Characterization of an Abnormal Fibrinogen Osaka V with the Replacement of γ-Arginine 375 by Glycine

THE LACK OF HIGH AFFINITY CALCIUM BINDING TO D-DOMAINS AND THE LACK OF PROTECTIVE EFFECT OF CALCIUM ON FIBRINOLYSIS*

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Prolonged thrombin time was completely corrected by the addition of millimolar concentrations of calcium in a new abnormal fibrinogen, Osaka V. Analysis of lysyl endopeptidase digests of Aa−, Bβ−, or γ-chains by high performance liquid chromatography, and the following amino acid sequence analysis of relevant peptides revealed that about 50% of the γ-chain has a replacement of γ-arginine 375 by glycine. When fibrinogen was digested with plasmin in the presence of millimolar concentration of calcium, the amount of fragment Dγ was about 50% of the normal control, and the rest was further cleaved to fragment Dγ1, Dγ2, or Dγ3 with an apparent Mγ of 62,000. Plasmic digestion of cross-linked fibrin in the presence of calcium resulted in the appearance of an abnormal fragment with an apparent Mγ of 123,000 as well as fragments Dγ1, Dγ2, and Dγ3, concomitant with the decrease of D dimer. The γ-remnant of the abnormal fragment proved to be a cross-linked complex of the normal Dγ  γ-remnant and residues 374–406/411 of the abnormal γ-chain. The number of high affinity Ca2+-binding sites for the normal fibrinogen and fibrinogen Osaka V obtained by equilibrium dialysis was 2.88 (about 3) and 1.85, respectively, and that for the abnormal molecules was calculated as 0.9 (about 1) from their relative amounts in the samples, suggesting the lack of two Ca2+-binding sites in the D-domains. These data suggest that the normal structure of the COOH-terminal portion of the γ-chain including residue 375 is required for the full expression of high affinity calcium binding to D-domains, the ability to be protected by calcium against plasmic digestion, and fibrin polymerization. During these studies, we found that the NH2-terminal amino acid of the γ-remnant in fragments D or D dimer which were obtained after prolonged digestion with plasmin is γ-Met19.

Most of the abnormal fibrinogens have prolonged thrombin-clotting time and impaired polymerization of fibrin monomers, and some of them have been described to have additional abnormalities in fibrinogen mobility (1–12). Fibrinogens Houston (2), Bern I (4), Haifa (8), Kyoto I (10), and Vlissingen (11) exhibit accelerated fibrinolysis. The γ-Asn308 → Lys exchange in fibrinogen Kyoto I causes a conformational change in the γ-chain, accelerated cleavage of the γ-Lys306–Ala307 and γ-Lys302–Phe303 bonds by plasmin, and the generation of a new plasmin cleavage site between Lys306 and Gly308 with the normal calcium binding properties of fragment Dγ (10). Fibrinogens Bern I (4), Haifa (8), and Vlissingen (11) have been reported to lack the ability to be protected by calcium against further attack by plasmin (13, 14), but the relationship between the lack of protective effect and structural or functional abnormalities has not been fully studied for these abnormal fibrinogens.

In this report we describe a new γ-chain variant in an abnormal fibrinogen, Osaka V, with a single-amino acid substitution of glycine for arginine at position 375 which is characterized by lack of high affinity calcium binding to D-domains, lack of the ability to be protected by calcium against further plasmic digestion, and complete correction of defective fibrinogen clotting by calcium.

EXPERIMENTAL PROCEDURES

Materials—The reversed-phase HPLC1 column was from the following sources: TSK gel TMS-250 from Toyoda Soda (Tokyo, Japan) and Cosmosol SC-18 from Nakarai (Kyoto, Japan). Chelex 100 was obtained from Bio-Rad. Human plasminogen was purified as described (15). Streptokinase was obtained from AB Kabi, and containing albumin was removed with Blue Sepharose CL-6B (Pharmacia LKB Biotechnology Inc.). Lysyl endopeptidase and dithioerythritol were obtained from Wako Chemical Co. (Osaka, Japan), and 4-vinyl-pyridine was purchased from Aldrich. Pharmalyte was obtained from Pharmacia, and Nonidet P-40 was purchased from Sigma. Affinity purified goat anti-mouse IgG horseshadish peroxidase conjugate and peroxidase substrate (4-chloro-1-naphthol) were obtained from Bio-Rad. 4CaCl2 (27.68 mCi/mg) and scintillation fluid, Bio-fluor, were from Du Pont-New England Nuclear. Polyvinylidene difluoride membranes (Clear Blot Membrane-P) were obtained from Atto (Tokyo, Japan).

Coagulation Studies of Plasma and Fibrinogen—Coagulation studies of plasma were performed according to standard procedures as described previously (16). Fibrinogen was purified from citrate- or citrate dextrose-plasma using lysine-Sepharose 4B chromatography,

1 The abbreviations used are: HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid.

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calcium binding to fibrinogen

OsaKA V WITH γ-Arg275—> Gly

gelatin-Sepharose 4B chromatography, and fractionation by ammo-
nium sulfates as previously described (16). Purified fibrinogen (10- 20 mg/ml) in 0.3 M NaCl was stored at -80 °C until use. Release of fibrinopeptides A and B was examined by HPLC and by the ratio of consumption of Aα- and Bβ-chains to the γ-chain, respectively, on SDS-PAGE as described (16). Polymerization of prepurified fibrin monomer was studied as described by Grafnek et al. (17) using their second method. Fibrin monomer (40 μg) in 30 μl of 20 mM acetic acid was mixed with 0.57 ml of 50 mM Tris-HCl, 71 mM NaCl, pH 7.4, containing various concentrations of CaCl₂ and the absorbance at 350 nm was continuously monitored at room temperature.

γ-Chain Digestion with Lysyl Endopeptidase—Reduced and pyri-
dylythylated (18, 19) fibrinogen was prepared as follows. Fibrinogen was dialyzed against 0.5 M Tris-HCl, 6 mM guanidine HCl, 10 mM EGTA, pH 8.5, at 4°C for 2 h, treated with dithioerythritol (0.9 mg/mg fibrinogen), flushed with nitrogen, and incubated at 37°C for 3 h. The mixture was treated with 4-vinyl-pyrrolidone (3.1 mol/mol dithioerythritol) at room temperature for 2 h, dialyzed against water at 4°C for 18 h, and further dialyzed against 50 mM Tris-HCl, 4 mM urea, pH 9.0. HPLC separation of fibrinogen chains (21) was performed as described (10). Purified γ-chain dissolved in 50 mM Tris-HCl, 4 mM urea, pH 9.0, was digested with lysyl endopeptidase (1 μg/mg γ-chain) at 37°C for 18 h, and fractionated on a Cosmosil 5C18-P reversed-phase HPLC column as described (10).

Plasmatic Digestion of Fibrinogen and Cross-linked Fibrin—Fibrino-
gen (25 mg/ml) in 50 mM Tris-HCl, 4 mM urea, pH 7.0, was incubated with various concentrations of CaCl₂ or with 10 mM EGTA at 37°C for 30 min and treated with 0.1 mg/ml human plasminogen and 3000 units/ml streptokinase for 18 h at 37°C. Cross-linked fibrin was prepared by adding 5 NIH units/ml bovine thrombin and 0.2 units/ml human factor XII (20) to 1 mg/ml of fibrinogen in 50 mM Tris-HCl, 0.135 M NaCl, 5 mM CaCl₂, pH 7.4, and incubated at 37°C for 2 h and 18-50% solvent system B for the following 1 h at a flow rate of 0.5 ml/min was employed, and the column effluent was monitored at 214 nm.

SDS-PAGE, Immunoblotting, Electroblotting, and Isoelectric Fo-

cusing—SDS-PAGE was performed as described previously (21) ac-
cording to the method of Laemmli (22). Transfer of proteins from

cations. 6.5% gels contained 8% (v/v) Pharmalyte for pH 3-10, 3%

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TABLE I

| Sample | Studies for fibrinogen function |
|--------|--------------------------------|
| Plasma | Fibrinogen (mg/dl) |
|        | Thrombin time method |
|        | 90 | 150-350 |
|        | Turbidimetric method |
|        | 218 | 150-350 |
|        | Immunochemical method |
|        | 250 | 150-350 |
|        | Thrombin time(s) |
|        | Without calcium ions |
|        | 14.3 | 9.7 |
|        | With calcium ions |
|        | 4.7 | 4.5 |
| Fibrinogen | Thrombin time(s) |
|        | Without calcium ions |
|        | 23.7 | 8.7 |
|        | With calcium ions |
|        | 5.3 | 5.7 |
|        | Reptilasea time(s) |
|        | Without calcium ions |
|        | 37.3 | 10.0 |
|        | With calcium ions |
|        | 45.3 | 5.2 |
Calcium Binding to Fibrinogen Osaka V with \(\gamma\text{-Arg}^{375} \rightarrow \text{Gly}\)

**Fig. 1. Effect of calcium on fibrin monomer polymerization.** Polymerization of fibrin monomer prepared by the addition of thrombin to fibrinogen was studied in the presence of 0, 0.05, 0.5, or 5 mM CaCl\(_2\). N, normal control; OV, fibrinogen Osaka V.

**Fig. 2. Effect of calcium on plasmic digestion of fibrinogen as analyzed by SDS-PAGE.** A, plasmic digests of fibrinogen in the presence of 1 mM CaCl\(_2\) (lanes 1, 2, 5, and 6) or 10 mM EGTA (lanes 3, 4, 7, and 8) were electrophoresed on 10% Laemmli gels. Lanes 1, 3, 5, and 7, normal control; lanes 2, 4, 6, and 8, fibrinogen Osaka V; lanes 1–4, nonreduced samples; lanes 5–8, reduced samples. The locations of the fragments are indicated. D\(_1\) (Deate), D\(_2\), D\(_{20}\), and D\(_{29}\), fragments with apparent \(M\), values of 117,000, 87,000, 62,000, and 39,000, respectively; D\(_{1}/\gamma\) and D\(_{2}/\gamma\), \(\gamma\)-chain remnant of fragment D\(_1\) and D\(_2\), with apparent \(M\), values of 42,000 and 30,000 respectively; /\(\beta\), \(\beta\)-chain remnant. The \(\alpha\)-chain remnant migrates at the dye front in lanes 5–8. B, fibrinogen was digested with plasmin in the presence of 0.01 (lanes 1 and 5), 0.1 (lanes 2 and 6), 1 (lanes 3 and 7) or 10 mM CaCl\(_2\) (lanes 4 and 8), and electrophoresed. Upper panel: NR, nonreduced samples; lower panel: R, reduced samples. Lanes 1–4, normal; lanes 5–8, Osaka V.

As shown in Table II, the amino acid sequence of OVN corresponded to residues 374–380 of the normal \(\gamma\)-chain, and that of OVA was the same as OVN except for the substitution of glycine for arginine at residue 375. If the assumption that peptides OVN and OVA have about the same extinction coefficient is allowed, the ratio of the amount of the normal \(\gamma\)-chain to the abnormal (mutant) \(\gamma\)-chain in the whole propositus fibrinogens will be 0.48:0.52. We could not find the other abnormal peptide peaks in lysyl endopeptidase digests of propositus \(\gamma\)-chain. The HPLC elution pattern of the lysyl endopeptidase digest of the propositus A\(\alpha\)- or B\(\beta\)-chains was the same as that of the normal control (data not shown).

Thus, fibrinogen Osaka V was demonstrated to be from a heterozygous dysfibrinogenemia in which the abnormal \(\gamma\)-chain, \(\gamma\)-Osaka V, has a substitution of glycine for arginine.
at residue 375, constitutes about 50% of the whole γ-chains and has a COOH-terminal region probably not protected by calcium against plasmic digestion.

Abnormal Plasmic Digests of Cross-linked Fibrin in the Presence of Calcium—Propositus fibrinogen showed normal cross-linking abilities of its α- and γ-chains (data not shown). Cross-linked fibrin was digested with plasmin in the presence of calcium and analyzed on SDS-PAGE. In the normal control (Fig. 4, lane 1), fragments D-dimer and E are the main products, with trace amount of fragments D3, Dα, and Dδ. A small amount of fragments with apparent M, values of around 123,000 (designated UA) were also noted, the nature of which remained unknown but might be the same as the U fragments reported by Gaffney et al. (29), a minor fragment formed during plasmic digestion of cross-linked fibrin (Fig. 5 of Ref. 13), or fragment D with M, of 102,000 (30). In contrast, considerable amounts of a fragment with an apparent M, of 123,000 and fragments D3, Dα, and Dδ were generated in the propositus sample concomitant with the decrease of fragment D-dimer (Fig. 4, lane 2). As shown in lanes 4 and 7 of Fig. 4, the propositus sample contained an abnormal γ-remnant with an apparent M, of 46,600 (designated Abn./γ) which is 4,000 higher than the normal fragment D1 γ-remnant (Fig. 4, lanes 5 and 8). Two-dimensional (nonreduced→reduced) SDS-PAGE revealed that this abnormal γ-remnant is the constituent of a fragment with an apparent M, of 123,000 (not shown). Amino acid sequences of the first 5 residues of the Abn./γ were analyzed after electroblotting. Two sequences of

| Cycle | Residue | pmol | Residue | pmol |
|-------|---------|------|---------|------|
| 1     | Thr     | 83   | Thr     | 69   |
| 2     | Arg     | 114  | Gly     | 106  |
| 3     | Trp     | 37   | Trp     | 38   |
| 4     | Tyr     | 66   | Tyr     | 120  |
| 5     | Ser     | —    | Ser     | —    |
| 6     | Met     | 28   | Met     | 46   |
| 7     | Lys     | 6    | Lys     | 8    |

*It was not possible to quantitate recovery.

†Corresponding residues of the γ-chain.

Calcium Binding to Fibrinogen Osaka V with γ-Arg^275 → Gly

![Fig. 3. HPLC analysis of the lysyl endopeptidase digests of the γ-chains. Purified γ-chains were digested with lysyl endopeptidase and separated by reversed-phase HPLC. For these chromatograms, peptides obtained from 70 μg of the γ-chains were injected. OVN is the peak with the same elution time as NN (the corresponding normal peak); OVA is the new peak.](image)

![Fig. 4. SDS-PAGE and immunoblotting of plasmic digests of cross-linked fibrin in the presence of calcium. Lanes 1-5, stained for protein; lanes 6-8, stained with γ-chain monoclonal antibody using immunoblotting as described under "Experimental Procedures"; lanes 1 and 2, nonreduced samples; lanes 3-8 reduced samples; lanes 1, 3, and 6, normal control; lanes 2, 4, and 7, fibrinogen Osaka V; lanes 5 and 8, normal fragment D1 is shown for comparison. D-D, D-dimer; UA, undefined or abnormal fragment(s) with apparent M, values of around 123,000; Abn./γ, abnormal γ-remnant with an apparent M, of 46,000.](image)

![Fig. 5. Calcium binding to fibrinogen determined by Scatchard analysis of equilibrium dialysis data. r, moles of Ca^2+ bound/mole of fibrinogen; C, free Ca^2+ concentration in moles/liter. ○—○, normal control; ⬤—⬤, fibrinogen Osaka V.](image)
ogen and fibrinogen Osaka V is 2.88 (about 3) and 1.85, respectively, with comparable dissociation constants of high affinity binding (3.2 and 3.4 μM, respectively).

As Fibrinogen Osaka V is heterozygous, it is important to know the relative amounts of normal and mutant fibrinogen in the samples to assess the number of calcium-binding sites obtained. Replacement of Arg (basic amino acid) by Gly (neutral) at residue 375 of γ-Osaka V led us to analyze fibrinogen by isolectric focusing, which was the only method able to differentiate the abnormal γ-chain from the whole γ-chains. The normal γ-chain was mainly resolved into three lines (Fig. 6, lanes 2 and 5) as previously reported (25).

However, the amounts of these three lines from the propositus γ-chains were reduced, and another three lines with increased negative charge were detected as shown by the schematic representation of Fig. 6 (lanes 4 and 6). Although too closely moving lines did not allow us to measure the intensity of each line densitometrically, the relative amounts of normal and mutant γ-chains in the propositus γ-chains seemed to be almost equivalent (Fig. 6, lanes 1 and 3).

If 52% of the propositus fibrinogen were abnormal molecules (Fig. 3, OVA) and the remaining 48% of normal molecules had the same number of Ca⁺⁺-binding sites as the normal control, the number of Ca⁺⁺-binding sites of the abnormal molecules is calculated as 0.9 ((1.85 – 2.88 × 0.48)/0.52), suggesting that the abnormal molecule in fibrinogen Osaka V lacks 1.98 (about 2) high affinity Ca⁺⁺-binding sites.

**DISCUSSION**

Heterozygous abnormal fibrinogen Osaka V is characterized by the correction of defective fibrinogen clotting with physiological concentrations of calcium; lack of protective effect of calcium on fibrinogen or cross-linked fibrin against further plasmic digestion; defective calcium binding to high affinity sites; and a single-amino acid substitution of glycine for arginine at position γ-375. This substitution can arise from a point mutation involving a single nucleotide change in the codon (CGG) responsible for position γ-375 (31): the codon is most likely altered from CGG to GGG. A considerable number of abnormal fibrinogens are known with defective γ-chains in which the molecular abnormalities have been elucidated, but their abnormalities have been limited to the sequence spanning γ-Arg Gly (10, 11, 32–44). It has been shown that a fibrin γ-chain polymerization site resides in the COOH-terminal portion (45–47) and that the native tertiary γ-chain structure is necessary for the expression of the polymerization site (28, 48). These reports are supported by the discovery of fibrinogen Osaka V with a single amino acid substitution at position γ-375.

Human fibrinogen has three high affinity calcium-binding sites (49–52). Two of them are located in the two D-domains (10, 47, 53), especially in residues 311–336 of the γ-chain (52), and the third site is located in the NH₂-terminal disulfide knot (54, 55). The existence of low affinity binding sites in human fibrinogen is also suggested (52, 56). The role of calcium bound to each site is not clarified yet. 2.88 (about 3) of Ca⁺⁺-binding sites for the normal fibrinogen (Fig. 5) is in good agreement with previous reports (49–52). In intact fibrinogen Osaka V, 1.85 binding sites were obtained. The ratio of the relative amounts of the normal fibrinogen to the mutant fibrinogen in whole fibrinogen Osaka V was about 1:1 which was obtained by isoelectric focusing of reduced fibrinogen (Fig. 6) and HPLC of lysyl endopeptidase digests of γ-chains (Fig. 3). 0.9 (about 1) of Ca⁺⁺-binding site and the lack of 1.98 (about 2) of binding sites calculated for the mutant fibrinogen molecule in fibrinogen Osaka V will probably be presumed to be caused by a lack of high affinity calcium binding to γ-chains in D-domains. Fibrinogen Osaka V is the first case of an abnormal fibrinogen with defective calcium binding which is not the result of Aα-chain degradation (51). Defective calcium binding caused by a single amino acid substitution has been reported only in mutant chicken skeletal myosin light chain (57).

The mechanism by which calcium ion enhances the polymerization rate of normal fibrin monomers is still unresolved, although potentiative effects of calcium on the binding of NH₂-terminal peptides of fibrin α- or β-chains to fibrinogen have been advanced as one of the explanations (58). Defective fibrinogen clotting is partially corrected via the binding of physiological concentrations of calcium in most abnormal fibrinogens, but its almost complete correction is very rare and has been observed only in three cases, fibrinogens Baltimore III (γ-Asn⁴⁰⁸→Ile) (40, 59), Milano I (γ-Asp⁴⁰⁹→Val) (43, 60) and Bern I (4). The mechanism for such a correction despite the presence of abnormal molecules in these three fibrinogens and fibrinogen Osaka V remains unknown. Thrombin-induced aggregation of fibrinogen Bern I was markedly delayed at 10 μM calcium, where only the high affinity calcium-binding sites of normal fibrinogen are occupied, but was normal at 5 mM calcium, where the molecule is saturated with calcium (4), and such was also the case with fibrinogens Milano I (60) and Osaka V (Fig. 1). Importantly, normal aggregation of fibrinogen at millimolar concentration of calcium was not affected by the presence (fibrinogen Baltimore III) (40) or absence (fibrinogen Osaka V) of high affinity calcium binding to D-domains. Aggregation of normal fibrin was also significantly enhanced by increasing the calcium concentration from 0.1 to 1 mM (61) or from 20 μM to 5 mM (52). These observations suggest that enhancement of fibrin polymerization is due to calcium bound not to the high affinity binding sites in D-domains but to some low affinity binding sites.

It has been shown that calcium has a protective effect in the plasmic digestion of fibrinogen (13), and purified fragment D₁ is degraded to fragments D₂ and D₃ by plasmin if bound calcium is chelated with EGTA (47, 62). Dang et al. (52) showed that this protective effect is obtained by 20 μM calcium during the digestion of fibrinogen and proposed that
this effect is due to calcium bound to the high affinity D-domain sites. 47% of propositus fibrinogen remained as fragment D₁ during plasmic digestion in the presence of calcium, but the remaining 53% was further digested (Fig. 2). If susceptibility of the normal and mutant fibrinogen to plasmin was not modified by each other, 53% of abnormally digested fibrinogen will probably reflect 52% of abnormal molecules with γ-Osaka V (Fig. 3, OVA) which lacks high affinity calcium binding to D-domains. The lack of protective effect of calcium on the plasmic degradation of abnormal molecules in fibrinogen Osaka V will thus be caused by lack of high affinity calcium binding to the abnormal D-domains. The lack of protective effect has been reported in fibrinogens Bern I (4), Haifa (8), and Vlissingen (11), and defective calcium binding is presumed except for fibrinogen Haifa.

On the other hand, calcium bound to low affinity sites also seems to have some effects on the plasmic degradation of fibrinogen. Millimolar concentration of calcium are required for the full expression of the protective effect under the experimental condition of Haverkate and Timan (13). Five mM calcium exerted a partial protective effect on fibrinogen Bern I while 1 mM calcium had no effect at all (4). In fibrinogen Osaka V, the ratio of smaller fragments decreased with increase of calcium concentration in the range of 0.1-10 mM (Fig. 2B). These phenomena will not be explainable without considering the role of calcium bound to low affinity sites.

Special attention should be paid to fragment D₂ which has been shown to have one high affinity calcium binding site, although the dissociation constants are somewhat larger than that of fragment D₁ (47, 62). If this is the case, fragment D₂ produced by plasmic digestion of abnormal fibrinogen which lacks abnormal residues 374-411 in γ-Osaka V should regain calcium binding ability. Nieuwenhuizen et al. (62) reported that the conversion of fragment D₂ to D₁ stops on addition of excess (over EGTA) calcium during the plasmic digestion of fragment D₁; and Purves et al. (14) also showed that the plasmic digestion of fragment D₂ as well as D-dimer in the presence of EGTA is inhibited by readdition of excess calcium to a free calcium concentration of 5 mM, although these effects could have been caused by calcium bound to low affinity sites. We tried to purify fragment D₂ from the abnormal molecule, but this was left for future analysis as it requires much more technical assistance. The dissociation constants are somewhat larger than that of fragment D₁ but at variance with most reports in which γ-Ala^{374} or γ-Ser^{374} is the main NH₂-terminal amino acid for the γ-remnant of fragments D (47, 48, 66-68) and D-dimer (48). Long term digestion of fibrinogen with plasmin under controlled experimental conditions will result in the cleavage of the γ-Lys^{386}Met^{389} bond.

In summary, fibrinogen Osaka V with γ-Arg^{375} → Gly provides insights into the tertiary y-chain structure necessary for the expression of calcium-binding sites and the role of calcium bound to high affinity or low affinity binding sites in fibrin polymerization and fibrinolysis.

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Addendum—After this paper was submitted for publication, a conference abstract dealing with fibrinogen Vlissingen appeared. This shows defective calcium binding to fibrinogen (69), and this work has subsequently been published (70).

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