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Aspartame and aspartame derivatives effect human thrombin catalytic activity

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Abstract

The study of small Asp-Phe analogs was undertaken since this dipeptide sequence is critical in fibrinogen recognition and catalysis. The inhibition of clotting activity by Asp-Phe-methyl ester (aspartame), formyl-Asp-Phe-methyl ester and acetyl-Asp-Phe was biphasic in all cases, indicating the presence of at least two binding sites. The N-terminally blocked derivatives are stronger inhibitors than aspartame. In contrast, tosyl-Gly-Pro-Arg-*p′* -nitroanilide hydrolysis was inhibited minimally by Asp-Phe-methyl, ester [K_i(app)=98 mM]. Acetyl-Asp-Phe inhibition of thrombin amidase activity was biphasic, tenfold stronger and appeared to be strongly cooperative. These results are discussed with respect to the inhibition of α-thrombin by ATP.

Keywords: Thrombin; Aspartame; Aspartyl-phenylalanine-methyl ester; Fibrinogen; Fibrinogen recognition site

1. Introduction

Thrombin (E.C. 3.4.21.5) is a unique serine protease in its highly specific interactions with its primary polypeptide substrate fibrinogen, where only two Arg-Gly linkages are susceptible to its action. There is strong evidence for the essential presence of Phe at position P_9 and Asp at P_{10} of the Aα-chain of human fibrinogen for α-thrombin catalyzed conversion to fibrin [1–4]. The nature of this dipeptide sequence, an anionic group at P_{10} followed by an apolar group at P_9, has a striking similarity to adenine nucleotides, e.g., ATP, which are potent inhibitors of the thrombins [5,6]. In view of these studies, the effect of terminally blocked derivatives of Asp-Phe directly on α-thrombin was examined.

2. Experimental

2.1. Methods

Purified human α- and γ-thrombins were generous gifts from Dr. John W. Fenton II, State Department of Health, Albany, NY. α-Thrombin specific activity was routinely 3000 NIH units mg^{-1} and 95–98% by NPGB (*p*-nitrophenyl-*p′* -guanidinobenzoate) active site titration. The enzyme was stored at −72 °C and thawed immediately before use. Thrombin concentrations were determined spectrophotometrically at 280 nm, ε_{280}=1.83 mg/ml/cm in 0.1N NaOH using a molecular weight of 36,600 Da [7]. Kinetic assays were performed at 25 °C unless stated otherwise. Kinetic and equilibrium constants were derived by computer best fit by nonlinear or linear regression analysis.

2.2. Fibrinogen clotting activity

α-Thrombin clotting activity was measured on a BBL Fibrometer at 37 °C according to Fenton et al. [7]. Clotting assays contained 4.44 g l^{-1} PEG 6000 (polyethylene glycol,

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Dow Chemical), 0.12 M NaCl, 7.8 mM imidazole (pH 7.4), 7.6 mM $CaCl_2$, 2.67 mg/ml fibrinogen (75–95% clottable, Miles Laboratories) and an α-thrombin concentration sufficient to produce 12–45 s clotting times (4–8 units, 25 nM). Each clotting time was the average of four determinations. Relative activity (% units) was calculated from a standard curve of clotting time versus enzyme concentration. The inclusion of PEG 6000 in our clotting assay was chosen for its accelerating effect on the rate of fibrin polymerization, whereby thrombin-catalyzed hydrolysis is usually rate-limiting.

2.3. Amidase hydrolysis

Amidase activity was determined spectrophotometrically at 405 nm [8] by following the rate of release of *p*-nitroanilide at 405 nm from Tosyl Chromozym TH™ (tosyl-Gly-ProArg-*p*-nitroanilide-HCl, Diagnostica Stago, France, lot #20) at pH 8.3, 50 mM Tris–HCl, 0.1 M NaCl. Thrombin (ca. 1-nM final concentration) was incubated in the sample cuvette and the reaction started by the addition of 3×10^{-5} M Tosyl Chromozym TH™.

2.4. Chemicals

All intermediates and products were analyzed for purity by thin layer chromatography on silica gel sheets (Eastman Kodak) in 2:1 (v/v) chloroform and methanol. R_f values are reported for products as homogeneous on thin layer chromatography (visualization with iodine or 0.2% ninhydrin in acetone). All melting points (MP) are uncorrected. Newly synthesized compounds were identified by ^{1}H NMR. All chemical shifts, δ, are relative to DSS (sodium 2,2-dimethyl-2-silapentanesulfonate) in D_2O at pH 7.0.

2.5. Formyl-L-Asp-L-Phe-methyl ester (formyl-aspartame)

Formyl-aspartame was synthesized according to the general procedure of du Vigneaud et al. [9]. Acetic anhydride (2.5 ml) was added dropwise to a solution of 0.5 g (1.7 mmol) L-Asp-L-Phe-methyl ester (Sigma, lot #122F-0884) in 5 ml of 90% formic acid at 4 °C. After stirring for 1 h at room temperature, 10-ml ice water was added. Solvent evaporation in vacuo produced an oil which crystallized with the addition of ether to yield formyl-L-Asp-L-Phe-methyl ester. Removal of a small amount of contaminating Asp-L-Phe-methyl ester was accomplished by column chromatography of the product on Dowex AG I ×8 anion exchange resin (Bio-Rad) at pH 7.0. Formyl-Asp-Phe-methyl ester was eluted with 0.05N HCl and the solvent was removed by rotary evaporation. The product crystallized from ether and methanol and was washed with ether. Formyl-L-Asp-L-Phe-methyl ester: yield 72%; MP 138–141; R_f=0.36; ninhydrin negative; δ (ester CH_3)=3.80 ppm; δ (formyl CHO)=8.09 ppm.

2.6. Acetyl-Ala-Phe-methyl ester

(D,L)-Ala-L-Phe (Sigma, lot #123F-0005) and L-Ala-L-Phe (Sigma, lot #63F-0003; R_f=0.118) were acetylated according to the method of du Vigneaud and Meyer [10]. Eight additions of 1 ml (0.5 ml) 2N NaOH and 0.1 ml (0.05 ml) acetic anhydride were made to 500 mg D,L-Ala-Phe (or 250 mg L-Ala-Phe) dissolved in 1.6 ml (0.8 ml) 2N NaOH, respectively. After stirring at room temperature for 80 min, 3 ml (1.5 ml) of 6N H_2S0_4 were added, respectively. After 24 h at 4 °C, crystals of product acetyl-Ala-Phe were collected and washed with ether. L-Acetyl-Ala-L-Phe: yield 76%; MP 217–220; ninhydrin negative; R_f=0.56; δ (acetyl CH_3)=1.96 ppm. (D,L)-acetyl-Ala-L-Phe: yield 90%; MP 203–206; ninhydrin negative; R_f=0.44; δ (acetyl CH_3)=1.96 ppm. The acetylated peptides were subsequently esterified after the method of Brenner and Huber [11] as described by Applewhite et al. [12]. Freshly distilled thionyl chloride (11.9 g, 0.1 mol) was added dropwise to 75-ml methanol with stirring. The solution was made up to 100 ml with additional methanol. Fifty milliliters of this solution was added to the acetyl-(D,L)-Ala-L-Phe and 25 ml was added to the L isomer above. Reaction mixtures were stirred at room temperature until the esterification was complete as judged by thin layer chromatography (about 1 h) and neutralized by the addition of solid sodium bicarbonate. The product was extracted with chloroform and crystallized overnight from ether and pentane at 4 °C. Acetyl-(D,L)-Ala-L-Phe methyl ester: yield 33%; MP 172–176; ninhydrin negative; R_f=0.737; 0 δ (ester CH_3)=3.73 ppm; δ (acetyl CH_3)=1.99 ppm. Acetyl-L-Ala-L-Phe-methyl ester: yield 24%; MP 134–136; ninhydrin negative; R_f=0.87; δ (ester CH_3)=3.79 ppm; δ (acetyl CH_3)=2.00 ppm.

2.7. Acetyl-L-Asp-L-Phe

L-Asp-L-Phe methyl ester (1 g, 3.4 mmol) was dissolved in 2.42 ml 2N NaOH and 0.8 ml water and cooled on an ice bath. Eight additions of 2 ml 2N NaOH and 0.2 ml acetic anhydride were made, with cooling and stirring between additions. After standing at room temperature for 40 min, there was no longer any reaction with ninhydrin; 6.14 ml 6N H_2S0_4 was added which resulted in the hydrolysis of the methyl ester [10]. The product crystallized after standing at 4 °C overnight and was recrystallized from water. Acetyl-L-Asp-L-Phe: yield 64%; MP 191–194; ninhydrin negative; R_f=0.55; δ (acetyl CH_3)=1.96 ppm.

3. Results

3.1. Inhibition of fibrinogen clotting activity

Fig. 1A and B shows Dixon plots for inhibition of α-thrombin clotting activity by aspartame (L-Asp-L-Phe methyl ester) (○), acetyl-L-Ala-L-Phe methyl ester (△),

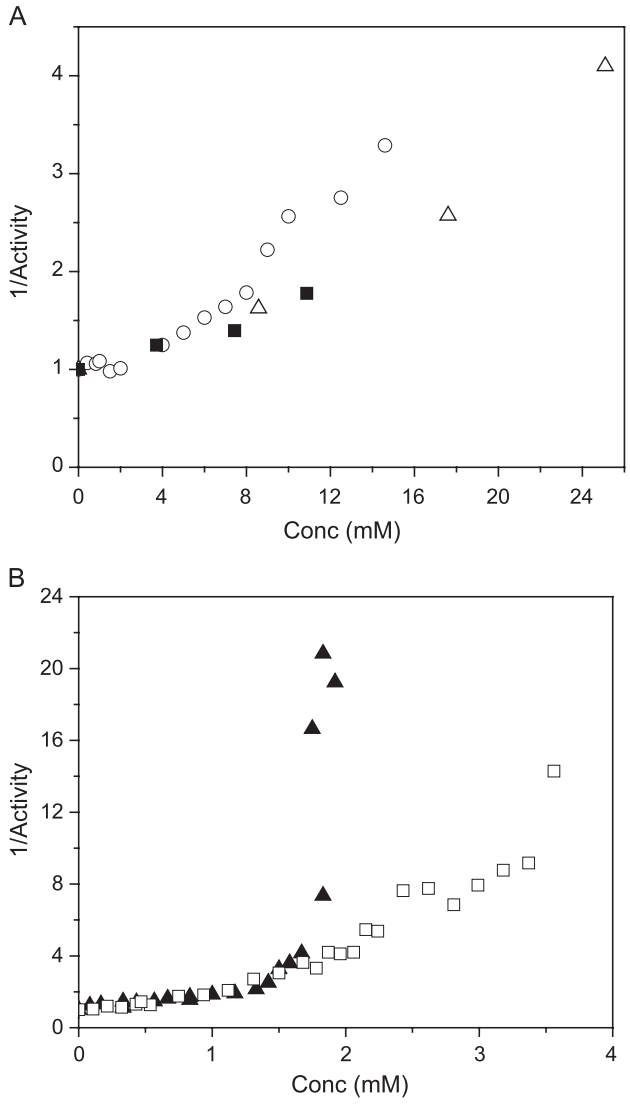

Fig. 1. Dixon plots of inhibition of α-thrombin catalyzed fibrinogen clotting activity. (A) Aspartame (L-Asp-L-Phe methyl ester) (○), acetyl-L-Ala-L-Phe methyl ester (Δ), acetyl-(D,L)-Ala-L-Phe methyl ester (■). (B) Acetyl-L-Asp-L-Phe (▲), formyl-L-Asp-L-Phe methyl ester (□).

acetyl-(D,L)-Ala-L-Phe methyl ester (■) (Fig. 1A), acetyl-L-Asp-L-Phe (▲) and formyl-L-Asp-L-Phe methyl ester (□) (Fig. 1B), respectively. The alanyl derivatives were relatively poor inhibitors (I_{50}~13.5 mM) versus the Asp-containing derivatives; the latter exhibited biphasic inhibition suggesting contributions to binding (at least two sites) by the aspartyl carboxylate group side chain (Fig. 1B). In comparison to the neutral zwitterion, Asp-Phe-methyl ester, the net negatively charged N-terminally blocked peptide derivatives were much stronger inhibitors (Fig. 1B). Esterification of the carboxyl terminal Phe did not appear to affect the observed inhibition.

Conery and Berliner [5] have reported the inhibition of α-thrombin catalyzed fibrinogen clotting and tosylarginine methyl ester (TAME) hydrolysis by ATP. Berliner et al. [6] later described even more complex behavior by ATP with thrombin. Consequently, it was of interest to compare these peptide derivatives with this mononucleotide. The similarities on clotting activity by formyl-L-Asp-L-Phe methyl ester (□) and acetyl-L-Asp-L-Phe (▲) compared to ATP (▽) are apparent in Fig. 2. In all three cases, inhibition was biphasic and could be approximately fit by a simple two-site inhibition model without cooperativity. The kinetic similarity between ATP and the Asp-Phe peptide derivatives in inhibiting α-thrombin, as well as their overall structural similarities, is suggestive of mutual binding sites.

3.2. Inhibition of amidase activity

The inhibition of α-thrombin catalyzed Tosyl Chromozym TH™ (tosyl-Gly-Pro-Arg-pNA) hydrolysis by formyl-L-Asp-L-Phe methyl ester (□) and acetyl-L-Asp-L-Phe (●) is illustrated in Fig. 3. Again, the inhibition by aspartame was weak, K_i (app)=98 mM, and could not be fully examined due to solubility limitations. Acetyl-Ala-Phe-methyl ester did not inhibit even at concentrations up to 16 mM (data not shown). The biphasic inhibition by acetyl-Asp-Phe appeared to be highly cooperative in nature. In comparison, ATP (▽) inhibition was observed at concentrations similar to those of acetyl-Asp-Phe. However, at lower ATP concentrations, activation of peptide hydrolysis by α-thrombin was observed (Fig. 3). If the allosteric site for ATP activation of Tos-Gly-Pro-Arg-pNA hydrolysis was the fibrinogen recognition site, inhibition of clotting might have been expected. Note from Fig. 2 that biphasic inhibition of clotting by ATP at two sites was observed. The inhibition of γ-thrombin hydrolysis of Tosyl Chromozym TH™ by formyl-Asp-Phe-methyl ester was also biphasic at similar concentrations (data not shown).

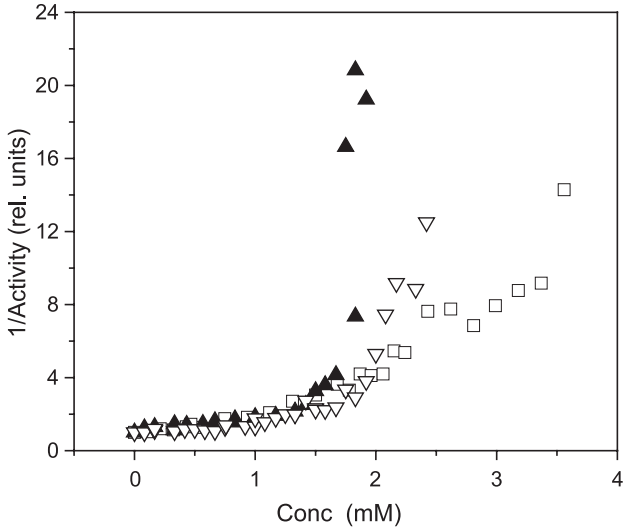

Fig. 2. Comparison of Dixon plots of inhibition of α-thrombin clotting activity as inhibited by formyl-L-Asp-L-Phe methyl ester (□), acetyl-L-Asp-L-Phe (▲) and ATP (▽).

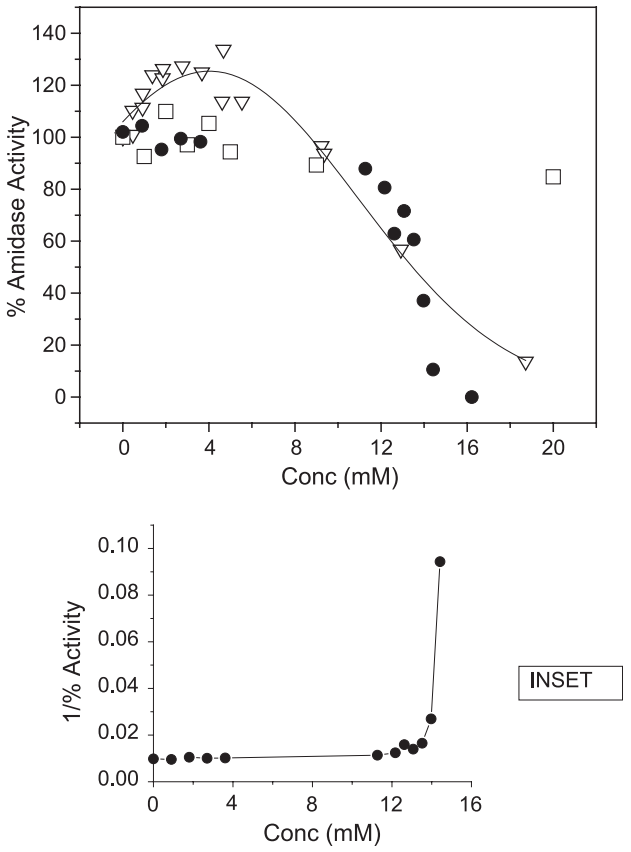

Fig. 3. Inhibition of α-thrombin catalyzed hydrolysis of Tosyl Chromozym TH™ (tosyl-Gly-Pro-Arg-pNA) by formyl-L-Asp-L-Phe methyl ester (□), acetyl-L-Asp-L-Phe (●) and ATP (▽). Inset: Dixon plot of acetyl-L-Asp-L-Phe inhibition (●).

3.3. Effect of Asp-Phe derivatives on fibrinogen

The binding of small peptides to fibrinogen that contain a Gly-Pro-Arg sequence prevents both polymerization of fibrin monomers [13,14] and fibrinogen binding to human platelet receptors [15]. The possibility here that these Asp-Phe derivatives were inhibiting α-thrombin clotting activity by binding, in part, to fibrinogen was examined by several methods:

(1) In the presence of 4.2 mM Asp-Phe-methyl ester, resulting in 52% inhibition of fibrinogen clotting at limiting (0.4 mg/ml) fibrinogen concentrations, clotting activity was linear with α-thrombin concentration (Fig. 4). At high α-thrombin concentrations, a rate comparable to that in the absence of Asp-Phe-methyl ester was achieved (Fig. 4).

(2) In addition, Asp-Phe-methyl ester did not bind to fibrinogen or fibrin monomer covalently liganded to Sepharose 4B, even in the presence of clotting assay buffer containing calcium [16]. Other ligands known to interact with fibrinogen do bind to fibrinogen-Sepharose 4B [17]. Hence, no evidence exists for the formation of an inactive complex between Asp-Phe methyl ester and fibrinogen in the presence or absence of calcium.

(3) Human fibrinogen contains three tight binding sites for Ca^{2+} (K_d=1.95×10^{-5} M) [18,19]. Additional free Ca^{2+} may also play a role in regulating fibrinogen conversion to fibrin [20] as it reduces clotting times significantly [21–25]; thus, we included 7.55 mM calcium in the clotting assays. This effect may be due to enhancement of thrombin cleavage of fibrinogen [26], fibrin monomer aggregation [23,25,27,28] or fibrin monomers released from thrombin [29]. While it was possible that the chelation of calcium by Asp containing peptides in clotting assays might contribute to the overall inhibition observed, at low inhibitor concentrations,

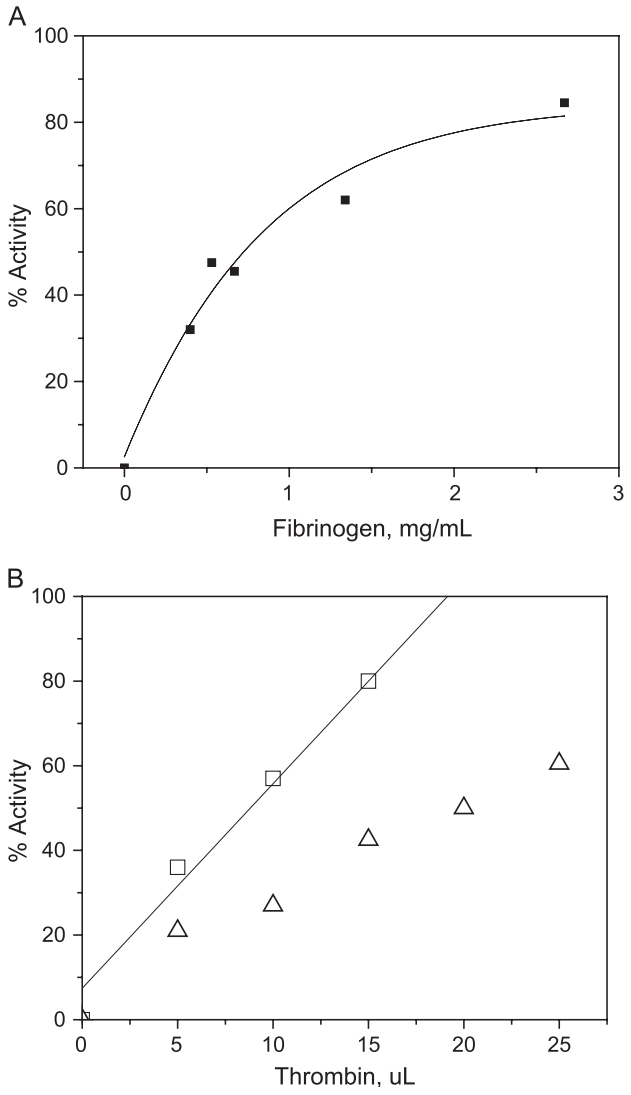

Fig. 4. (A) Dependence of α-thrombin clotting activity on fibrinogen concentration. Standard clotting curve with varying fibrinogen concentration. Note that standard clotting assays are 2.67 mg/ml fibrinogen. (B) In the presence of 0.5 mM L-Asp L-Phe methyl ester (□) and 4.2 mM L-Asp-L-Phe methyl ester (Δ). Fibrinogen concentration was 0.4 mg/ml.

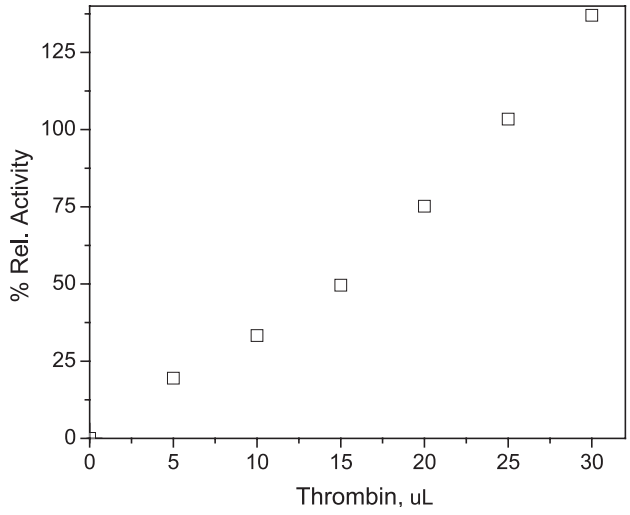

Fig. 5. Linearity of clotting activity with α-thrombin concentration in the presence of 2.5 mM formyl-L-Asp-L-Phe methyl ester activity in the absence of inhibitor (♦). Fibrinogen concentration was 2.67 mg/ml.

this was unlikely due to a limiting amount of active fibrinogen. In addition, the inhibition of Tos-Gly-Pro-Arg-pNA hydrolysis was observed in the absence of calcium.

(4) An attempt to observe the displacement of proflavin from α-thrombin by formyl-Asp-Phe-methyl ester resulted in the precipitation of α-thrombin at a concentration of the inhibitor of 5 mM (data not shown). In view of this result, it seemed possible that the peptide derivatives could be inactivating α-thrombin in the kinetic experiments. In the presence of 2.52 mM formyl-Asp-Phe-methyl ester, which inhibited clotting activity by 80%, clotting activity was linear with enzyme concentration (Fig. 5).

(5) Reversibility of clotting inhibition was demonstrated with formyl-Asp-Phe-methyl ester. At the higher concentrations of acetyl-Asp-Phe required to inhibit amidase activity, inhibition by this peptide was also reversible in dilution experiments (data not shown).

4. Discussion

Scheraga et al. [1–4] have demonstrated the importance of the Phe and Asp moieties, i.e. residues P_9 and P_{10}, respectively, from the Arg-Gly cleavage site in the Aα chain of fibrinogen in binding to α-thrombin. The inclusion of Phe at P_9 and Asp at P_{10}, respectively, in synthetic fibrinogen-like peptides 14 residues in length contributed to both increased k_{cat} and decreased K_m for peptide hydrolysis by α-thrombin. This confirmed the high degree to which these residues are conserved among different fibrinogen species [30–33]. Addition of the Asp10-Phe9 sequence to the F_2 peptide resulted in a fivefold increase in peptide binding, while k_{cat} was increased by 2 orders of magnitude, both residues contributing to the kinetic parameters. In addition, Ni et al. [36,37] examined the interactions between these peptides and thrombin by NMR. The authors proposed the following alternatives in explaining their results: (1) there was an interaction between Asp10-Phe9 and α-thrombin; (2) there was an interaction between Asp10 and residue(s) in the P_{10}–P_4 sequence of fibrinogen which is important in forming the fibrinogen chain structure for thrombin hydrolysis by introducing (distant) residues like Phe9 to the region of hydrolytic cleavage; and (3) a direct contribution to binding to thrombin and indirect contribution due to proper structuring of the fibrinogen molecule, i.e. a combination of (l) and (2) above. In support of (2), short peptides in which D-Phe "is folded back over," the residues next to the cleavable Arg are hydrolyzed more rapidly than the corresponding L-Phe peptides lacking this structural feature [34,35].

The results reported here suggest that an interaction between Asp-Phe directly with thrombin may also contribute to fibrinogen binding. A distinction between the two inhibitory sites for Asp-Phe derivatives is impossible at this juncture without X-ray confirmation [38,39]. However, the highly cooperative inhibition by acetyl-Asp-Phe of Tosyl Chromozym TH™ (Tos-Gly-Pro-Arg-pNA) hydrolysis indicates that binding to one site influenced binding to the second site. Biphasic inhibition by these peptide derivatives was also observed with γ-thrombin, which cannot clot fibrinogen, suggesting that these potential fibrinogen recognition site(s) might be conserved in the α- to γ-thrombin conversion.

The structures of formyl-Asp-Phe-methyl ester and acetyl-Asp-Phe are not dissimilar to ATP. Both peptides and the nucleotide have aromatic rings adjacent to negatively charged functionalities with the same distance relationships as judged from molecular modeling. The inhibition of α-thrombin clotting activity by ATP at comparable concentrations was also biphasic. However, the effect on amide hydrolysis clearly involved an allosteric site since activation was observed, as well as an inhibitory site which overlapped the active site proflavin-binding region [5]. On the other hand, formyl-Asp-Phe-methyl ester was unable to displace thrombin from ATP-agarose (data not shown).

In order to dispel any concerns about the consumption of aspartame and hemostatis, we note that aspartame inhibition of α-thrombin probably has little physiological consequence since the levels required far exceed that ingested, even in overdose amounts. Furthermore, aspartame is immediately hydrolyzed in the blood by esterases [40,41]. In addition, free aspartate inhibition of fibrinogen clotting would be much weaker than that of aspartame itself, which has an I_{50} of 9 mM.

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