Platelet Receptor Recognition Site on Human Fibrinogen. Synthesis and Structure–Function Relationship of Peptides Corresponding to the Carboxy-Terminal Segment of the γ Chain†

Marek Kloczewiak, Sheila Timmons, Thomas J. Lukas, and Jacek Hawiger*

ABSTRACT: Binding of fibrinogen to human platelets depends on the interaction of the γ-chain carboxy-terminal segment with specific receptors exposed by different agonists such as ADP, epinephrine, and thrombin. The functions of a series of synthetic peptides encompassing the sequence of the 15 carboxy-terminal residues of the γ chain were investigated in this study. Both pentadecapeptide (γ397–411) and dodecapeptide (γ400–411) inhibited binding of ^{125}I-fibrinogen to ADP-treated platelets, with the concentration causing 50% inhibition (IC_{50}) being 28 μM. In comparison, decapeptide (γ402–411) was almost 4 times less active (IC_{50} = 106 μM), thus suggesting that the two histidine residues (γ400–401) are required for a full inhibitory effect. A heptapeptide (γ405–411) had a similar effect (IC_{50} = 102 μM) whereas a pentapeptide (γ407–411) was even less inhibitory (IC_{50} = 190 μM), indicating that the lack of lysine (γ406) further diminishes the reactivity of the platelet recognition site on the γ chain of human fibrinogen. The heptapeptide (γ400–406) containing two histidine residues and derived from the dodecapeptide by proteolytic degradation with trypsin had very low inhibitory activity. The synthetic peptides inhibited fibrinogen-supported platelet aggregation in the same order of decreasing reactivity: pentadecapeptide = dodecapeptide > decapeptide = heptapeptide > pentapeptide. Modified synthetic pentadecapeptides bearing tyrosine or cysteinyltyrosine at the amino terminal were prepared to provide a means for radiolabeling and for formation of molecules of higher valency. Tyrosyl-γ397–411 and the dimer cystinyl-(tyrosyl-γ397–411)$_2$ obtained by the formation of a disulfide bond between two single peptides had the same inhibitory activity toward the fibrinogen receptor on platelets. Radiolabeled tyrosyl-pentadecapeptide exhibited specific binding to human platelets which was inhibited by the dodecapeptide (γ400–411). A polyvalent conjugate of cysteinyl-tyrosyl-γ397–411 with human serum albumin was able to induce aggregation of ADP-stimulated platelets which was blocked by pentadecapeptide (γ397–411) or dodecapeptide (γ400–411). Furthermore, monospecific antibody Fab fragment directed against the peptide, encompassing residues γ385–411, partially inhibited the platelet-aggregating function of the synthetic pentadecapeptide–albumin conjugate. Thus a polyvalent peptide conjugate functioned as a synthetic fibrinogen substitute in the platelet aggregation system. In conclusion, the continuous sequence of the 12 amino acid residues at the carboxy-terminal end constitutes the platelet recognition site for the γ chain of human fibrinogen. This segment binds to specific platelet receptors and is involved in the aggregation of platelets.

Human fibrinogen, a clottable plasma protein (M_r 340 000), is composed of three pairs of nonidentical polypeptide chains, α, β, and γ, and serves as a substrate for thrombin (Doolittle, 1973). Fibrinogen is also one of two main adhesive plasma proteins required for platelet function, the other being von Willebrand factor (Shattil & Bennett, 1980). Binding of fibrinogen to its specific platelet receptor(s) is essential for the aggregation of platelets induced by a number of agonists such as ADP, epinephrine, and thrombin (Mustard et al., 1978; Marguerie et al., 1979; Bennett & Vilaire, 1979; Hawiger et al., 1980; Peerschke et al., 1980; Niewiarowski et al., 1981). The nature of the platelet receptor for fibrinogen has been attributed to glycoproteins IIb and IIIa forming a calcium-dependent complex on the membrane of stimulated platelets (Bennett et al., 1982; Kornecki et al., 1982) and in an isolated system (Leung & Nachman, 1982). On the other hand, localization of the platelet receptor recognition site on human fibrinogen has been attributed to the γ and α chains of human fibrinogen (Hawiger et al., 1982a) and pinpointed to the last 15 amino acid residues on the carboxy-terminal segment of the γ chain (Kloczewiak et al., 1982, 1983a). In the present study, the structure–function relationship of this segment of the γ chain was investigated in regard to its interaction with the platelet receptor. By synthesizing peptides of an increasing number of amino acid residues, patterned after the carboxy-terminal segment of the γ chain, we compared the role of these residues in the interaction with the platelet receptor. Thus, we sought to establish the minimal sequence of the carboxy-terminal segment of the γ chain required for its interaction with the platelet receptor and to determine which amino acid residues play a key role in its recognition. A preliminary account of these studies was published in abstract form (Kloczewiak et al., 1983b).

Materials and Methods

Human fibrinogen (Kabi, Sweden) was used. Na^{125}I was purchased from Amersham and *N*-tosyl-L-phenylalanine chloromethyl ketone–trypsin (TPCK–trypsin)[1] from Worthington. Boc-amino acids were from Peninsula (San Carlos,

† From the Division of Experimental Medicine, New England Deaconess Hospital and Harvard Medical School, Boston, Massachusetts 02215, and the Departments of Pathology, Medicine, and Pharmacology, Vanderbilt University and Howard Hughes Medical Institute, Nashville, Tennessee 37232. *Received June 29, 1983; revised manuscript received October 17, 1983.* This work was supported by research grants from the National Institutes of Health, U.S. Public Health Service (HL-30649, HL-30648, and GM 30861).

* Correspondence should be addressed to this author at the Division of Experimental Medicine, New England Deaconess Hospital and Harvard Medical School.

[1] Abbreviations: Boc, *tert*-butoxycarbonyl; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CNBr, cyanogen bromide; IC_{50}, concentration of peptide causing 50% inhibition of binding; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

0006-2960/84/0423-1767$01.50/0

CA). Dicyclohexylcarbodiimide (DCC), trifluoroacetic acid (TFA), and aminomethylpoly(styrene) (1% divinylbenzene) were from Pierce Chemical Co.

Peptide Synthesis. Peptides were synthesized by solid phase methods (Barany & Merrifield, 1980) in a manual shaker apparatus (Chipco Mfg., San Francisco, CA). The aminomethyl resin (0.45 μmol/g) was derivatized with Boc–Val-4-(oxymethyl)phenylacetic acid as described (Mitchell et al., 1976). The general solid phase synthesis protocol used the following: 50% TFA in dichloromethane for deprotection; 5% triethylamine for neutralizations; and a 2–3 fold excess of preformed Boc-amino acid symmetric anhydrides for coupling, except for glutamine and histidine where direct DCC-mediated couplings were used. The level of resin substitution, the completeness of coupling, and deprotections were measured by a quantitative ninhydrin reaction (Sarin et al., 1981). Protecting groups and the peptide–resin link were cleaved by reaction in liquid HF–anisole (9:1 v/v) for 1 h at 0 °C. After evaporation of HF, the resin was washed twice with anhydrous ethyl ether. Crude peptides were extracted with 10% acetic acid (3 × 15 mL) and freeze-dried. Lyophilized peptides were dissolved in 10% acetic acid, and insoluble material was filtered and purified by high-pressure liquid chromatography (HPLC). A Beckman 340 chromatograph with Beckman 112 pumps and a Whatman preparative column (Partisil 10 ODS-3 Magnum 9) were used. The column with adsorbed peptide was eluted with 0.1% TFA at 1.5 mL/min until the absorbency at 214 nm returned to the base line, and then a linear gradient of acetonitrile from 0 to 80% concentration with 0.1% TFA was applied for 100 min. The main peptide peak was collected and freeze-dried. The purity of isolated peptides was checked on an analytical HPLC (Ultrasphere ODS, 0.46 × 25 cm, Altex) column using the same solvent system. A linear acetonitrile gradient from 0 to 80% was applied for 40 min at a flow rate of 1 mL/min.

Cysteine-containing peptides were converted to cystine-cross-linked peptides by oxidation with potassium ferricyanide [$K_3Fe(CN)_6$]. Cysteine-containing peptide at a concentration of approximately 10 mg/mL dissolved in 0.2 M Tris-HCl buffer, pH 7.4, was mixed with a 2 molar excess of $K_3Fe(CN)_6$, shaken, and incubated at room temperature for 2 h. After acidification with acetic acid, the peptide was mixed with AG 1-X8 (Bio-Rad) resin to remove inorganic reagents. The supernatant was rechromatographed on the analytical column described above and the resulting main peptide peak collected and freeze-dried.

Labeling of Fibrinogen and Synthetic Peptides with ^{125}I. Fibrinogen and synthetic peptides containing tyrosine were iodinated with ^{125}I by using the standard iodine monochloride method of McFarlane (1963). The specific radioactivity of ^{125}I-fibrinogen was 3×10^7 cpm/mg and of ^{125}I-tyrosyl-pentadecapeptide was 1.1×10^6 cpm/mg.

Amino Acid Analysis. Amino acid analyses of synthetic peptides were performed on an LKB 4400 automated amino acid analyzer. The peptide samples were hydrolyzed at 108 °C with 6 N hydrochloric acid for 24 h. The cystine concentration was determined by oxidation of the cystinyl-(tyrosyl-γ397–411)$_2$ peptide with performic acid as described by Hirs (1967).

Concentrations of the peptide stock solutions were determined by amino acid analysis.

Enzymatic Digestion of Synthetic Dodecapeptide. The dodecapeptide (11 mg) dissolved in 1 mL of 0.1 M Tris-HCl buffer, pH 8.0, was degraded with 0.1 mg of TPCK–trypsin for 24 h at 37 °C. The reaction was stopped by the addition of 0.1 mL of glacial acetic acid. The whole mixture of degradation products was applied on an analytical reverse-phase C18 HPLC column. The linear gradient of acetonitrile in 0.1% TFA (0.32% of acetonitrile/min) was applied until elution of the first broad peak containing peptide material ended (approximately 60 min). Three pools from the initial, middle, and last part of the peak were collected and analyzed for amino acid composition.

Acetylation of Synthetic Dodecapeptide. A 0.2-mL aliquot of 0.5 mM dodecapeptide in 0.05 M Na_2HPO_4, pH 8.0, was reacted for 1 h with 1 μL of acetic anhydride at room temperature. The reaction mixture was acidified with 0.02 mL of glacial acetic acid and applied on an analytical HPLC column (reverse-phase C18 column described above). An acetonitrile gradient (2%/min) in 0.1% TFA was applied for 40 min. Elution times of modified and nonmodified dodecapeptides were 16 and 14 min, respectively. Reaction of the modified peptide with trinitrobenzenesulfonic acid (Mokrash, 1967) showed that approximately 80% of its amino residues were acetylated.

Calculation of Average Hydrophobicity. The distribution of hydrophobic groups (average hydrophobicity) in the sequence of the γ chain (Henschen et al., 1980) was calculated by the method of Rose (1978). From these data, values for the 27 carboxy-terminal residues of human fibrinogen γ chain were obtained.

Preparation of the Polyvalent Synthetic Pentadecapeptide–Albumin Conjugate. Human serum albumin (Miles) dissolved (10 mg/mL) in 0.1 M Na_2HPO_4, pH 8.0, was mixed with a 10-fold molar excess of a 17.9 mM solution of *N*-succinimidyl [(*p*-azidophenyl)dithio]propionate (Pierce) in dioxane in order to introduce additional sulfhydryl residues. Reaction was carried out at 4 °C in the dark for 1 h. The mixture was dialyzed in the dark against several changes of distilled water with 1 mM mercaptoethanol and then against water. At this stage of the procedure, the protein concentration was determined with Bradford reagent (Bradford, 1976). The content of sulfhydryl residues was determined with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959). Approximately two sulfhydryl residues were introduced per albumin molecule. The synthetic peptide cysteinyl-tyrosyl-γ397–411 was added to the modified albumin in 10-fold molar excess in 0.05 M Tris-HCl buffer, pH 7.4. Free sulfhydryl residues were oxidized with potassium ferricyanide as described above. After oxidation, the whole mixture was acidifed to pH 3 with acetic acid and mixed for a few minutes with AG 1-X8 resin to remove organic reagents. After the resin was spun down, the supernatant was dialyzed against 0.15 M NaCl. A control sample of albumin without peptide was processed in the same way. No free sulfhydryl residues could be detected in oxidized conjugates or in the control sample.

Binding Studies. Human platelets were separated from plasma proteins by stepwise albumin gradient centrifugation and Sepharose 2B gel filtration of platelet-rich plasma (Timmons & Hawiger, 1978). All experiments were performed with platelets suspended in 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) balanced salt buffer, pH 7.35, containing 0.1% dextrose and 0.35% albumin. Binding of ^{125}I-fibrinogen or ^{125}I-labeled tyrosyl-pentadecapeptide and inhibition of binding to platelets were done at room temperature without stirring in a final volume of 0.5 mL which contained 1×10^8 platelets.

Fitting Inhibition Curves of Binding. Inhibition curves of ^{125}I-fibrinogen binding to platelets by dodeca-, deca-, hepta-, and pentapeptides were fitted to the equation $y = 100 \exp[-a$

Table I: Amino Acid Sequence of the Carboxy-Terminal Segment of Human Fibrinogen γ Chain and Designed Sequences of Synthetic Peptides[a]

	CNBr ↓ SP ↓ FXIII ↓ FXIII ↑
γ384-411	M-K-I-I-P-F-N-R-L-T-I-G-E-G-Q-Q-H-H-L-G-G-A-K-Q-A-G-D-V-COOH (25 20 15 10 5)
γ397-411	G-Q-Q-H-H-L-G-G-A-K-Q-A-G-D-V-COOH
Y-γ397-411	Y-G-Q-Q-H-H-L-G-G-A-K-Q-A-G-D-V-COOH
C-Y-γ397-411	C-Y-G-Q-Q-H-H-L-G-G-A-K-Q-A-G-D-V-COOH
	Tr ↓
γ400-411	H-H-L-G-G-A-K-Q-A-G-D-V-COOH
γ400-406[b]	H-H-L-G-G-A-K
γ402-411	L-G-G-A-K-Q-A-G-D-V-COOH
γ405-411	A-K-Q-A-G-D-V-COOH
γ407-411	Q-A-G-D-V-COOH

[a] The sequence of γ384-411 is based on data reported by Henschen et al. (1980); arrows show the positions of residues involved in CNBr cleavage and enzymatic reaction with factor XIIIa (FXIII), staphylococcal protease (SP), and trypsin (Tr). [b] Tryptic fragment of γ400-411.

Table II: Amino Acid Composition of Synthetic Peptides of Sequences Corresponding to the Carboxy-Terminal Segment of the γ Chain[a]

	peptide					
amino acid	penta (488.0)	hepta (687.0)	deca (914.0)	dodeca (1188.1)	pentadeca (1501.1)	tyrosyl-pentadeca (1664.2)
aspartic acid	1.00	0.94	1.01	1.04	1.06	1.00
glutamic acid	1.05	0.95	1.04	1.07	2.82	3.16
glycine	0.99	1.03	3.11	3.12	4.16	3.91
alanine	0.99	1.83	1.94	2.06	1.86	2.01
valine	0.99	0.92	0.98	1.08	0.95	1.07
leucine		0.02	0.99	1.00	0.94	1.04
tyrosine		0.02	0	0	0	0.75
histidine		0.10	0.01	1.70	1.81	2.06
lysine		0.91	0.98	0.95	0.95	1.06
	5[b]	7[b]	10[b]	12[b]	15[b]	16[b]

[a] Values are given in moles of amino acid per mole of the peptide; molecular weights calculated from the amino acid sequence are given in parentheses beneath the name of the peptide. [b] Total residues rounded to the nearest integer.

exp(−*bc*)] describing the growth function. In this equation, *a* and *b* were the parameters to be fitted, *c* was the concentration of the peptide (micromolar), and *y* was the inhibition of fibrinogen binding (percent). The function to be minimized was the sum of the squares of residuals. The computer program based on Rosenbrock's search multivariable minimization algorithm (Rosenbrock, 1968) was used for this purpose.

Platelet Aggregation. Platelet aggregation was carried out by using human platelets separated from the plasma proteins as in binding studies (see above) and as described previously (Kloczewiak et al., 1982). The staphylococcal clumping reaction with human fibrinogen in the presence of synthetic peptides was performed as described (Hawiger et al., 1982b).

Preparation of Fab Antibody Fragments against Peptide γ385–411. Fibrinogen and fragment D_1, which were prepared as previously described (Kloczewiak et al., 1982), were coupled to aminohexyl-Sepharose 4B (Pharmacia) in 0.1 M phosphate buffer, pH 8.5, with dimethyl adipimidate (Pierce) (Smith & Loh, 1978).

The CNBr-derived 27 carboxy-terminal residue peptide (γ385–411) was prepared as previously described (Kloczewiak et al., 1982) and coupled to AH-Sepharose 4B (12 mg of peptide per 15 mL of AH-Sepharose 4B) with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (Sigma) at pH 4.5 (Cuatrecasas, 1970).

Sera from rabbits immunized with fragment D_1 of human fibrinogen were passed through a fragment D_1–Sepharose 4B affinity column to isolate monospecific antibodies against fragment D_1. Antibodies were eluted with 0.1 M glycine hydrochloride buffer, pH 2.4. After neutralization, they were applied to an affinity column containing peptide γ385–411 coupled to Sepharose 4B, equilibrated with 0.05 N Tris-HCl buffer with 0.3 M NaCl, pH 7.4. Approximately 30% of anti-fragment D_1 antibody was bound to this column. Fab fragments were obtained by proteolytic degradation with papain and subsequent ion-exchange chromatography on a CM-Sepharose CL-4B column in conditions described by Porter (1959). A pool of Fab fragments was passed through the fibrinogen–Sepharose 4B column to isolate the population of Fab molecules which binds to fibrinogen.

Results

Chemical Characterization of Synthetic Peptides. The carboxy-terminal 27-residue peptide derived from cyanogen bromide cleavage of human fibrinogen γ chain was shown to bear the recognition site for the platelet receptor (Kloczewiak et al., 1982). Subsequently, we isolated a pentadecapeptide resulting from digestion of the parent 27-residue peptide with staphylococcal protease (Kloczewiak et al., 1983a). This peptide had a similar reactivity toward the platelet receptor for fibrinogen. Therefore, we synthesized a series of peptides, patterned after the known sequence of the carboxy-terminal segment of the human fibrinogen γ chain (Henschen et al., 1980), designed to delineate the functional role of key amino acid residues in reactivity toward the platelet receptor (Table I).

The amino acid compositions of synthetic peptides containing 5, 7, 10, 12, and 15 amino acid residues of the sequence starting with the carboxy-terminal valine (γ411) are given in Table II. They were in excellent agreement with data pre-

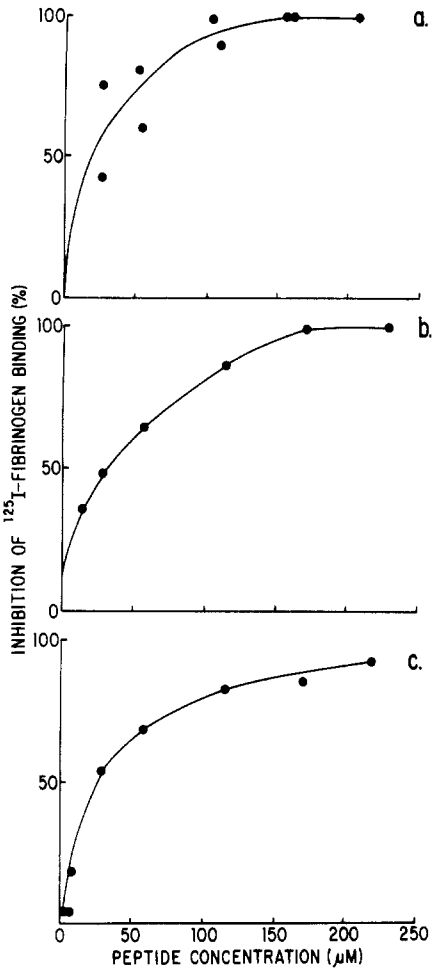


FIGURE 1: Inhibitory effect of synthetic peptides on binding of ^{125}I-fibrinogen (0.17 μM) to human platelets (1×10^8 cells/0.5 mL) which were treated with ADP (5 μM). (a) Inhibition curve for γ397–411; (b) inhibition curve for tyrosyl-γ397–411; (c) inhibition curve for cystinyl-(tyrosyl-γ397–411)$_2$. Inhibition of ^{125}I-fibrinogen binding was calculated after subtracting the value for binding of ^{125}I-fibrinogen to platelets which were not treated with ADP. The inhibitory concentration of tested peptides causing 50% inhibition of ^{125}I-fibrinogen binding (IC_{50}) is similar for the three peptides.

dicted from the known sequence of the γ chain (Henschen et al., 1980). In addition to these peptides, two synthetic peptides containing tyrosine or cysteinyltyrosine at the amino terminal were prepared (Tables I and II). The amino acid analysis of the performic acid oxidized cystinyl-(tyrosyl-γ397–411)$_2$ peptide showed the presence of one cysteine residue per molecule of pentadecapeptide (data not shown), in addition to the residues present in tyrosyl-pentadecapeptide (Table II).

Solid phase sequencing of the protected pentadecapeptide also showed the correct sequence with two histidines (data not shown).

Analytical HPLC chromatography of purified peptide showed the presence of one peptide component in each preparation. The only exception was the pentapeptide which showed one major peak and a very closely eluting second minor peak. The possible explanation of this apparent heterogeneity could be partial cyclization of the amino-terminal glutamyl residue which would change the polarity of the peptide.

Effect of Synthetic Peptides on the Binding of ^{125}I-Fibrinogen to the Platelet Receptor. The inhibitory potency of all seven synthetic peptides toward the binding of ^{125}I-fibrinogen to the platelet receptor was determined. The concentration of each peptide causing 50% inhibition (IC_{50}) of ^{125}I-fibrinogen binding was derived from binding inhibition

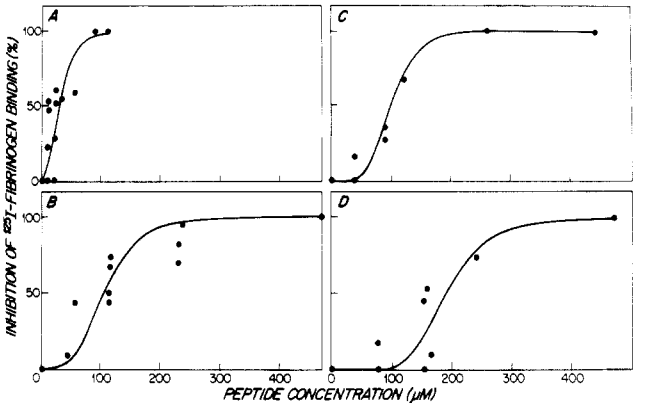


FIGURE 2: Concentration-dependent inhibition of ^{125}I-fibrinogen (0.17 μM) binding to human platelets (1×10^8/0.5 mL) by synthetic peptides of decreasing length corresponding to the carboxy-terminal sequence of the γ chain: dodecapeptide (γ400–411) (A); decapeptide (γ402–411) (B); heptapeptide (γ405–411) (C); pentapeptide (γ407–411) (D). See the legend of Figure 1 for calculation of the inhibition of binding, and see the text for values corresponding to IC_{50} for each tested peptide and for the curve-fitting equation.

curves as shown in Figures 1 and 2.

Inhibition of ^{125}I-fibrinogen binding to its receptor on human platelets was concentration dependent. Pentadecapeptide (γ397–411) was inhibitory with IC_{50} = 28 μM a value similar to that for a related peptide possessing an additional tyrosine residue (tyrosyl-γ397–411). Likewise, addition of a cysteine residue to this peptide to form a cystine-linked dimeric peptide, cystinyl-(tyrosyl-γ397–411)$_2$, did not change significantly the inhibitory potency of such a peptide (Figure 1).

The group of synthetic peptides of decreasing length was analyzed in terms of their inhibitory potency toward binding of ^{125}I-fibrinogen to the platelet receptor (Figure 2). The dodecapeptide (γ400–411) had IC_{50} = 28 μM, identical with that of the pentadecapeptide. The inhibitory potency of other peptides decreased with shortening of the peptide length. The decapeptide (γ402–411) was almost 4 times less potent (IC_{50} = 106 μM) than the dodecapeptide (γ400–411). The heptapeptide (γ405–411) had IC_{50} = 102 μM, similar to the decapeptide, but the pentapeptide (γ407–411) was less reactive (IC_{50} = 190 μM). Whereas the dodecapeptide inhibited completely ^{125}I-fibrinogen binding at 80–100 μM, the pentapeptide required a much higher concentration (500 μM) for complete inhibition.

Experimental data points, with a sigmoidal growth function fitted to them (shown in Figure 2), were derived from at least three independent experiments. Theoretical curves presented were obtained for one of the local minimum set of parameters *a* and *b* (see Materials and Methods for details). The inherent variability of platelets from different individuals contributed to a wider scatter of data points, thus preventing us from obtaining the absolute minimum during the search minimization process. However, in all of the cases, *a* and *b* sets gave very similar IC_{50} values; the basic differences were the slopes of the theoretical curves.

Acetylation of dodecapeptide (γ400–411) abolished its inhibitory activity. At a concentration of 80 μM, no inhibition of fibrinogen binding could be observed (results not shown).

Enzymatic Degradation of Dodecapeptide. Since the dodecapeptide (γ400–411) has one bond susceptible to trypsin, enzymatic degradation was done to see whether the resulting smaller peptides retained reactivity toward the platelet receptor. Amino acid analysis of the three collected pools is presented in Table III. Chromatography on the same analytical HPLC column showed (gradient of acetonitrile 2%/

Table III: Amino Acid Compositions of Three Pools from the Tryptic Hydrolysate of the Carboxy-Terminal Dodecapeptide Compared with Theoretical Compositions Derived from the Known Sequence γ400–411[a]

	pool 1		pool 2		
			mol/M_r 718.1		
amino acid residue	mol/ M_r 488.0	γ407–411	2A	2B	γ400–406
aspartic acid	0.78	1	0.04	0.06	0
glutamic acid	0.81	1	0.03	0.06	0
glycine	0.80	1	1.99	2.04	2
alanine	0.97	1	1.04	1.04	1
valine	0.77	1	0.04	0.06	0
leucine	0.21	0	1.05	1.03	1
histidine	0.38	0	1.92	1.88	2
lysine	0.21	0	1.03	1.08	1
		5[b]			7[b]

[a] Values are given in moles of amino acid per mole of the peptide corresponding to the known sequence (Henschen et al., 1980); molecular weights are based on the known sequence. [b] Total.

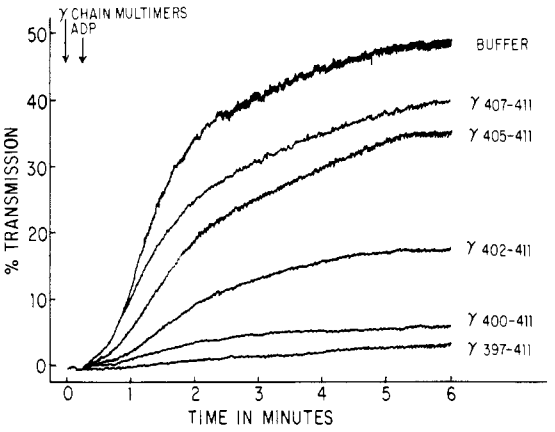


FIGURE 3: Inhibition of γ-chain-induced (7 μM) platelet aggregation in the presence of ADP (5 μM) by synthetic peptides (60 μM) of decreasing length as indicated at each aggregation tracing. Platelets (1×10^8/0.5 mL) separated from plasma proteins were tested in an aggregometer at 37 °C with stirring (900 rpm).

min) that the elution time of pool 1 was 12.0 min and that of the synthetic pentapeptide (γ407–411) was 12.3 min. Elution times of pools 2A and 2B were 12.8 and 13 min, respectively, which was different from the elution time of the synthetic dodecapeptide (14 min).

Data obtained from HPLC elution times and from amino acid analysis (Table III) proved that pool 1 contains peptide(s) encompassing five carboxy-terminal residues of the dodecapeptide. It is probably partially contaminated with peptide from pool 2A which corresponds to the seven-residue amino-terminal part of the dodecapeptide (Table I). Pool 2B contains the same amino-terminal heptapeptide. The inhibitory activity of these peptides (pools 1, 2A, and 2B) toward binding of ^{125}I-fibrinogen to the platelet receptor was measured. None exhibited activity comparable to the starting dodecapeptide (γ400–411). In particular, the seven-residue amino-terminal peptide which corresponds, in terms of its composition, to γ400–406 had low inhibitory activity at a concentration of 1 mM.

Effect of Synthetic Peptides on Platelet Aggregation. The pentadecapeptide (γ397–411) and dodecapeptide (γ400–411) at equimolar concentrations (60 μM) fully inhibited ADP-induced γ-chain-dependent aggregation of platelets as judged by the slope and T_{max} values of aggregation tracings (Figure 3). The aggregation system contained 60 μM albumin. The inhibitory effect of the decapeptide (γ402–411, 60 μM) was apparently diminished when compared to the effect of the

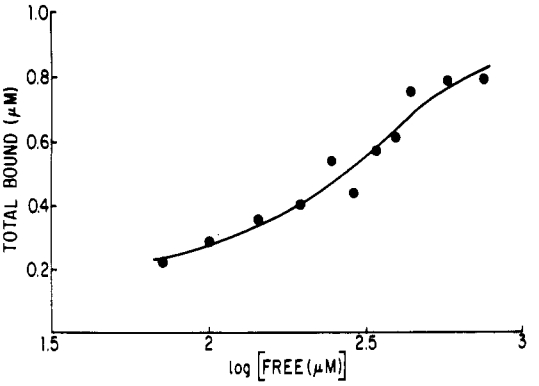


FIGURE 4: Concentration-dependent binding of ^{125}I-tyrosyl-pentadecapeptide to ADP-stimulated (10 μM) platelets (1×10^8/0.5 mL). Results are presented as the concentration of total peptide bound vs. the logarithm of the free peptide concentration (Klotz, 1982).

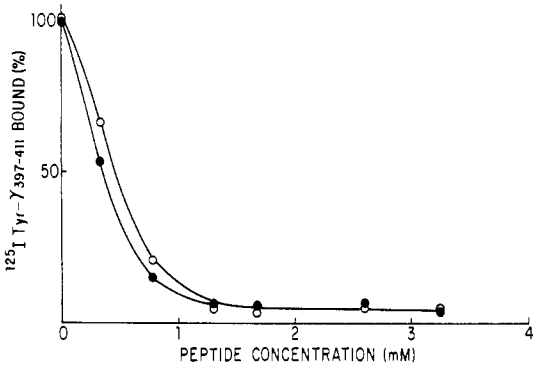


FIGURE 5: Concentration-dependent inhibition of binding of ^{125}I-tyrosyl-γ397–411 (80 μM) to ADP (○) and thrombin (●) stimulated platelet (1×10^8/0.5 mL) by the dodecapeptide (γ400–411).

dodecapeptide. The heptapeptide (γ405–411) and pentapeptide (γ407–411), both used at concentrations of 60 μM, inhibited platelet aggregation to a much lesser degree (Figure 3).

Direct Binding of ^{125}I-Labeled Tyrosyl-Pentadecapeptide to Platelet Receptor and Its Inhibition by Dodecapeptide. The binding of radiolabeled peptide was concentration dependent (Figure 4). Data shown as bound ligand concentration plotted against the logarithm of free ligand concentration according to Klotz (1982) produced a sigmoid curve with an inflection point corresponding to the dissociation constant (K_d) of 3×10^{-4} M. This binding was specific as determined by inhibition with unlabeled dodecapeptide (γ400–411) using ADP- or thrombin-stimulated platelets (Figure 5). In this experiment, 0.88 nmol of ^{125}I-tyrosyl-pentapeptide bound to 10^8 platelets. Dodecapeptide in concentrations of 460 and 360 μM inhibited 50% binding (IC_{50}) of ^{125}I-tyrosyl-pentadecapeptide (80 μM) induced by ADP and thrombin, respectively.

Platelet Aggregating Function of the Polyvalent Synthetic Pentadecapeptide–Albumin Molecule. Assuming that the platelet aggregating function of human fibrinogen requires more than one receptor recognition site, we conjugated a synthetic peptide, cysteinyl-tyrosyl-γ397–411, to human serum albumin to prepare a polyvalent substitute. The peptide–albumin conjugate added to platelets separated from plasma proteins caused platelet aggregation in the presence of 10 μM ADP (Figure 6). In the absence of ADP, the peptide–albumin conjugate did not aggregate platelets. Aggregation of ADP-treated platelets by a polyvalent synthetic peptide–albumin conjugate at a concentration of 2.5 μM was prompt and biphasic. It was abolished by the prior addition of unconjugated monovalent pentadecapeptide (Figure 6) or dodecapeptide

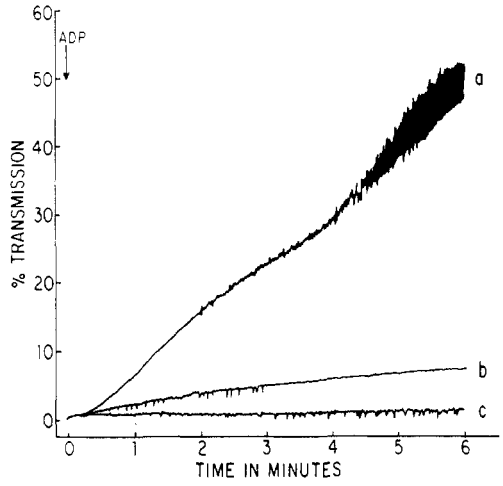


FIGURE 6: Aggregation of ADP-treated platelets induced by the cysteinyl-tyrosyl-pentadecapeptide–albumin conjugate (2.5 μM) in the presence of buffer (a), in the presence of pentadecapeptide (γ397–411, 60 μM) (c), and in the presence of monospecific antibody Fab fragment against γ385–411 (1.8 μM) (b). For details of platelet aggregation, see the legend to Figure 3.

(results not shown in Figure 6). A parallel control of cross-linked albumin, prepared in the same way, did not aggregate ADP-treated platelets.

To confirm the specificity of the interaction of the polyvalent synthetic pentadecapeptide–albumin conjugate with platelets, monovalent Fab antibody fragments directed against the carboxy-terminal segment of the human fibrinogen γ chain (γ385–411) were used. These antibody Fab fragments at a concentration of 1.8 μM completely blocked binding of ^{125}I-fibrinogen (0.17 μM) to ADP-stimulated platelets (results not shown). They inhibited substantially aggregation of ADP-treated platelets induced by a polyvalent conjugate of synthetic pentadecapeptide with albumin (Figure 6).

Distribution of Hydrophilic and Hydrophobic Groups in the γ-Chain Segment Bearing the Platelet Receptor Recognition Site. Calculation of the average hydrophobicity of the segment of the γ chain encompassing the platelet receptor recognition site can provide useful information about its nature. Whereas the preceding segment of the γ chain encompassing 10 residues (γ385–394) is highly hydrophobic, having an average hydrophobicity substantially higher than the calculated mean value for the whole γ chain (1.17 kcal/mol), the 15-residue carboxy-terminal segment bearing the platelet receptor recognition site is much more hydrophilic, with the notable exception of valine at the carboxy terminus of the γ chain (Figure 7).

Discussion

Our understanding of the mechanism of the fibrinogen–platelet interaction depends on defining the recognition site for the platelet receptor on human fibrinogen and on determining the chemical nature of platelet receptors. Earlier experiments indicated that the main site interacting with platelet receptors is present on the γ chain of human fibrinogen and that a much less reactive site is localized on the α chain (Hawiger et al., 1982a). We showed that the γ-chain site was encompassed within the 27-residue carboxy-terminal fragment resulting from cyanogen bromide cleavage (Kloczewiak et al., 1982). The staphylococcal protease generated 15-residue carboxy-terminal peptide retained the platelet receptor recognition site, whereas it was without effect on the polymerization of fibrin monomer (Kloczewiak et al., 1983a). With this information at hand, we synthesized a series of peptides

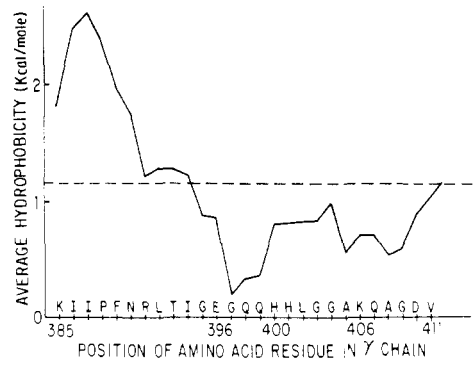


FIGURE 7: Pattern of average hydrophobicity in the γ chain carboxy-terminal segment (γ385–411). The broken line represents the middle value of the average hydrophobicity of the whole γ chain.

encompassing the carboxy-terminal segment of the γ chain. This segment of the γ chain is endowed with other important biological functions such as the cross-linking donor and acceptor sites for the factor XIIIa catalyzed reaction (Chen & Doolittle, 1971) and the recognition site for the staphylococcal clumping receptor (Strong et al., 1982). The latter characteristic aided us in the design and screening of synthetic peptides active in the staphylococcal clumping test prior to, or parallel with, checking their effect on the binding of ^{125}I-fibrinogen to platelets.

The data presented herein indicate that the most reactive synthetic peptides were a pentadecapeptide (γ397–411) and a dodecapeptide (γ400–411), both of which inhibited ^{125}I-fibrinogen binding at an IC_{50} of 28 μM. These compare favorably to results with the natural γ-chain fragment encompassing residues 397–411 derived from proteolysis (IC_{50} = 38 μM). The lack of two glutamine residues (γ398–399) in the dodecapeptide apparently did not diminish the reactivity of this peptide toward the fibrinogen receptor on human platelets. Since one of these two glutamine residues is the acceptor site for factor XIIIa catalyzed cross-linking of the γ chain (Chen & Doolittle, 1971), the high reactivity of the dodecapeptide indicates that enzymatic cross-linking is not responsible for the interaction of the pentadecapeptide with platelets.

Synthesis of modified peptides with tyrosine or cysteinyl-tyrosine residues attached to the amino-terminal end of the pentadecapeptide was aimed to accomplish the following two goals: (1) iodination of the peptide to facilitate the study of direct binding of the peptide to platelets and (2) easy construction of derivatized peptides. Oxidation of the cysteinyl-tyrosyl-pentadecapeptide leads to the formation of the dimeric molecule cystinyl-(tyrosyl-γ397–411)$_2$ which could enhance the inhibitory activity or interact more tightly with the platelet receptor. Our results with such a divalent peptide indicate that its reactivity toward the platelet receptor has not been increased.

The reactivity of the carboxy-terminal segment of the γ chain toward platelet receptors depends to a significant extent on two histidine residues (His-400 and His-401). Removal of these residues leads to a pronounced decrease in inhibitory activity. However, histidine residues are not absolutely required for the inhibitory activity. Even the short pentapeptide possessed inhibitory activity (IC_{50} = 190 μM) stronger than the unrelated tetrapeptide, Gly-Pro-Arg-Pro (IC_{50} = 2000 μM),[2] which corresponds to data for this unrelated tetrapeptide

[2] S. Timmons, M. Kloczewiak, J. Hawiger, and R. F. Doolittle, unpublished results.

previously reported by Plow & Marguerie (1982). It seems reasonable to assume that the biological activity of the carboxy-terminal segment of the γ chain depends mainly on the histidine residues 400–401, but the interaction with the receptor for fibrinogen on platelets involves other functional residues situated along the length of the dodecapeptide such as lysine-406 and valine-411. According to findings reported previously by Strong et al. (1982), no inhibitory effect of five- and seven-residue peptides in the interaction with the staphylococcal clumping receptor was observed, while the longer peptides were inhibitory.

Results with ^{125}I-tyrosyl-pentadecapeptide (Figures 4 and 5) showed that binding of the peptide is saturable and could be displaced by the unlabeled dodecapeptide. From these preliminary data, we can estimate that the dissociation constant for the peptide–platelet interaction will be approximately 3×10^{-4} M, which is 3 orders of magnitude higher than the dissociation constant for the fibrinogen–platelet interaction (Marguerie et al., 1979; Bennett & Vilaire, 1979; Hawiger et al., 1980).

It is important to note that the pentadecapeptide in both forms, monomeric (γ397–411) and dimeric [cystinyl-(tyrosyl-γ397–411)$_2$], did not induce platelet aggregation although direct and specific binding of ^{125}I-tyrosyl-pentadecapeptide has been demonstrated in this study. However, polyvalency was achieved by coupling the synthetic peptide cysteinyl-tyrosyl-γ397–411 to human serum albumin. This endowed the conjugate with a platelet aggregating function mimicking that of the native human fibrinogen molecule. Because platelet aggregation induced by the conjugate was blocked by monovalent pentadecapeptide or dodecapeptide as well as by monospecific antibody Fab fragments directed against the carboxy-terminal segment of human fibrinogen γ chain (γ385–411), we conclude that the minimal structure of the γ chain encompassing residues 400–411 is involved in the aggregating function of human fibrinogen.

The platelet receptor reactive residues encompass a highly hydrophilic part of the γ chain which was postulated by Chen & Doolittle (1971) to be exposed to the surrounding solvent, thus being easily accessible to enzymes (factor XIII, plasmin, and staphylococcal protease) and to staphylococcal clumping factor receptor (Hawiger et al., 1982b; Strong et al., 1982). The predicted secondary structure (data not shown) indicates that the neighboring hydrophobic segment (γ385–390) forms a stretch of continuous β structure whereas the hydrophilic part has no definite secondary structure. It may be possible that a loop fitting the platelet receptor is formed and stabilized by the salt-bridge formation between the ϵ-amino residue of Lys-406 and the carboxyl residue of Val-411 (or Asp-410). Additional experimental evidence favoring this hypothesis is that modification of lysine (Lys-406) with acetic anhydride abolished the inhibitory reactivity of the dodecapeptide. Whether functional reactivity of the platelet recognition site at the carboxy-terminal end of the γ chain is influenced by the additional sequence present in the γ' variant of human fibrinogen (Francis et al., 1980; Wolfstein-Todel & Mosesson, 1980) remains to be established.

In summation, our structure–function studies utilizing a family of synthetic peptides indicate that the site recognizing the platelet receptor is localized on the 12 carboxy-terminal residues of the γ chain of human fibrinogen. The structural continuity of this segment is essential for the optimal interaction of human fibrinogen with the platelet receptor.

Acknowledgments

We acknowledge the advice and support for this study by Russell F. Doolittle and D. Martin Watterson. The excellent assistance of Kristina A. Lyscars in the preparation of the manuscript is also appreciated.

Registry No. γ397–411, 80755-86-8; Y-γ397–411, 89088-46-0; C-Y-γ397–411 dimer, 89088-47-1; γ400–411, 89105-94-2; γ402–411, 89088-48-2; γ405–411, 89088-49-3; γ407–411, 80755-85-7.

References

Barany, G., & Merrifield, R. B. (1980) in *The Peptides, Analysis, Synthesis, Biology* (Gross, E., & Meinhoper, J., Eds.) Vol. 2, pp 1–284, Academic Press, New York.
Bennett, J. S., & Vilaire, G. (1979) *J. Clin. Invest. 64*, 1393.
Bennett, J. S., Vilaire, G., & Cines, D. B. (1982) *J. Biol. Chem. 257*, 8049.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Chen, R., & Doolittle, R. F. (1971) *Biochemistry 10*, 4486.
Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059.
Doolittle, R. F. (1973) *Adv. Protein Chem. 27*, 1.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 7a.
Francis, C. W., Marder, V. J., & Martin, S. E. (1980) *J. Biol. Chem. 255*, 5599.
Hawiger, J., Parkinson, S., & Timmons, S. (1980) *Nature (London) 283*, 5743.
Hawiger, J., Timmons, S., Kloczewiak, M., Strong, D. D., & Doolittle, R. F. (1982a) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2068.
Hawiger, J., Timmons, S., Strong, D. D., Cotrell, B. A., Riley, M., & Doolittle, R. F. (1982b) *Biochemistry 21*, 1407.
Henschen, A., Lottspeich, F., Southan, C., & Topfer, P. E. (1980) in *Proteins of the Biological Fluids* (Peeters, H., Ed.) pp 51–56, Pergamon Press, Oxford.
Hill, A. V. (1910) *J. Physiol. (London) 40*, 190.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 59.
Kloczewiak, M., Timmons, S., & Hawiger, J. (1982) *Biochem. Biophys. Res. Commun. 107*, 181.
Kloczewiak, M., Timmons, S., & Hawiger, J. (1983a) *Thromb. Res. 29*, 249.
Kloczewiak, M., Timmons, S., Lukas, T., & Hawiger, J. (1983b) *Clin. Res. 31*, 534A.
Klotz, I. M. (1982) *Science (Washington, D.C.) 217*, 1247.
Kornecki, E., Tuszynski, G. P., & Niewiarowski, S. (1982) *Circulation, Suppl. 66*, 51.
Leung, L. L. K., & Nachman, R. L. (1982) *J. Clin. Invest. 78*, 542.
Lineweaver, M., & Burk, D. (1934) *J. Am. Chem. Soc. 56*, 658.
Marguerie, G. A., Plow, E. F., & Edgington, T. S. (1979) *J. Biol. Chem. 254*, 5357.
McFarlane, A. S. (1963) *J. Clin. Invest. 42*, 346.
Mitchell, A. R., Erickson, B. W., Ryabstav, M. N., Hodges, R. S., & Merrifield, R. B. (1976) *J. Am. Chem. Soc. 98*, 7357.
Mokrash, L. C. (1967) *Anal. Biochem. 18*, 64.
Mustard, J. F., Packham, M. A., Kinlough-Rathbone, R. L., Perry, D. W., & Rogoeczi, E. (1978) *Blood 52*, 453.
Niewiarowski, S., Budzynski, A. Z., Morinelli, R. A., Brudzynski, T. M., & Stewart, G. J. (1981) *J. Biol. Chem. 256*, 917.
Peerschke, E. I., Zucker, M. S., Grant, R. A., Egan, J. J., & Johnson, M. M. (1980) *Blood 55*, 841.
Plow, E. F., & Marguerie, G. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3711.
Porter, R. R. (1959) *Biochem. J. 73*, 119.
Rose, G. D. (1978) *Nature (London) 272*, 586.
Rosenbrock, H. H. (1960) *Comput. J. 3*, 175.

Sarin, V. K., Kent, S. B. H., Tam, J. P., & Merrifield, R. B. (1981) *Anal. Biochem. 117*, 147.
Shattil, S. J., & Bennett, J. S. (1980) *Ann. Intern. Med. 94*, 108.
Smith, A. P., & Loh, M. M. (1978) *Biochemistry 17*, 1761.
Strong, D. D., Laudano, A. P., Hawiger, J., & Doolittle, R. F. (1982) *Biochemistry 21*, 1414.
Timmons, S., & Hawiger, J. (1978) *Thromb. Res. 12*, 297.
Wolfstein-Todel, C., & Mosesson, M. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5069.

Reaction Mechanism of Phosphoenolpyruvate Carboxylase. Bicarbonate-Dependent Dephosphorylation of Phosphoenol-α-ketobutyrate†

Nobuyuki Fujita, Katsura Izui, Tokuzo Nishino, and Hirohiko Katsuki*

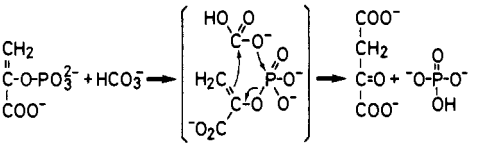
ABSTRACT: Phosphoenolpyruvate carboxylase (EC 4.1.1.31) of *Escherichia coli* was found to catalyze the cleavage reaction of phosphoenol-α-ketobutyrate, a potent competitive inhibitor with the substrate, to yield inorganic phosphate and α-ketobutyrate. The rate of phosphate liberation was about $^1/_{20}$th of that in the normal reaction with phosphoenolpyruvate. Although HCO_3^- and Mg^{2+} were the necessary components in this reaction as in the normal reaction, no CO_2 fixation could be detected. When the reaction was carried out in the presence of [^{18}O]HCO_3^-, multiple incorporations of ^{18}O atoms into the liberated phosphate molecule were observed. The molar proportions of phosphate having one, two, and three ^{18}O atoms were 70, 25, and 5%, respectively. No multiple but only one ^{18}O atom incorporation was observed when phosphoenolpyruvate was used as a substrate. These results suggest that the liberation of phosphate can proceed without CO_2 fixation, being not consistent with the concerted mechanism [Maruyama, H., Easterday, R. L., Chang, H. C., & Lane, M. D. (1966) *J. Biol. Chem. 241*, 2405–2412] but essentially consistent with the current stepwise mechanism [O'Leary, M. H., Rife, J. E., & Slater, J. D. (1981) *Biochemistry 20*, 7308–7314].

Phosphoenolpyruvate (PEP)[1] carboxylase (EC 4.1.1.31) catalyzes the following reaction in the presence of a divalent metal ion: PEP + HCO_3^- → oxaloacetate + P_i. The reaction is highly exergonic (Tchen et al., 1955), and the reverse reaction is not demonstrated. This enzyme, which is widespread in all higher plants and many kinds of bacteria (Utter & Kolenbrander, 1972), plays a role in replenishing oxaloacetate to the tricarboxylic acid cycle (Ashworth & Kornberg, 1966). The enzyme of Enterobacteriaceae such as *Escherichia* and *Salmonella* families is known to be regulated by many kinds of metabolites (Utter & Kolenbrander, 1972; Morikawa et al., 1980).

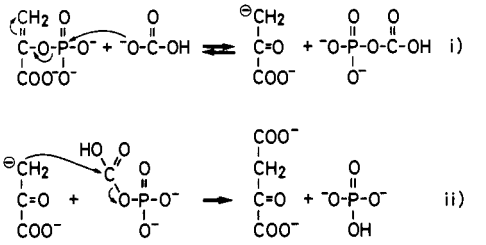
Scanty studies have been made on the mechanism of the enzyme reaction, since neither partial reaction nor exchange reaction has been found. In 1966, Maruyama et al. revealed with the peanut enzyme that ^{18}O was incorporated from [^{18}O]HCO_3^- into inorganic phosphate and oxaloacetate in a ratio of 1:2. They proposed the "concerted mechanism" shown in Scheme I. After a long blank, some studies that argue against their proposal have recently appeared. O'Leary et al. (1981) pointed out that the reaction was hardly explainable in terms of the concerted mechanism, on the basis of the results of precise determination of carbon isotope effect of bicarbonate ions in the reaction with the maize enzyme. Hansen & Knowles (1982) studied the reaction catalyzed by the wheat enzyme with [(*S*)-^{16}O,^{17}O]thiophosphoenolpyruvate as a substrate in [^{18}O]H_2O and found that inversion of the configuration at phosphorous atom occurred in the reaction. Both groups of investigators proposed an alternative reaction mechanism (shown in Scheme II) in which carbonic phosphoric anhydride and enolate anion of pyruvate were formed as an intermediate. This mechanism seems attractive because the question how the carbon atom of bicarbonate, which is inherently a poor electrophile, is activated to accomplish C–C bond formation is well explainable. However, no direct demonstration of the formation of enolate anion of pyruvate and carbonic phosphoric anhydride, which was found in other enzyme reactions (Powers & Meister, 1976; Wimmer et al., 1979), has been made.

In the present study with the *Escherichia coli* enzyme, we found that PEKB, a potent competitive inhibitor (Silverstein,

Scheme I: Postulated "Concerted Mechanism" for PEP Carboxylase



Scheme II: Postulated "Stepwise Mechanism" for PEP Carboxylase



† From the Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606, Japan. *Received July 26, 1983*. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (58580106).

[1] Abbreviations: PEP, phosphoenolpyruvate; PEKB, phosphoenol-α-ketobutyrate; DNPH, 2,4-dinitrophenylhydrazone; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-1774$01.50/0