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MECHANISM OF INHIBITION OF THROMBIN-INDUCED PLATELET AGGREGATION BY PYRIDOXAL PHOSPHATE

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Summary

Thrombin and ADP-induced platelet aggregation are reversibly inhibited by pyridoxal phosphate. Sodium borohydride converts Schiff bases formed between pyridoxal phosphate and amino groups to covalent bonds. When platelets treated with sodium borohydride and pyridoxal phosphate are resuspended in fresh platelet-poor plasma, they recover their response to thrombin, but not to ADP. Thus Schiff base formation between pyridoxal phosphate and platelet surface amino groups does not block thrombin aggregation. The loss of thrombin potency as an aggregating agent is due to interaction between pyridoxal phosphate and thrombin. This is evidenced by spectrophometric determination of adduct formation and loss of hydrolytic action on p-tosyl-L-arginine methyl ester.

Blood platelets are activated by various endogenous agonists (e.g., ADP, epinephrine, collagen and thrombin) to change shape, aggregate and secrete their granular contents. There is evidence that separate platelet membrane sites interact with the different agonists (1-4). We found that pyridoxal phosphate (PLP), the coenzyme form of vitamin B_6, inhibits aggregation of platelets exposed to ADP but not to epinephrine, arachidonic acid or A23187. On the other hand PLP also inhibits thrombin-induced aggregation (5). PLP may inhibit thrombin by acting on a site common to both ADP and thrombin, by acting on a separate site or by reacting directly with thrombin to inactivate it. The present report demonstrates that the mechanism responsible for the inhibition by PLP of thrombin-induced aggregation is by adduct formation between PLP and thrombin. Corollaries to this finding are 1) ADP and thrombin act on separate platelet membrane sites, and 2) formation of Schiff bases with platelet surface amino groups does not interfere with the action of thrombin on platelets.

Materials and Methods

Human platelet-rich plasma was prepared as previously described (6). Platelets in plasma treated with EDTA (7.5 mM) were centrifuged at 365 xg for

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15 min and resuspended either in a calcium and albumin-free Tyrodes solution (NaCl, 136; KCl, 2.7; NaH_2PO_4, 0.4; $NaHCO_3$, 1.2; MgCl, 1.19 and glucose, 5.5; in mM) or in platelet-poor plasma obtained by centrifuging platelet-rich plasma at 2000 xg for 15 min. Platelet aggregation was measured at 37°C by methods previously described (6).

Pyridoxal phosphate, imipramine, and p-tosyl-L-arginine methyl ester HCl (TAME) and sodium borohydride were obtained from Sigma Chemical Co., St. Louis, Missouri. 5-hydroxy [side chain-2-^{14}C] tryptamine creatinine sulfate, 58 mCi/mmole [^{14}C]-serotonin was obtained from Amersham/Searle Corp., Arlington Heights, Illinois. Human thrombin was obtained from two sources. Commercial human thrombin was purchased from Ortho Diagnostics, Raritan, New Jersey. Highly purified thrombin (specific activity = 2,513 units/mg) was the gift of Dr. John W. Fenton, II, New York State Department of Health, Albany, New York. Thrombin and TAME were dissolved in distilled water and adjusted to pH 7.4 with 1N NaOH. Pyridoxal phosphate was initially dissolved in a few drops of 1N NaOH, made up to volume with distilled water, kept on ice and stored in the dark. The final pH was adjusted to 7.4.

To determine the extent of platelet release, [^{14}C]-serotonin (0.34 uM) was added to platelet-rich plasma and incubated for 1 hour at 23°C. Imipramine (2 uM) was added to prevent re-incorporation of serotonin and incubation continued for 5 min. The extent of release was calculated as the percentage of the total platelet content of [^{14}C]-serotonin appearing in the supernatant fraction 3 min following stimulation.

Protein concentration was determined according to the method of Bradford (1976) (7) with the use of a BioRad Protein assay kit (BioRad Laboratories, 2200 Wright Avenue, Richmond, California 94804).

Results and Discussion

In agreement with earlier reports (8-9) we confirm that pyridoxal phosphate (PLP) inhibits thrombin-induced platelet aggregation. Aggregation in the presence of 3 mM PLP became reversible and was further inhibited by 6 mM PLP while 9 mM PLP completely inhibited aggregation, but appeared not to inhibit shape change (Fig. 1). The effect of PLP to inhibit thrombin-induced aggregation was also seen with platelets resuspended in buffer (Table 1). In addition, both platelets in plasma or resuspended in buffer exhibited a dose-dependent inhibition of [^{14}C]-serotonin release when increasing amounts of PLP were added (Table 1).

We investigated the direct effect of PLP on the ability of thrombin to act on platelets. Thrombin treated with PLP and then dialyzed to eliminate free PLP showed a decreased ability to aggregate platelets in plasma. Thus, approximately six times the control amount of thrombin was necessary to obtain the same initial velocity and extent of aggregation. Spectrophotometric studies

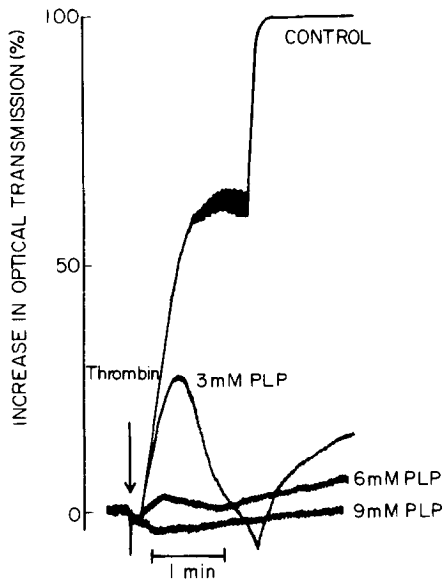

Fig. 1. Effect of pyridoxal phosphate (PLP) on thrombin-induced aggregation of platelets in platelet-rich plasma. The plasma was incubated with PLP (final concentrations shown above) for 1 min at 37°C. Thrombin (0.25 units/ml) was added at the arrow. In the absence of added PLP, aggregation ensued until clot formation altered the record. Increasing amounts of PLP inhibited the action of thrombin.

TABLE I. EFFECT OF PYRIDOXAL PHOSPHATE (PLP) ON THROMBIN-INDUCED PLATELET AGGREGATION AND SECRETION.[1]

PLP	Platelet-rich Plasma[2]		Resuspended Platelets[3]	
mM	% Agg.	% Release	% Agg.	% Release
0.0	100	100 (45)	100	100 (55)
0.5	58	8	88	100
2.0	44	2	50	50
4.0	38	1	29	25
5.0	7	0	11	20
10.0	2	0	3	9

[1]Mean of three experiments.
[2]Thrombin (0.25 units/ml).
[3]Thrombin (0.125 units/ml).
Numbers in parenthesis indicate the percent of the total [^{14}C]-serotonin taken up that is released by thrombin alone.

of PLP-treated thrombin indicated a characteristic change in the spectrum resulting in the appearance of a new absorption maximum at about 325 nm (Fig. 3). The induction of this peak by PLP has been previously observed in spectral studies of enzymes, e.g., malate dehydrogenase (10), and appears to involve the

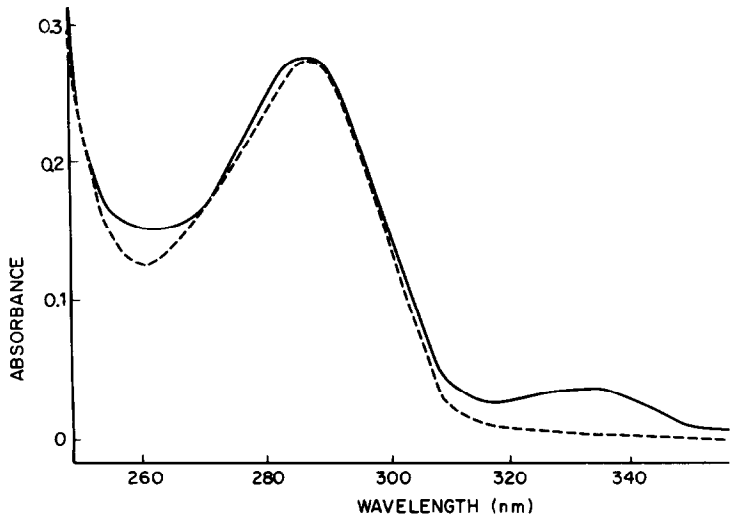

Fig. 2. The effect of pyridoxal phosphate on the absorption spectra of thrombin. Highly purified thrombin (2,513 units/mg) was incubated for 30 min at 37°C with 0.1M pyridoxal phosphate (solid line) or with 0.155M NaCl (dashed line). The solutions were dialyzed at 4°C for 12 hrs against 0.155M NaCl to eliminate free pyridoxal phosphate. Protein concentration of each solution was 240 ug/ml.

formation by PLP of an azolidine ring with a lysine and histidine residue at the enzyme active site resulting in the loss of enzyme activity. Modification of thrombin's histidine residues results in loss of catalytic activity (11). Modification of thrombin lysine groups by PLP results in loss of figrinogen clotting activity as well as a decreased sensitivity to heparin in the antithrombin III/thrombin reaction (12). Indeed, we observed an inhibition by PLP of the ability of thrombin to hydrolyze the synthetic substrate, tosyl arginine methyl ester (Fig. 3). These results indicated that PLP directly inhibits thrombin thereby decreasing the potency of thrombin as an aggregating agent.

Since PLP could inhibit ADP-induced platelet aggregation by interacting with platelet surface amino groups (5), we tested whether the inhibition by PLP of thrombin-induced aggregation could be attributed to a similar platelet surface interaction. Fig. 4 (a-c) shows that the resuspension in platelet-poor plasma of platelets previously treated with both PLP and $NaBH_4$ produced an irreversible inhibition of ADP-induced aggregation whereas such prior treatment of platelets did not inhibit thrombin-induced aggregation (Fig. 4 d-f). Our find-

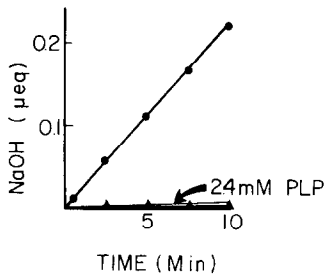

Fig. 3. Effect of pyridoxal phosphate (PLP) on the hydrolytic (esterase) activity of thrombin. Esterase activity was determined according to Sherry and Troll (1954) (13). TAME (p-tosyl-L-arginine methyl ester HCl) (40 mM) and saline (●) or 2.4 mM PLP (▲) was incubated at 40°C at pH 7.4. The addition of thrombin, at a final concentration of 2 units/ml, initiated the reaction. The extent of hydrolysis of TAME was followed on a recording pH Stat by measuring the addition of microequivalents (uEq) of 0.005N NaOH to maintain constant pH. The total volume was 4.05 ml.

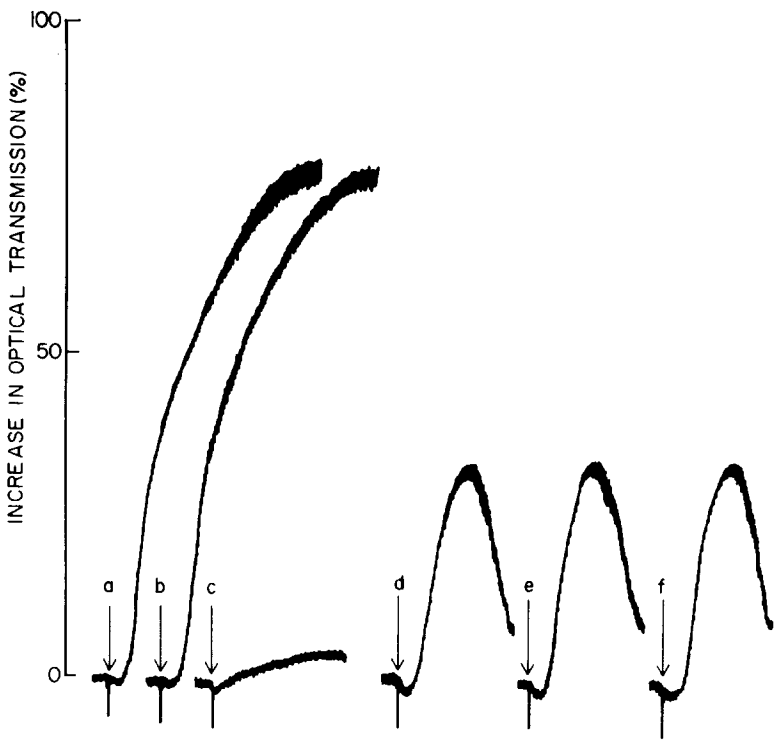

Fig. 4. Effect of ADP and thrombin on resuspended platelets previously treated with pyridoxal phosphate plus sodium borohydride. The curves show the aggregation induced by 10^{-5}M ADP (a,b,c) or thrombin (0.1 unit/ml) (d,e,f) after treatment of platelet-rich plasma followed by resuspension of platelets in platelet-poor plasma. Curves (a) and (d) indicate treatment with 0.15M NaCl; curves (b) and (e) indicate treatment with 4 mM pyridoxal phosphate; and curves (c) and (f) indicate treatment with 4 mM pyridoxal phosphate plus 5 mM sodium borohydride.

*ings therefore demonstrate that thrombin and ADP act on separate platelet re-*ceptor sites. In addition the results show that the formation of Schiff bases involving platelet surface amino groups does not interfere with the action of thrombin on platelets.

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