

NMR Experiments. Purified peptides (8 mM) were dissolved in 0.15 M NaCl and 2 mM sodium phosphate containing 20% D_2O. The pH* was adjusted to the desired value with NaOH. The pH meter reading was not corrected for isotope effects because a preliminary study showed that the pH values of solutions prepared by adding 20% D_2O or H_2O to 2.5 mM sodium phosphate differed by less than 0.1 unit over the range 6.5–7.5 and less than 0.2 unit at pH 6.0, 8.0, and 8.5. Proton NMR spectra of peptides (500 μL) were measured with a Bruker AMX-600 spectrometer (Billerica, MA) interfaced to an X.32 computer. Chemical shifts were referenced to the H_2O resonance at 4.80 ppm at 20 °C, the temperature dependence of the H_2O resonance being 0.012 ppm/°C (Basus, 1989). Water suppression was achieved by low-power selective irradiation of the H_2O resonance during the relaxation delay (1.5–2.0 s) and the mixing time for ROESY spectra. All spectra were processed using FELIX 2.0 or FELIX 2.05 software from Hare Research, Inc. (Bothell, WA) running on a Silicon Graphics Personal Iris 4D/35TG (SGI Express, Mountain View, CA).

One-dimensional spectra were acquired as 16K or 32K data points. The data were apodized by an exponential window

function with 0.2 Hz of line broadening prior to Fourier transform. The data were baseline-corrected using a fourth-order polynomial. Two-dimensional experiments were recorded in the phase-sensitive mode using time-proportional phase incrementation (TPPI) (Marion & Wüthrich, 1983; Rance et al., 1983) and quadrature detection (Bodenhausen et al., 1984) and with the carrier frequency set to the frequency of H_2O. Five hundred twelve t_1 increments were accumulated as 2K complex data points. Time data were multiplied by a squared sine bell window function shifted by $\pi/2$ in the t_2 and t_1 dimensions. The data were zero-filled to give a final frequency matrix of 1K × 1K real points. The data were baseline-corrected along each column (ω_1) using a fourth-order polynomial. Phase-sensitive HOHAHA (Davis & Bax, 1985; Bax & Davis, 1985a) was recorded using the MLEV-17 sequence preceded and followed by 2.5-ms trim pulses. A spin-lock time of 100 ms, with an effective radiofrequency field of 6.8 kHz corresponding to a 37-μs 90° pulse, was used. The relaxation delay was 1.5 s and 16 accumulations were collected for each t_1.

Phase-sensitive ROESY (Bothner-By et al., 1986; Bax & Davis, 1985b) was recorded using a low radiofrequency continuous wave spin-lock field. The mixing time was 200 ms. The relaxation delay was 2.0 s and 128 scans were collected for each t_1. The temperature of the sample was 303 K and its pH* was 4.2.

Peptide Digestion Studies. Analysis of the plasma digestion products of the T-11 peptide (SFLLRNPNDKY) was performed by incubating T-11 (2 mM final concentration) in PPP for various times at 37 °C and then precipitating the plasma proteins with 9–10% trichloroacetic acid at 4 °C for at least 30 min. After centrifugation at 12000g at 22 °C for 3 min, the supernatant was analyzed by reverse-phase HPLC (C8; Applied Biosystems 300 RP Aquapore; 220- × 4.6-mm column); elution was with 0.1% trifluoroacetic acid with a 0–60% acetonitrile gradient. In experiments to assess the effects of peptidase inhibitors, citrated PPP, pH 7.4, was preincubated with various concentrations of amastatin (Sigma), bestatin (Boehringer-Mannheim), puromycin (Sigma), or 1,10-phenanthroline (Aldrich) for 30 min before the T-11 peptide (2 mM) was added. After 2–5 h at 37 °C, the plasma proteins were precipitated as above and the supernatant was analyzed by HPLC. Inhibition of digestion was calculated from the percentage of peptide remaining in the intact T-11 peak.

To determine the amino acid sequence of the digestion products of T-11 and to assess their ability to aggregate platelets, the peptide (2 mM) was incubated with citrated PPP for 2 h at 37 °C, plasma proteins were precipitated with trichloracetic acid, and the supernatant after centrifugation was both analyzed and fractionated on the analytical reverse-phase column. Peak fractions were dried in a vacuum centrifuge (Speed-Vac, Savant, Farmingdale, NY) and dissolved in either buffer (0.15 M NaCl and 0.01 M Tris/HCl, pH 7.4) for aggregation studies or 0.1% trifluoroacetic acid for amino acid analysis. Quantitative amino acid analysis of the peptides was performed with commercial reagents and equipment (Pico-Tag workstation and C-18 Pico-Tag column; Waters–Millipore, Bedford, MA). The amino acid sequence could then be inferred from the amino acid content of each peak.

When the data obtained in the above HPLC studies established that T-11 was undergoing N-terminal hydrolysis in plasma, subsequent studies were conducted with procedures and reagents previously utilized to characterize peptide metabolism by human serum aminopeptidase M (EC 3.4.11.2) (Palmieri et al., 1989; Ward et al., 1990; Wang et al., 1991). For these studies, the standard reaction (600 μL) consisted of serum (20 μL), Hanks' balanced salt solution (pH 7.2), and T-11 substrate (50 μM) incubated at 37 °C. Studies of T-11 hydrolysis by intact human umbilical vein endothelium were conducted on confluent monolayers of cells in 24-well dishes as previously reported (Palmieri et al., 1989). Spent medium was removed and the cells were washed twice with 2 mL of Hanks' salt solution. T-11 (50 μM) was then incubated in a final volume of 1.0 mL of this buffer, with and without amastatin (100 μM). At various time points up to 4 h, 100-μL samples were removed from the cells, immersed in a boiling water bath for 5 min, cooled, and centrifuged for 3 min; the supernatant was then analyzed by HPLC. Inhibitors were preincubated with serum or cells and buffer for 10 min at 37 °C. K_m and V_{max} were determined by measuring reaction velocity over a range of substrate concentrations (25–100 μM) and plotting $1/V$ vs $1/[S]$.

T-11 substrate and the initial metabolite, des-Ser^1-T-11, were separated and quantitated on a reverse-phase column (retention times of 11.3 and 10.6 min, respectively; Vydac, 10-μm C_{18}-μBondapak, 1.9 × 30 cm) at a constant flow rate of 2 mL/min using a linear gradient from 95% solvent A (0.05% TFA)/5% solvent B (0.04% TFA in acetonitrile) to 35% solvent A/65% solvent B over a 21-min period. N-Terminal hydrolysis was calculated by the decrease in T-11, with the sample in which the aminopeptidase was inhibited by amastatin (100 μM) serving as the control. Integration of sample peak areas and quantitation of substrate against the last-run standard (run every sixth injection) were automatically calculated by the data module (Palmieri et al., 1989; Ward et al., 1990; Wang et al., 1991).

RESULTS

Platelet Aggregation. When added to PRP at pH 7.0, the 14 amino acid peptide of Vu et al. (1991a) (T-14) could initiate the platelet shape change at concentrations of ~1 μM and platelet aggregation at ~2 μM; full aggregation occurred at doses ≥5 μM (Figure 1A). A pattern of aggregation followed by disaggregation was generally observed at doses that did not produce full aggregation. Control peptides in which the S and F in the first two positions were either reversed (T14-FS) or omitted (T-12) did not induce shape change or aggregation of citrated PRP even at 85 μM, consistent with the findings of Vu et al. (1991a). Aggregation induced by T-14 was completely inhibited by preincubating the platelets with a near-saturating concentration of monoclonal antibody 10E5, which is directed at the GPIIb/IIIa receptor (Coller et al., 1983) (data not shown). The pH dependence of aggregation induced by T-14 was tested by adjusting the pH of PRP from blood anticoagulated with either ACD-A or citrate with NaOH or HCl to achieve a range of PRP pH values between 7.0 and 8.1. Aggregation was maximal at pH ~7.0–7.2 and showed progressive diminution as the pH increased to 8.1 (Figure 1B). The pH dependence of aggregation induced by T-14 contrasts with the pH dependence of aggregation induced by thrombin, with the latter inducing more rapid aggregation at pH 8.1 than at pH 7.2 (Figure 1B).

An 11 amino acid peptide lacking the three C-terminal amino acids of T-14 (T-11) induced platelet aggregation as well or better than the T-14 peptide, with several experiments suggesting that it is nearly twice as potent (Figure 1). Aggregation induced by the T-11 peptide demonstrated the same pH dependence as did the T-14 peptide, being consid-

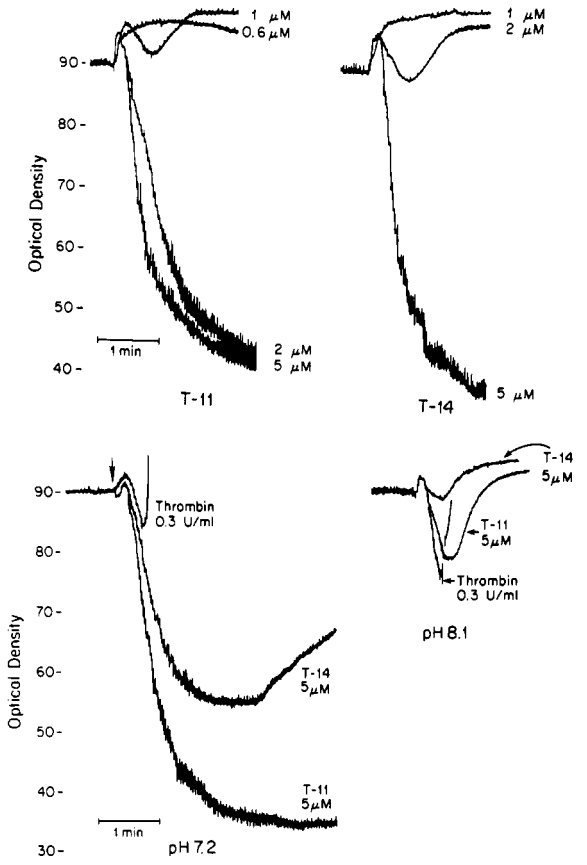

FIGURE 1: (A, top panel) Platelet-rich plasma (PRP) was prepared from blood anticoagulated with ACD-A and adjusted to 326 000 platelets/μL. The pH of the PRP was 7.0. Aggregation was initiated by adding 13 μL of either T-11 or T-14 in 0.15 M NaCl, 0.01 M Tris/HCl, and 0.05% sodium azide, pH 7.4, to 450 μL of PRP to achieve the indicated concentrations. (B, bottom panel) Citrated PRP (0.45 mL; 317 000 platelets/μL) was adjusted to either pH 7.2 or 8.1 with HCl or NaOH and aggregation was initiated with 13 μL of T-14 (168 μM), 13 μL of T-11 (168 μM), or 14 μL of partially purified human thrombin (10 units/mL), giving final concentrations of 5 μM, 5 μM, and 0.3 unit/mL. The upward deflection in the thrombin-induced aggregation tracings is due to the onset of clot formation.

erably greater at pH 7.0–7.2 than at pH 8.0. T-11 and T-14 dose comparison studies at different pH values indicated that it requires ~1.5–2 times as much peptide to induce the same aggregation response at pH 8.1 as it does at pH 7.0–7.2.

NMR Structural Studies. To assess potential pH-dependent structural alterations in the T-11 peptide, 600-MHz proton NMR studies were performed on purified T-11. Specific sequential assignments of individual resonances were obtained from the combination of HOHAHA and ROESY

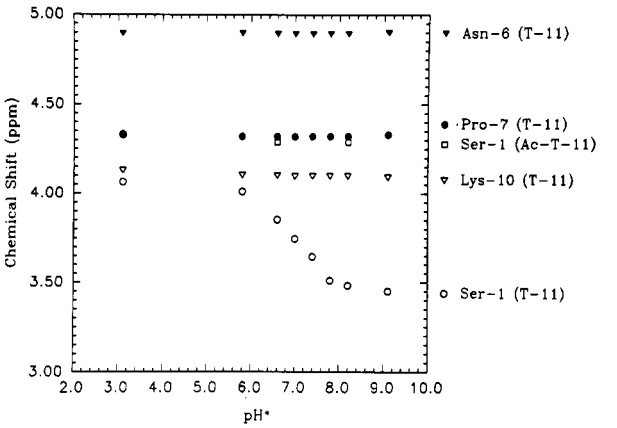

FIGURE 2: ^{1}H NMR pH* titration of selected α protons of T-11 and Ac-T-11. pH titration was performed at 37 °C for T-11 and 30 °C for Ac-T-11 as described under Materials and Methods. T-11: solid triangles, Asn-6; solid circles, Pro-7; open triangles, Lys-10; open circles, Ser-1. Ac-T-11: open squares, Ser-1.

experiments (manuscript in preparation) following the procedure described by Wüthrich and co-workers (Table II) (Billeter et al., 1982; Wüthrich, 1983; Wüthrich et al., 1982, 1984). On the basis of these assignments, it was possible to follow changes in the chemical shifts of specific protons as a function of pH*. Titration of T-11 showed that most of the nonexchangeable protons did not exhibit a pH*-dependent change in chemical shift (Figure 2), but the α_{ser} underwent a dramatic pH*-dependent change that could be fitted to the equilibrium equation for a single ionizable group.

In order to identify which ionizable group might be responsible for the pH* dependence of the α_{ser} chemical shift and to assess whether the pH-dependent platelet-aggregating activity and the pH*-dependent structural changes detected by NMR were related, we studied the Ac-T-11 peptide, in which the amino group on serine was acetylated and thus unable to ionize. Ac-T-11 did not aggregate platelets over the entire range of pH 7.2–8.1, and the α_{ser} did not display any pH*-dependent change in chemical shift (Figure 2).

Digestion of Peptides by Plasma Peptidase. Preincubation of T-14 or T-11 in plasma at 37 °C led to progressive loss of platelet-aggregating ability and the results with the T-11 peptide are shown in Figure 3A. Incubation times as short as 10 min were sufficient to produce significant inhibition. To assess the molecular basis of this loss of activity, T-11 was incubated in plasma for various periods of time and the integrity of the peptide was analyzed by reverse-phase HPLC (Figure 3B). The control sample containing plasma but no peptide had a peak that eluted at ~22 min, and this peak was also present in the other samples obtained throughout the exper-

Table II: Chemical Shift Values of Peptide T-11 in H_2O[a]

	NH	CαH	CβH	CγH	CδH	other
Ser-1		3.76	3.79			
Phe-2		4.64	3.11, 3.03			7.24 (H2,6), 7.30 (H4), 7.35 (H3,5)
Leu-3	8.11	4.29	1.57	1.57	0.93, 0.88	
Leu-4	8.17	4.27	1.51	1.51	0.88, 0.83	
Arg-5	8.22	4.28	1.77, 1.71	1.57, 1.54	3.13	NH 7.38, 7.10
Asn-6	8.61	4.92	2.89, 2.69			
Pro-7		4.35	2.29, 1.98	1.98	3.81, 3.77	
Asn-8	8.38	4.66	2.83, 2.71			
Asp-9	7.92	4.54	2.67			
Lys-10	8.15	4.12	1.55	1.14	1.55	ε2.89
Tyr-11	8.11	4.52	3.14, 2.88			7.15 (H2,6), 6.83 (H3,5)

[a] Measured at pH* 7.0 and 30 °C; given in parts per million. Data are accurate to 0.01 ppm except for the Ser-1 values, which are accurate only to 0.05 ppm because of overlapping resonances.

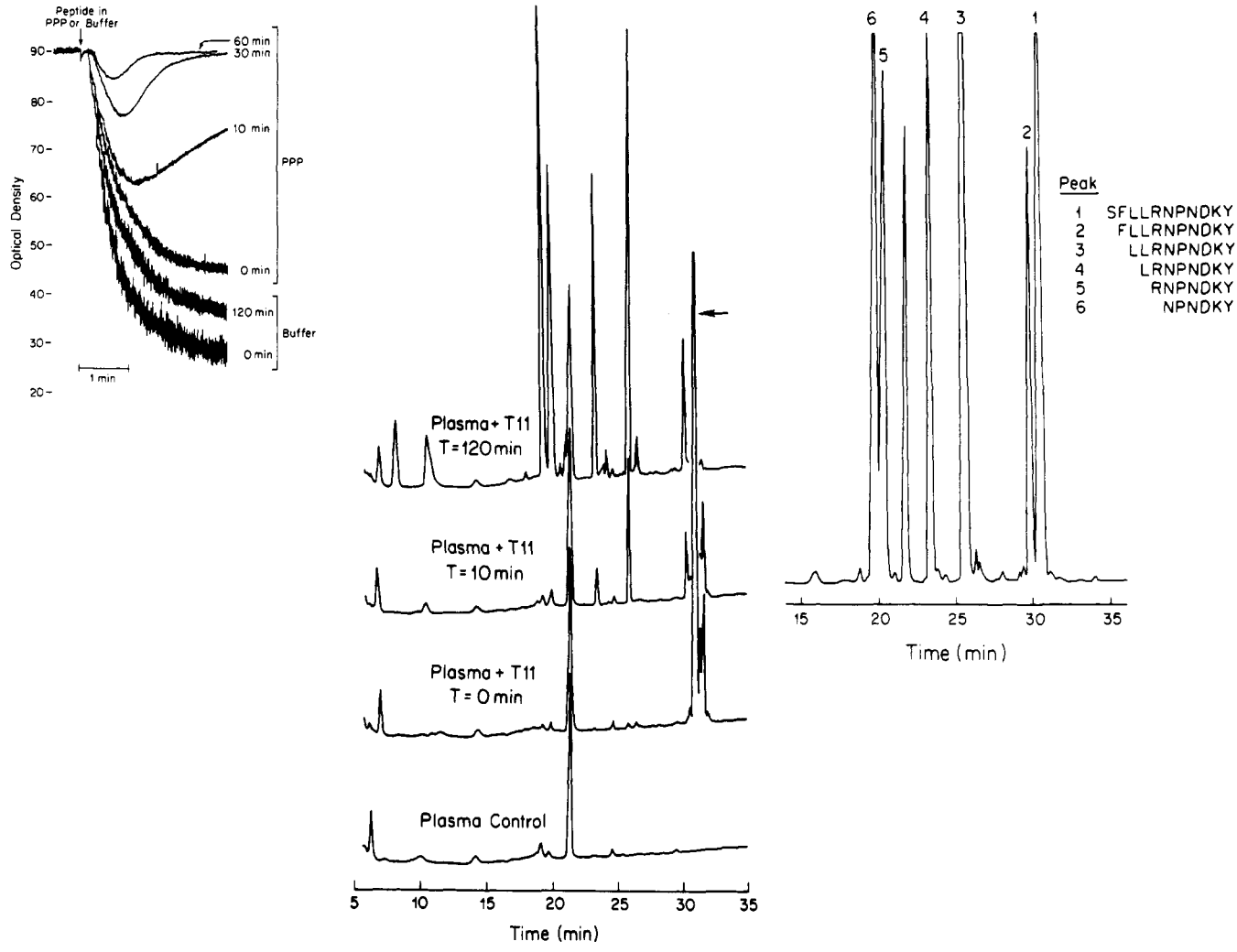

FIGURE 3: (A, left panel) Citrated platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared and the PRP was adjusted to 343 000 platelets/μL. T-11 (20 mM) was added to 200 μL of PPP or buffer to a final concentration of 2 mM. After the indicated incubation times, the peptide was diluted 1/8 in PPP or buffer and then 13 μL of this solution was added to 450 μL of PRP. There was little loss of aggregating activity when T-11 was incubated for 120 min with buffer, but within 10 min of incubation in PPP, T-11 lost a significant amount of aggregating activity; within 60 min, little activity remained. (B, center panel) T-11 peptide (2 mM) was incubated with PPP for various times up to 2 h at 37 °C. After precipitation of plasma proteins with trichloroacetic acid, the supernatant was analyzed by reverse-phase HPLC. Plasma without peptide (control) was treated in the same manner. The plasma control contained a peak eluting at ~22 min and this was present in all the chromatograms. At $T = 0$ min the intact T-11 peptide peak eluting at ~31 min dominated the chromatogram. At $T =$ 10 and 120 min there was a progressive decrease in the 31-min peak (obscured by the overlapping $T = 0$ peak) until at $T = 120$ this peak only extended up to the arrow on the side of the $T = 0$ peak. New peaks eluting at earlier time points appeared by 10 min and became very prominent by 120 min. (C, right panel) T-11 peptide (2 mM) was incubated with citrated PPP for 2 h at 37 °C, and after precipitation of plasma proteins, the supernatant was analyzed by HPLC. Peaks were subjected to amino acid analysis, making it possible to deduce the amino acid sequence of the peptide in each peak. The unlabeled peak at ~22 min was present in the plasma control and so was not analyzed further.

iment. The T-11 peptide in plasma at $T = 0$ had a dominant peak at ~31 min (corresponding to the elution of the T-11 peptide in buffer) with two small peaks eluting immediately thereafter. After the peptide was incubated for 10 min in plasma, the 31-min peak diminished (not observable in Figure 3B because the 31-min peak from the $T = 0$ sample obscures the peaks in the $T = 10$ and $T = 120$ samples) and new peaks appeared at ~30, 26, and 23 min. After 120 min of incubation, the ~31-min peak declined further (reaching only to the height indicated by the arrow), the peaks at ~30, 26, and 23 min all increased in size, and two major new peaks appeared at ~20 and 19 min. In addition, two minor new peaks at ~10 and 7 min also appeared. Figure 3C contains the chromatogram of another 120-min incubation of T-11 in plasma at 37 °C in which the peptide peaks were collected and analyzed for amino acid content and ability to aggregate platelets. Peak 1 contained the intact T-11, whereas peaks 2–6 contained a series of peptides characterized by the sequential loss of one N-terminal amino acid. Only the intact peptide was able to aggregate platelets, indicating that the removal of just the N-terminal serine was sufficient to abolish aggregating activity. In another experiment, the peak at 7 min was found to contain the sequence NDKY, whereas the peak at 10 min was identified as phenylalanine.

The sequential loss from T-11 of N-terminal amino acids suggested the activity of a plasma aminopeptidase. To further evaluate this activity, the inhibitors amastatin, bestatin, puromycin, and 1,10-phenanthroline were tested for their ability to inhibit plasma-mediated cleavage of T-11 in two separate experiments. Amastatin was the most potent inhibitor, producing 53% inhibition of cleavage at 0.3 μM, 70% inhibition at 1 μM, and 91% at 10 μM. Bestatin produced only 34% inhibition even at 100 μM and 39% at 1 mM. Puromycin was tested only at 10 mM; it produced 77% inhibition. 1,10-Phenanthroline inhibited only 14% of the cleavage at 1 mM but inhibited 77% at 20 mM.

Incubation of T-11 (50 μM) with serum resulted in degradation of T-11 (12.5 ± 0.3 nmol min^{-1} mL^{-1}, $n = 6$) and

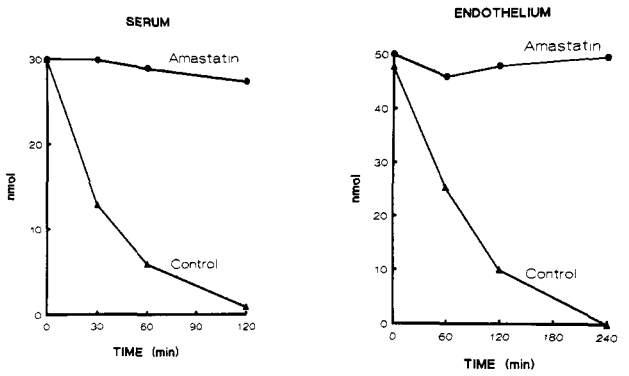

FIGURE 4: T-11 (50 μM) degradation by (A, left panel) human serum and (B, right panel) umbilical vein endothelium either under control conditions (no inhibitor) or in the presence of amastatin (100 μM). Separation and quantitation of substrate and products were assessed by HPLC on a Vydac C_{18}-μBondapak column as described under Materials and Methods.

the production of des-Ser^1-T-11. N-Terminal hydrolysis was proportional to the time of incubation (0–120 min) and the amount of serum added (20–50 μL). On the basis of experiments employing T-11 at 25, 50, and 100 μM, K_m and V_{max} values were 91.2 ± 14.7 μM and 35.1 ± 4.0 nmol min^{-1} mL^{-1} (n = 3). Purified aminopeptidase M was able to digest T-11 in a manner indistinguishable from cleavage by plasma and serum; in one experiment the K_m and V_{max} values were 133 μM and 8.5 μmol min^{-1} mg^{-1}.

The data in Figure 4A show that digestion of T-11 in serum was virtually complete at 120 min and that 100 μM amastatin nearly completely inhibited the digestion. In contrast, no inhibition of digestion occurred with the angiotensin-converting enzyme inhibitor captopril at 10 μM (data not shown). Endothelial cells also have the ability to rapidly cleave the amino-terminal serine from T-11 (0.40 ± 0.06 nmol min^{-1} (10^5 cells)$^{-1}$, n = 3) (Figure 4B). Amastatin (100 μM) inhibited this cleavage nearly completely.

To assess whether plasma aminopeptidase M inhibits platelet aggregation induced by T-11 or thrombin, PRP was preincubated with 10 μM amastatin, a concentration shown to nearly completely inhibit aminopeptidase M. Aggregation induced by T-11 was consistently enhanced, often converting reversible platelet aggregation into irreversible aggregation (Figure 5). In contrast, amastatin did not affect thrombin-induced aggregation or aggregation induced by other platelet agonists.

DISCUSSION

The successful cloning of the thrombin receptor by Vu et al. (1991a) has ushered in a new phase of investigation of thrombin-induced platelet activation with enormous potential for improving our understanding of hemostasis and thrombosis. The speed with which their discovery has been confirmed and extended attests to the intense interest in this subject.

Our studies have focused on two observations: (1) platelet aggregation induced by T-14 and T-11 peptides decreases as the pH of the PRP increases from pH 7.0 to 8.2, which is the reverse of the pH-dependence of thrombin-induced aggregation, and (2) the T-14 and T-11 peptides rapidly lose activity when incubated in normal plasma. Our detailed studies of these phenomena provide insights into the mechanism and structure–function relationships of thrombin receptor activation.

The slight increase in thrombin-induced platelet aggregation over the pH range from 7.0 to 8.2 was not unexpected since Martin et al. (1975) found a slight increase in thrombin-induced release of calcium from washed platelets over the pH range from 7.0 to 8.0, and one of us and others previously demonstrated that platelet aggregation induced by other agonists that activate the GPIIb/IIIa receptor increases as the pH rises from 7.4 to 7.8–8.0 (Coller et al., 1976). It was surprising, therefore, that aggregation induced by the T-14 and T-11 peptides was approximately twice as great at pH 7.2 as at pH 8.1. This suggested that a pH-dependent change in the activating peptide or the receptor's binding site for the peptide was responsible for the observation.

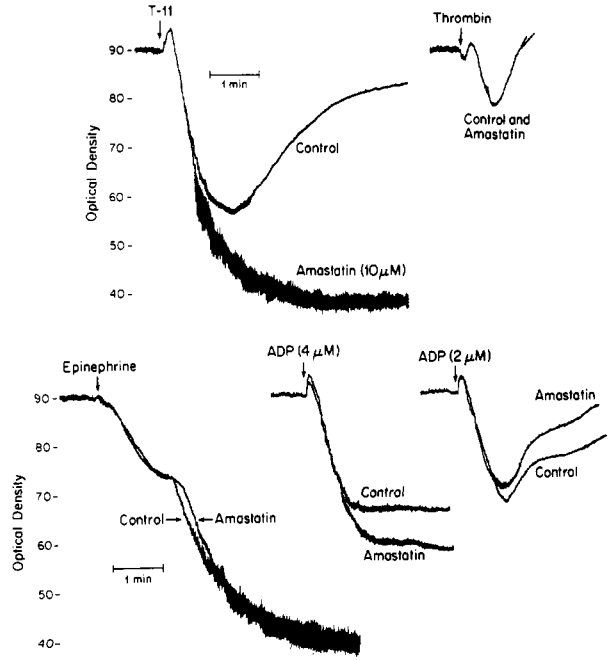

FIGURE 5: Blood was anticoagulated with ACD-A, and PRP was prepared and then adjusted to 333 000 platelets/μL. The PRP was adjusted to pH 7.4 and incubated with either amastatin (10 μM) or the buffer used to dissolve the amastatin (0.15 M NaCl and 0.01 M Tris/HCl, pH 7.4) for 15 min at 22 °C. The PRP was then adjusted to pH 7.7 immediately before testing with T-11 (3.8 μM), ADP (2 and 4 μM), epinephrine (4 μM), or purified human thrombin (0.3 unit/mL).

The effect of pH on the structure of the activating T-11 peptide was studied with 600-MHz proton NMR. A specific and dramatic change in the chemical shift of the α_{ser} was identified over the pH* range between 6.0 and 8.0, going from ~4.0 to ~3.5 ppm. This change probably reflects the ionization of the amino group on serine since acetylation of serine's amino group abolished the pH-dependent change in the chemical shift. Since acetylation also abolished the platelet-aggregating activity of T-11, collectively, these data are consistent with there being a requirement for a positively charged amino group on the N-terminal serine in order to induce platelet aggregation.

The observed pK* for the amino group (~7.1) is somewhat lower than expected for an N-terminal amino group (~8.0), suggesting possible electrostatic and/or dipolar (e.g., hydrogen bond) interactions between the N-terminus and either a backbone carbonyl group or side-chain groups of lysine or arginine. This interpretation appears to be supported by the value of the α_{ser} chemical shift after acetylation (4.30 ± 0.05 ppm), which does not correspond to the chemical shift of the α_{ser} in T-11 when the latter is either nearly fully protonated (at pH 3.1, the chemical shift is 4.07 ± 0.01 ppm) or nearly fully unprotonated (at pH 9.2, the chemical shift is 3.46). This suggests that the acetylation eliminates both the ionizable equilibrium and the interactions that reduce the pK*. Ad-

ditional NMR studies are now underway to more precisely define the structure–activity relationships in T-11 and related peptides. At present we have no method to assess pH-dependent changes in the receptor's peptide binding region and so it remains possible that these contribute to the observed pH-dependent changes in aggregating activity. However, we think this is less likely because thrombin-induced platelet aggregation, which relies on the same type of interaction, did not have the same pH dependence.

The pattern in PRP of platelet aggregation followed by disaggregation induced by the T-11 and T-14 peptides made us consider the possibility that the peptides are cleaved in plasma. Studies to test this hypothesis demonstrated rapid cleavage of the peptides with sequential loss of amino acids starting with the N-terminal serine. Most importantly, we found that loss of only a single amino acid, the N-terminal serine, was sufficient to abolish all aggregating activity. After these studies were performed, Vassallo et al. (1992) also reported the peptides lacking the N-terminal serine are inactive.

Although numerous aminopeptidases have been characterized (McDonald & Schwabe, 1986; Kenny, 1977), it is now well established that aminopeptidase M is the major enzyme responsible for the ability of plasma to cleave N-terminal neutral/basic amino acids from low molecular weight peptides (Ward et al., 1990; Ahmad & Ward, 1990). Aminopeptidase M is also present on vascular endothelium and smooth muscle cells (Palmieri et al., 1989) and in the microvasculature (Churchill et al., 1987; Hersh et al., 1987). Aminopeptidase M degrades opioid peptides and has been shown to play a physiologically significant role in the degradation of circulating angiotensins (Ahmad & Ward, 1990) and neurokinins (Wang et al., 1991; Ahmad et al., 1992). Aminopeptidase M differs from other aminopeptidases in its inhibitor profile, with amastatin a more potent inhibitor than bestatin or puromycin. Digestion of T-11 could be nearly completely inhibited in plasma and serum by micromolar concentrations of amastatin, but higher concentrations of the other inhibitors were required, and captopril had virtually no effect. Moreover, purified aminopeptidase M cleaved the T-11 peptide in a similar manner, and the kinetic parameters indicate that T-11 is a preferred substrate for the enzyme, being comparable in K_M to angiotensin (Ward et al., 1990). Collectively, these data demonstrate that aminopeptidase M is responsible for virtually all of the plasma/serum proteolytic activity. Endothelial cell aminopeptidase M also readily cleaved the T-11 peptide.

Adding amastatin to PRP consistently enhanced the aggregation induced by T-11, in some cases converting reversible aggregation into irreversible aggregation (Figure 5). This indicates that plasma aminopeptidase M acts rapidly enough on T-11 at (1–10) $\times 10^{-6}$ M to inhibit platelet aggregation. This observation needs to be considered when designing and interpreting experiments using thrombin receptor peptides to activate platelets or other cells in the presence of serum or plasma, especially when long incubations are involved. If aminopeptidase M can also digest the new N-terminal serine produced by thrombin cleavage of the intact thrombin receptor, it presumably could inactivate the tethered ligand, thus affording a potential control mechanism. From a quantitative standpoint, on the basis of our data with the T-11 peptide, there should be sufficient aminopeptidase M since Brass et al. (1992) most recently reported that 1800 molecules of a monoclonal antibody directed against the thrombin receptor bound per platelet. If this accurately reflects the copy number of receptors per platelet, then at a whole blood platelet count of 2.5×10^8/mL and a hematocrit of 0.45, there would be a maximum of 1.4×10^{-9} mol of cleaved thrombin receptors/L of plasma, or 1/2600 the number of T-11 peptide molecules used in the experiment in Figure 5. To test whether aminopeptidase M affects thrombin-induced platelet activation, aggregation induced by thrombin was tested in the absence and presence of amastatin. No differences were detected. Thus, under the conditions used in the experiments, with bolus administration of a relatively high dose of thrombin to platelets, no effect of aminopeptidase M was observed. Possible explanations for the failure of aminopeptidase M to affect the aggregation include the inability of aminopeptidase M to digest the N-terminal amino acid on a large protein like the thrombin receptor or the inability of aminopeptidase M to gain access to the tethered ligand because the latter is so rapidly inserted into the activation pocket of the receptor. Our data do not unequivocally exclude an in vivo role for aminopeptidase M, however, since it remains possible that lower concentrations of thrombin may act on platelets in vivo in a manner that is more favorable to subsequent aminopeptidase M digestion.

The identification of T-11 cleavage activity on the surface of endothelial cells is consistent with the known tissue localization of aminopeptidase M (Palmieri et al., 1989). The localization of this enzyme on the endothelium places it in a strategic position to interact with the thrombin receptor on endothelial cells. Whether this facilitates its ability to act on the endothelial cell receptor tethered ligand after thrombin cleavage remains to be established.

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Registry No. T-14, 141923-36-6; T-14FS, 144108-74-7; T-11, 144108-75-8; Ac-T-11, 144108-76-9; T-12, 144108-77-0; Ser, 56-45-1; ADP, 58-64-0; aminopeptidase M, 9054-63-1; thrombin, 9002-04-4; epinephrine, 51-43-4.