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Disulfide Bond Reduction in Fibrinogen: Calcium Protection and Effect on Clottability†

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ABSTRACT: Fibrinogen contains 29 disulfide bonds. Limited reduction in buffers containing calcium led to cleavage of three of them: the two Aα442Cys–Aα472Cys intrapeptide disulfide bonds and the symmetrical Aα28Cys–Aα28Cys bond. The limited reduction did not affect clotting by thrombin. However, a prolongation of the thrombin clotting time occurred when the limited reduction took place in the absence of calcium. The bonds reduced under this condition included the three already mentioned and also the two γ326Cys–γ339Cys intrapeptide disulfide bonds located in the C-terminal ends of the γ-chain. N-Terminal analysis of thrombin-treated samples showed that thrombin cleavage occurred at the normal Aα16–Aα17 site in fibrinogen that was partially reduced in the presence of calcium. By contrast, thrombin cleaved at the Aα19–Aα20 site in fibrinogen that was partially reduced in the absence of calcium, rendering the protein unclottable by removing the Aα17Gly–18Pro–19Arg peptide. The loss of thrombin clottability may have also come from γ326Cys–γ339Cys disulfide bond reduction since the structure supported by this bond may be important for the function of the C-terminal polymerization site. In samples of the partially reduced fibrinogen lacking the Aα17–19 residues, gel formation occurred through an oligomerization mechanism catalyzed by factor XIII.

Fibrinogen, the clotting protein in blood, has a molecular weight of approximately 340000 and a dimeric chemical structure. Many details about human fibrinogen are known, including the amino acid sequence of its three polypeptide chains [for a review, see Furlan (1988) and Doolittle (1984)]. Each half of the molecule contains a set of Aα-, Bβ-, and γ-chains, which are linked together by seven interchain disulfide bridges. The disulfide bridges are located in clusters in the amino-terminal and middle regions of the three chains. There are also six intrachain disulfide bonds in each half-molecule: two in the middle region of the Bβ-chain, one in

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the middle region of the γ-chain, and one near the carboxy-terminal end of each of the chains. The two half-molecules are also covalently linked to each other at the amino-terminal regions by additional disulfide bonds, one of which is between the Aα-chains and two between the γ-chains. Altogether there are 58 cysteine residues in fibrinogen forming 29 disulfide bonds and no free sulfhydryl groups.

The overall arrangement of the polypeptide chains has been extensively studied. The six interconnected amino termini in fibrinogen form a globular structure at the center of the molecule, and the middle and carboxy-terminal regions of the three chains of each half-molecule extend outward from the two sides of the center nodule. The carboxy-terminal ends form independent globular structures. The carboxy-terminal structure of the longer Aα-chain is thought to extend further out and may be free-floating or folded into a structure found near the center nodule.

Interest in the chemical reactivity of disulfide bonds in fibrinogen developed quite early, not only in the context of clotting mechanisms (Lyons, 1945) but also in disulfide bond formation during fibrinogen biosynthesis (Doolittle et al., 1978; Bolyard & Lord, 1988) or disulfide exchange reactions. Early studies showed that limited reduction of fibrinogen by thioredoxin brought about changes in the protein's physical properties and clottability (Blombäck et al., 1974). More recently, a prolongation of the thrombin clotting time was demonstrated in partially reduced fibrinogen (Blombäck et al., 1985). The clotting time prolongation did not occur when partial reduction was done in buffers containing calcium ions. The present study was conducted to determine the sites of disulfide bond cleavage during limited reduction of fibrinogen and to see whether the cleavage of unique disulfide bonds led to the prolongation of the thrombin clotting time.

Materials and Methods

Proteins and Reagents. Human fibrinogen (Imco, Stockholm) was prepared as previously described (Blombäck et al., 1985). The fibrinogen solution (2% in 0.3 M NaCl) was dialyzed against 100 volumes of Tris–saline–EDTA buffer (TNE buffer)[1] during 3 h with three changes of outer fluid. The fibronectin present in this preparation was removed by affinity chromatography on gelatin–Sepharose (Procyk et al., 1985). The fibrinogen stock solution (about 12 mg/mL) was stored at −70 °C. Fibrinogen concentration was measured spectrophotometrically in alkaline urea (Blombäck et al., 1985) using $E(1\%, 1\text{ cm}) = 16.5$ at 282 nm. Triplicate determinations were done. Human factor XIII, thrombin, and plasmin were obtained from the Department of Blood Coagulation Research, Karolinska Institutet. Activated factor XIII (factor XIIIa) was prepared as described earlier (Procyk et al., 1985). Trasylol was from Bayer AG (West Germany).

Partial Reduction of Fibrinogen. Fibrinogen stock solution was diluted with TNE buffer to a final concentration of 7 mg/mL. For partial reduction in the presence of calcium, $CaCl_2$ was added (from a 1 M stock solution in water) to give a final molarity of 20 mM. Samples were incubated at 37 °C for 5 min (to allow for calcium binding to fibrinogen), and then DTT was added (from a concentrated stock solution in water) to the desired final molarity (usually 0.5–5 mM). Nitrogen was blown over the sample and the tube sealed and placed in a water bath at 37 °C for the duration of reduction (usually 5–20 min). For reduction in the absence of calcium, 2 M NaCl was added in place of the calcium to adjust the ionic strength of the sample to the same value as in samples with calcium.

Carboxymethylation of Partially Reduced Fibrinogen. The procedure was done in an inflatable glove chamber filled with nitrogen. The DTT was removed by gel filtration on a PD-10 column (Pharmacia, NJ). The column was equilibrated with nitrogen-saturated TNE buffer supplemented with either 20 mM $CaCl_2$ or 40 mM NaCl. The protein was collected at a predetermined elution volume, usually within 3 min after sample application. The pH of the eluate was adjusted to 8.1 by the addition of 2 M Tris, pH 8.5, and iodoacetic acid (Sigma, St. Louis, MO; recrystallized 3 times from petroleum ether before use) was added to give a final molarity of 1.6 mM. The iodoacetic acid stock solution (12 mM) was made in 0.1 N NaOH. Nitrogen was blown over the sample and the tube sealed and kept in the dark at room temperature for 45 min. Afterward, unreacted reagent was removed by gel filtration (PD-10 columns equilibrated in TNE buffer) and the sample used for analysis or clotting.

Determination of the Number of Disulfide Bonds Reduced. [^{3}H]Iodoacetic acid (Du Pont Co., Wilmington, DE) was added to the iodoacetic acid stock solution in order to tag thiol groups during the carboxymethylation step. The [^{3}H]iodoacetic acid (crystalline solid) was dissolved in 0.1 N NaOH and diluted with a solution containing unlabeled iodoacetic acid to give a final specific activity between 16 and 54 mCi/mmol. The number of cleaved disulfide bonds was determined from the radioactivity and protein content of the sample and expressed as moles of iodoacetic acid incorporated per mole of fibrinogen. Protein content was measured in alkaline urea (see above). For liquid scintillation counting, samples were neutralized with 0.2 M HCl prior to addition of Hydrofluor (National Diagnostics, Manville, NJ). Determinations were done in triplicate. The specific activity of the iodoacetic acid stock solution in each experiment was determined from carboxymethylation of fully reduced fibrinogen (with reduction and carboxymethylation done in the presence of 6 M guanidine hydrochloride).

The number of cleaved disulfide bonds in individual fibrinogen chains was derived from isolated chains and expressed as moles of iodoacetic acid incorporated per mole of polypeptide. The concentration of polypeptide was based on the amount of fibrinogen applied to the HPLC or FPLC column (see below).

The possibility that some reduced disulfide bonds may not react with the iodoacetic acid without unfolding the native molecule was evaluated in experiments where the carboxymethylation step was done in the presence of 5 M guanidine hydrochloride. Guanidine hydrochloride (crystals; Pierce, Rockford, IL) was added to a final molarity of 5 M immediately after removal of the DTT, but prior to the addition of the iodoacetic acid. The level of iodoacetic acid incorporation into the unfolded molecule was only about 5–15% greater than the level obtained in the absence of denaturant. This indicated that in native partially reduced fibrinogen the alkylating reagent had access to almost all of the sites where reduction occurred (see also Discussion).

Isolation of Fibrinogen Polypeptide Chains. Partially reduced fibrinogen was denatured by addition of 8.4 M guanidine hydrochloride–50 mM Tris, pH 8.2, buffer (4 M final concentration of guanidine, incubation for 30 min at 37 °C) and completely reduced with DTT (10 mM final concentration,

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TNE buffer, Tris–saline–EDTA buffer (0.05 M Tris, 0.1 M NaCl, and 1 mM EDTA, pH 7.4); Tris, tris(hydroxymethyl)aminomethane.

Table I: Effect of Mild Reduction of Fibrinogen on Clotting

reduction buffer[a]		mol of -SH	clotting	FPA released (% of total)	
[$CaCl_2$] (mM)	[DTT] (mM)	alkylated/mol of FBG[b]	time[c] (s)	after 0.2 h[d]	after 21 h[d]
0	0	0	109	87	90
0	1.0	5.2	262		
0	1.2		317		
0	5.0	9.2	>600	88	93
20	1.0	3.6	148		
20	5.0	6.1	142	86	99
20	10.0		138		

[a] Reduction was for 20 min in TNE buffer as noted in the presence or absence of calcium. See Materials and Methods for details. [b] Determined by incorporation of [^{3}H]iodoacetic acid into fibrinogen. FBG = fibrinogen. [c] Samples for clotting contained 2 mg/mL fibrinogen in TNE buffer, $CaCl_2$ (20 mM), and thrombin (0.2 unit/mL). [d] Time after addition of thrombin. FPA = fibrinopeptide A.

incubation for 30 min at 37 °C). Free thiols were blocked by 4-vinylpyridine (Aldrich, Milwaukee, WI) or iodoacetic acid (80 mM final concentration, incubation for 30 min, room temperature in the dark). Samples were dialyzed for several hours against HPLC or FPLC starting buffer (0.1% phosphoric acid or 10% acetonitrile–0.1% trifluoroacetic acid) for chain separation either on a Vydac C4 column (250 × 10 mm; Separations Group, Hesperia, CA), with a gradient of 15–50% acetonitrile in 0.1% phosphoric acid, or on a RPC PRO 10/10 column (Pharmacia, Piscataway, NJ), with a 30–42% acetonitrile–0.1% trifluoroacetic acid gradient. Recoveries were typically about 95%.

Amino Acid Sequencing. A 1-mg sample of the partially reduced and alkylated fibrinogen in TNE buffer was incubated with 0.2 unit/mL thrombin to remove the fibrinopeptides. After 1.5 h, the sample was denatured and reduced as for chain isolation, except that the remaining free sulfhydryls were alkylated with 4-vinylpyridine (88 mM final concentration, 30-min incubation at 22 °C). Samples were dialyzed against 10% acetonitrile–0.1% trifluoroacetic acid and applied to FPLC for separation of the polypeptide chains. Eluted fractions were concentrated (Speed-Vac Model SVC-100 HT; Savant Instruments, Farmingdale, NY) and subject to automated amino acid sequence analysis using a Model 477A protein sequencer (Applied Biosystems, Foster City, CA).

Electrophoresis. SDS–polyacrylamide slab gel electrophoresis was performed on 5–10% gels, 0.75 mm thick, in the Laemmli (1970) system. Gels were prepared for fluorography according to Bonner and Laskey (1974).

Other Measurements. Clotting time was determined spectrophotometrically (Procyk, 1985). Fibrinopeptide A release was measured by radioimmunoassay (Nossel et al., 1974) as described by Blombäck and Okada (1982).

Results

Incubation of fibrinogen with successively higher concentrations of DTT in buffer containing EDTA led to cleavage of disulfide bonds and a concomitant prolongation of the thrombin clotting time (Table I). Although clotting was delayed, the release of fibrinopeptide A, an indication of thrombin activity, was normal. Incubation of fibrinogen in buffer containing calcium and DTT also led to the cleavage of disulfide bonds, but without the accompanying prolongation of the thrombin clotting time. Fibrinopeptide A release was the same as in the experiments with EDTA. These results indicated that cleavage of possibly unique fibrinogen disulfide bridges during limited reduction in the absence of calcium may have affected the function of one (or more) polymerization

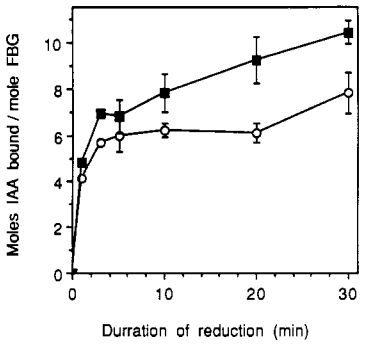

FIGURE 1: Number of -SH groups alkylated in partially reduced fibrinogen at various durations of reduction. Experimental protocol described under Materials and Methods. Reduction was with 5 mM DTT. (○) Reduced in the presence of calcium. (■) Reduced in the absence of calcium. Data points with error bars indicate average values and standard deviations of two to five experiments.

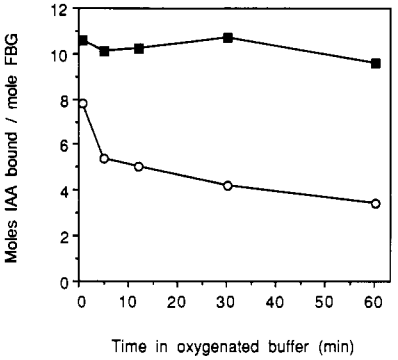

FIGURE 2: Number of -SH groups remaining in partially reduced fibrinogen after exposure to oxygen-saturated TNE buffer for various lengths of time. Reduction, in the presence of calcium (○) or absence of calcium (■), was with 5 mM DTT for 30 min, after which the DTT was removed by gel filtration. A constant stream of oxygen was bubbled through the eluted sample at a slow rate without causing foaming, and aliquots were removed for carboxymethylation with [^{3}H]iodoacetic acid at designated times. See Materials and Methods for details.

domains in fibrinogen. As reported previously (Blombäck et al., 1985), the very long clotting time of fibrinogen that was reduced in the absence of calcium could not be corrected by subsequent addition of calcium to the clotting mixture (not shown).

Half-molecules of fibrinogen or individual polypeptide chains were not observed in any of the partially reduced and alkylated samples. Typically, samples migrated as a single high molecular weight band of only slightly faster mobility than control (unreduced fibrinogen) in SDS electrophoresis (not shown). Similar results were obtained when the samples were dissolved in buffer containing 8 M urea and applied to electrophoresis (not shown).

During a relatively short period of reduction, in either the presence or the absence of calcium, about 4 mol of iodoacetic acid was incorporated per mole of fibrinogen, indicating that possibly two disulfide bridges were being affected (Figure 1). After longer periods of reduction (e.g., 20–30 min), more disulfide bonds were cleaved in fibrinogen when reduction was carried out in buffer containing EDTA rather than calcium (i.e., about 10 mol of iodoacetic acid/mol of protein versus 7 in the presence of calcium). Therefore, about five disulfide bonds were possibly being reduced in fibrinogen in the absence of calcium, whereas between three and four bonds were cleaved during reduction in the presence of calcium.

The re-formation of disulfide bonds was studied by incubating the partially reduced fibrinogen in oxygenated buffer (Figure 2). One disulfide bond was re-formed within 10 min

Table II: Incorporation of Iodoacetic Acid into Partially Reduced Fibrinogen

[$CaCl_2$] in buffer for reduction[a] (mM)	duration of reduction[a] (min)	mol of –SH alkylated/mol of polypeptide[b]		
		Aα-chain	Bβ-chain	γ-chain
0	5	2.0	0.1	0.7
0	10	2.3	0.2	1.1
0	20	2.5	0.2	1.6
20	5	1.7	0	0.5
20	10	1.9	0.1	0.6
20	20	2.2	0.2	0.6

[a] Reduction was with 5 mM DTT for the time indicated. Preincubation with calcium as noted. See Materials and Methods for details. [b] DTT was removed by gel filtration and the protein reacted with [^{3}H]iodoacetic acid. See Materials and Methods for details on chain separation and specific activity determination.

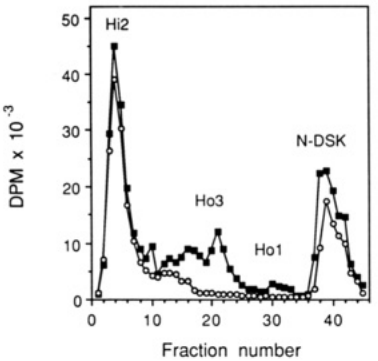

FIGURE 3: Analysis of CNBr digests of partially reduced and alkylated fibrinogen. Partial reduction in the presence (○) or absence (■) of calcium was with 5 mM DTT for 20 min. [^{3}H]Iodoacetic acid was used to block free thiol groups (see Materials and Methods). After treatment with CNBr (Blombäck et al., 1972), samples were analyzed by HPLC (Vydac C4 column, eluted with a gradient of 15–50% acetonitrile in 0.1% phosphoric acid). The position of fragments is noted.

in fibrinogen that was previously exposed to reducing reagent in the presence of calcium. By contrast, no disulfide bonds were re-formed in fibrinogen that was subject to partial reduction in the absence of calcium. The addition of calcium to the oxygenated buffer did not facilitate disulfide bond reformation in the sample that was partially reduced in the absence of calcium (not shown).

The data in Table II show that reduction of disulfide bonds occurred primarily at sites in the Aα- and γ-chains. The bonds linking these chains with the Bβ-chain, or the disulfide bonds in the Bβ-chain (e.g., Bβ394Cys–Bβ407Cys), apparently were not cleaved under these conditions. The Aα-chain was labeled to slightly over 2 mol of iodoacetic acid/mol of polypeptide under all conditions. By contrast, labeling of the γ-chain increased to almost 2 mol of iodoacetic acid/mol of polypeptide in fibrinogen that was partially reduced in the absence of calcium, but remained constant, at about 0.5 mol of iodoacetic acid/mol of polypeptide, in fibrinogen that was partially reduced in the presence of calcium.

Analysis of CNBr fragments of partially reduced and alkylated fibrinogen verified that the middle segments of fibrinogen [the Ho1 fragment; see Furlan (1988) for nomenclature] did not contain cleaved disulfide bonds, thus confirming that the interchain disulfide bridges between the Bβ-chain and the Aα- and γ-chains in this fragment were not susceptible to reduction (Figure 3). On the other hand, the amino-terminal N-DSK fragment (containing the three symmetrical disulfide bonds at 8γCys, 9γCys, and Aα28Cys, and the γ23Cys–Aα45Cys disulfide bond) and also the fragment

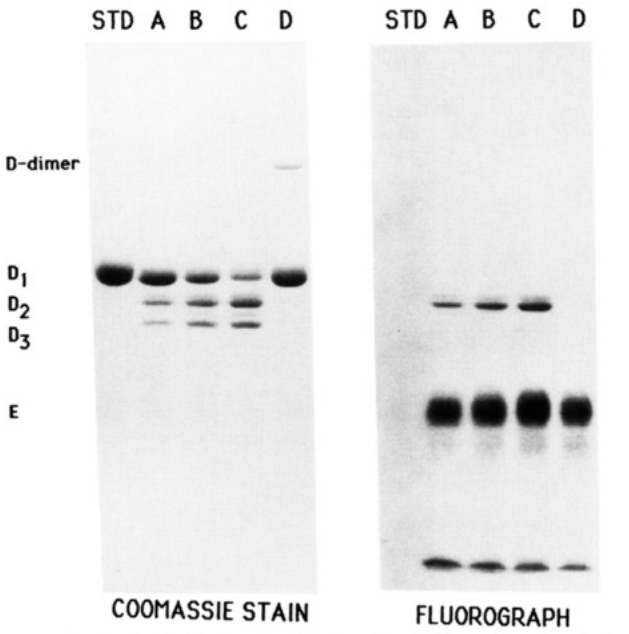

FIGURE 4: Analysis of plasmic digests of partially reduced and alkylated fibrinogen. Partial reduction was with 5 mM DTT in the absence of calcium for 5 (lane A), 10 (lane B), or 20 min (lane C) or in the presence of calcium for 20 min (lane D). [^{3}H]Iodoacetic acid was used to block free thiol groups (see Materials and Methods). Afterward, samples (1.5 mg/mL) were digested with plasmin (15 μg/mL) for 5 h at 37 °C in buffer containing 10 mM $CaCl_2$. Digestion was terminated by addition of 100 KIU/mL trasylol, and samples were dissolved in electrophoresis buffer without reducing reagent. Lanes contained 30 μg of protein. A standard sample (unreduced fibrinogen digested with plasmin; courtesy of Dr. B. Kudryk) was analyzed in the lane marked "STD." The position of fragments is shown at left.

of the carboxy-terminal region of the Aα-chain (the Hi2 fragment with the Aα442Cys–Aα472Cys disulfide bond) were radioactive in fibrinogen that was partially reduced in the presence of calcium. In fibrinogen that was partially reduced in the absence of calcium, the N-DSK and Hi2 fragments and, in addition, the fragment encompassing the carboxy-terminal end of the γ-chain (the Ho3 fragment with the γ326Cys–γ339Cys disulfide bond) were labeled. The radioactivity of the Ho3 fragment increased during longer periods of reduction, indicating that the γ326Cys–γ339Cys disulfide bond was being cleaved. By contrast, the Ho3 fragment did not become radiolabeled in fibrinogen that was partially reduced in the presence of calcium, even at high concentrations of DTT and after prolonged periods of reduction (not shown).

Plasmin digestion of the partially reduced and alkylated fibrinogen samples was carried out in buffer containing calcium in order to confirm the location of radioactivity in specific plasmic fragments. As shown in Figure 4, left panel, fibrinogen fragments D and E were obtained from both types of partially reduced fibrinogen. Fragment E appeared as a very faint band in the Coomassie-stained gel and was more visible in the fluorograph (right panel). In fibrinogen that was partially reduced in the absence of calcium, the intensity of the band representing fragment D_1 decreased (e.g., lanes B and C). The γ-chain segment of the D_1 fragment spans from residues γ86 to γ411 and was apparently being converted to the smaller fragment D_2 by plasmin cleavage at γ355Ser–γ356Lys, and this fragment was being converted to the even smaller fragment, D_3, through an additional cleavage at γ302Lys–γ303Phe (Henschen, 1981; Varadi & Scheraga, 1986). These interconversions were unexpected since they typically occur only after extensive plasmin digestion in the presence of EDTA and not in buffers containing calcium (Haverkate & Timan, 1977).

Table III: Susceptibility of N-Terminal Disulfide Bonds to Cleavage during Limited Reduction[a]

location of cysteine	disulfide bridge	reduction in presence of $CaCl_2$ (% PTH-CMCys[b])	reduction in presence of EDTA (% PTH-CMCys[b])
α28	symmetrical	94	88
α36	α36–β65	0	
α45	α45–γ23		0
γ8	symmetrical	18.0	6.5
γ9	symmetrical	10.5	9.0
γ19	γ19–β80	0	0
γ23	γ23–α45	1.5	3

[a] Reduction was with 5 mM DTT for 30 min in buffer containing either calcium or EDTA. The α- and γ-chains from the partially reduced fibrinogen were isolated and sequenced. See Materials and Methods for details. [b] Reported as recovery of PTH-(carboxymethyl)-cysteine (picomoles) per sum of PTH-(carboxymethyl)- and PTH-(pyridylethyl)cysteine recovered (picomoles) for a particular cycle, in percent. Data for the α-chain are the sum of recoveries from both cycles where the appropriate residue appeared (see Results). Values are the average of two experiments. CM = carboxymethyl.

Apparently, in fibrinogen that was partially reduced in the absence of calcium, the calcium ions added to the plasmin digestion buffer (10 mM) could no longer protect the fragment D_1 from further digestion.

The fluorograph in Figure 4 (right panel) showed that fragment E was labeled in all of the partially reduced fibrinogen samples, confirming that some disulfide bonds in the amino-terminal region (e.g., N-DSK) were reduced. In addition, some very low molecular weight fragments (e.g., those containing Aα442Cys and Aα472Cys) were also labeled and visible at the bottom of the gel. On the other hand, fragment D_1 was not labeled in either of the samples, indicating that the carboxy-terminal intrapeptide γ326Cys–γ339Cys disulfide bonds (and also the Bβ394Cys–Bβ407Cys linkage) were not reduced. Fragment D_2 was labeled and present only in samples of fibrinogen that were reduced in the absence of calcium. The increase in intensity of this band indicated that the γ326Cys–γ339Cys disulfide bridge was cleaved. As expected, fragment D_3 was unlabeled since the peptide region with γ326Cys and γ339Cys was missing in this fragment. The amount of fragment D_1 remaining in lanes A–C was proportional to the number of fibrinogen molecules in which γ326Cys–γ339Cys disulfide bond reduction and carboxymethylation with [^{3}H]iodoacetic acid had not yet occurred. Densitometric analysis of the Coomassie band intensities showed that this fraction constituted less than 15% of the total in samples reduced in the absence of calcium for 20 min (not shown).

Amino acid sequencing was used to determine which disulfide bonds in the amino-terminal region of fibrinogen were cleaved during limited reduction. Under the protocol used, exposed cysteines were carboxymethylated by iodoacetic acid, and the remaining disulfide bonds, subsequently cleaved during total reduction, were blocked with 4-vinylpyridine (see Materials and Methods). The recovery of PTH-(carboxymethyl)cysteine (retention time of 8.21 min) during a sequencing cycle indicated that the disulfide bond at that position was cleaved during the limited reduction. Recovery of PTH-(pyridylethyl)cysteine (retention time of 21.2 min) indicated that the disulfide bond was not affected by limited reduction. The data in Table III show that the symmetrical Aα28Cys–Aα28Cys disulfide bond, the bond on the 2-fold symmetry axis relating the two identical halves on the molecule, was almost totally reduced during the limited reduction in either the presence or the absence of calcium. There was a low amount of reduction of the symmetrical γ-chain disulfide bonds (up to 20% on occasion; see Discussion) and no indication of reduction at the other disulfide bonds of the amino-terminal regions of the Aα- and γ-polypeptide chains.

Prior to total reduction and pyridylethylation, thrombin was added to the partially reduced and alkylated fibrinogen to remove the fibrinopeptides and thus permit α-chain sequencing from residue 17 (i.e., 12 residues from the first cysteine at α28; see Materials and Methods). However, sequencing of the α-chain gave two different PTH-amino acids at each cycle: one PTH derivative at each cycle followed exactly the expected sequence of the α-chain, and a second PTH derivative was always three residues ahead of the current cycle. This indicated that the α-chains contained a heterogeneous N-terminal end, i.e., a population of chains beginning at the expected Gly17 residue and a second population beginning at the Val20 residue. The relative proportion of α-chains with N-terminal Val20 was small (less than 10% of total) in samples where partial reduction was done in the presence of calcium. However, for samples that were partially reduced in the absence of calcium, α-chains with N-terminal Val20 constituted more than 90% of the total. Amino acid sequencing of the β-chains from the partially reduced fibrinogen samples gave the expected N-terminal sequences (not shown).

Discussion

Mild reduction of fibrinogen in buffer containing calcium resulted in the cleavage of essentially three disulfide bonds: the symmetrical Aα28Cys–Aα28Cys bond and the two carboxy-terminal Aα442Cys–Aα472Cys disulfide bonds. The cleavages occurred rapidly, at a low concentration of DTT and without the addition of chaotropic reagents that would disrupt native structures in fibrinogen (see Materials and Methods). The reduced disulfide bonds may be located near or at the surface of the molecule, in structures readily accessible to the surrounding water. In fact, they are located in hydrophilic fragments of fibrinogen, i.e., N-DSK and Hi2 fragments.

Reduction of fibrinogen in buffer containing EDTA led to the cleavage of two additional disulfide bonds. These were the two carboxy-terminal γ326Cys–γ339Cys bonds located in a hydrophobic fragment of fibrinogen, the Ho3 fragment. The γ326Cys–γ339Cys disulfide bonds are part of the globular carboxy-terminal structure of the γ-chain and may be located at or near a surface of a pocket, or part of a substructure, which is accessible to small hydrophilic molecules such as DTT. EDTA binds to fibrinogen (Nieuwenhuizen et al., 1981). However, it is unlikely that EDTA binding may have caused irreversible structural changes in the fibrinogen molecule that would cause an increased susceptibility of the γ326Cys–γ339Cys disulfide bond to reduction. The concentration of EDTA used in the experiments (1 mM) was too low for this to occur (Perizzolo et al., 1985).

Calcium also binds to fibrinogen. Three high-affinity sites and between 14 and 20 weak calcium binding sites have been characterized (Marguerie et al., 1977). One of the high-affinity sites has been localized in the region of the γ-chain encompassing the γ326Cys–γ339Cys disulfide bond (Dang et al., 1985; Varadi & Scheraga, 1986). It is probable that reduction of the γ326Cys–γ339Cys disulfide bond occurred when calcium was removed from the site by EDTA. Thus, calcium binding appears to stabilize a conformation of the binding site region such that the γ326Cys–γ339Cys disulfide bond cannot be cleaved by DTT. This is a reasonable expectation since calcium binding is thought to cause small conformational effects in the region of the binding sites (Marguerie, 1977).

Disulfide bond protection by calcium has also been observed during the reduction of thrombospondin, a major platelet glycoprotein (Turk & Detwiler, 1986). The calcium binding sites in thrombospondin have been postulated to reside in a series of repeating amino acid sequences resembling calcium binding loop structures, although lacking the adjacent α-helical regions typically found in calmodulin-like calcium binding sites. Instead, the sites in thrombospondin are thought to be stabilized by disulfide bridges which are protected from reducing reagents in the presence of calcium (Lawler & Hynes, 1986).

Several properties of reduced fibrinogen indicate that the γ326Cys–γ339Cys disulfide bridge is important for the structure of the carboxy-terminal region of the γ-chain. Calcium ions no longer protect the fragment D_1 from further digestion to the smaller fragments D_2 and D_3 in fibrinogen that was partially reduced in the absence of calcium. Thus, it would appear that the calcium ions could no longer interact with calcium binding sites in fibrinogen that had the γ326Cys–γ339Cys disulfide bridge cleaved since the calcium binding region was irreversibly destablized upon cleavage of the disulfide bond. Preliminary studies of calcium binding in this fibrinogen indicate that only one high-affinity binding site is functional (unpublished observations), presumably the one in the central N-terminal domain of fibrinogen (Nieuwenhuizen et al., 1983).

An absence of a protective effect of calcium on fragment D_1 during plasmin digestion has also been observed in abnormal fibrinogens, such as fibrinogen Haifa (Soria et al., 1987). Initial findings suggested that the mutation in this fibrinogen may be located at position γ275Arg, which is outside the structural region supported by the γ326Cys–γ339Cys disulfide bond. However, since other abnormal fibrinogens with mutations at the γ275Arg site, e.g., Saga (Yamazumi et al., 1988), Osaka II (Terukina et al., 1988), or Tochigi (Yoshida et al., 1988), all showed normal protection of the fragment D_1 during plasmin digestion in the presence of calcium, it is possible that the site of the mutation in fibrinogen Haifa may be closer to the structure supported by the γ326Cys–γ339Cys disulfide bond. Interestingly, calcium binding appeared to be normal in fibrinogen Haifa (Soria et al., 1987); however, an amino acid substitution at one of the two Cys residues or at other residues in the region may still be possible.

The amino-terminal Aα28 symmetrical disulfide bond in fibrinogen showed no calcium protection during limited reduction, and this may imply that it is not associated with the amino-terminal calcium binding site (Nieuwenhuizen et al., 1983). The symmetrical disulfide bonds of the γ-chain appeared not to be cleaved as a result of the limited reduction/alkylation procedure. The alkylation step was done by first removing the DTT by gel filtration and then adding the iodoacetic acid to the eluted sample. When alkylation was done at high concentrations of iodoacetic acid and without removing the DTT, in either the presence or the absence of calcium, the symmetrical disulfide bonds of the γ-chain were cleaved to a greater extent than before (up to 70%, determined from PTH-carboxymethyl recovery during amino acid sequencing of the γ-chain; unpublished observation). This indicates that the symmetrical γ-chain disulfide bonds were susceptible to reduction under the conditions of the limited reduction; however, they would re-form immediately after removal of the DTT by gel filtration. It is possible that the γ8 and γ9 cysteine residues remain juxtaposed to each other in the compact structure of fibrinogen, even when not involved in a disulfide bridge, and therefore quickly re-form the disulfide bridge when exposed to oxygen in the buffer.

Cleavage of the Aα19Arg–20Val bond by thrombin in the fibrinogen that was subject to limited reduction in the absence of calcium was unexpected. The order of cleavage has not yet been established; however, it is clear that both the Aα17–19 and the Aα1–16 peptides were released. The antibody used to detect fibrinopeptide A release (Table I) confirmed the presence of the Aα16Arg carboxy terminal since it is specific for the Aα16Arg residue. On the other hand, amino acid sequencing of the α-chain demonstrated the 20Val N-terminal. Thus, both the Aα17–19 and the Aα1–16 peptides must have been present in the clotting mixture after the introduction of thrombin to the sample.

Thrombin cleavage of the Aα19Arg–20Val bond is known to occur in fragments of the fibrinogen Aα-chain, or in extensively denatured Aα-chains, but not in intact fibrinogen (Blombäck et al., 1967; Hageman & Scheraga, 1974; Lord & Fowlkes, 1989; Ni et al., 1989). Since the Aα17–19 residues are an important part of the "A" fibrin polymerization site (Blombäck et al., 1978; Laudano & Doolittle, 1978), it is likely that their removal by thrombin in fibrinogen that was partially reduced in the absence of calcium led to the observed clotting time prolongation (Table I). It is also possible that the loss of thrombin clottability may have come from γ326Cys–γ339Cys disulfide bond reduction since the structure supported by this bond may be important for the function of the C-terminal "a" polymerization site which is complementary to the "A" site in the N-terminal end of fibrinogen.

Although the quantity of the free Aα17–19 (i.e., Gly-Pro-Arg peptide) in the sample would not be sufficient to totally inhibit thrombin-induced clot formation (Laudano & Doolittle, 1978), it appeared that the association of fibrin monomers lacking the "A" (or "a") polymerization site(s) into protofibrils would be unlikely. Nevertheless, this fibrinogen formed a clot after some time. Fibrinogen can undergo polymerization and gel formation in a thrombin-independent mechanism involving the plasma transglutaminase factor XIII (Blombäck et al., 1985). This type of gelation does not require the removal of fibrinopeptides and presumably occurs without activation of the polymerization site that encompasses the Aα17–19 residues. Since the fibrinogen used in our studies contained traces of factor XIII (0.05 unit/mg; Procyk et al., 1985), we investigated the thrombin clottability of reduced fibrinogen samples in the presence of 5 mM iodoacetamide, a potent inhibitor of factor XIII (unpublished observations). Inclusion of iodoacetamide had no effect on the clottability of fibrinogen that was partially reduced in the presence of calcium (i.e., 95.7% recovery of protein in clot matrix). By contrast, iodoacetic acid prevented thrombin-induced gel formation in fibrinogen that was partially reduced in the absence of calcium, thereby confirming that this fibrin monomer with missing Aα17–19 residues and reduced γ326Cys–γ339Cys disulfide bonds clotted by an oligomerization mechanism dependent on factor XIII cross-linking.

Cleavage of the carboxy-terminal γ326Cys–γ339Cys disulfide bond was the only clear difference between the fibrinogen that was susceptible to thrombin cleavage at the Aα19–20 bond and the fibrinogen that was not. It appears that reduction of the γ326Cys–γ339Cys disulfide bond and the subsequent apparent lack of calcium binding affect the entire conformation of the molecule. It is possible that after the normal Aα16Arg–17Gly cleavage occurs, the structure of the new N-terminus of the α-chain is not stabilized and therefore susceptible to cleavage by thrombin. The reoxidation experiments (Figure 2) indicated that it was not possible to

re-form the γ326Cys–γ339Cys disulfide bond by reoxidation. Importantly, none of the cleaved disulfide bonds re-formed in the fibrinogen that was partially reduced in the absence of calcium. This implied that the region perturbed as a consequence of the reduction was not limited to just the area encompassing the disulfide loop in the C-terminal γ-chain structure, but also probably included the C-terminal α-chain structure and the central globular nodule. Ongoing studies employing differential scanning calorimetry will be useful for examining the perturbation of compact structure in fibrinogen after reduction of specific disulfide bonds.

The sites for factor XIII induced cross-linking in fibrinogen are not located in regions supported by disulfide bonds, and limited reduction of fibrinogen did not affect γ- or α-chain cross-linking by factor XIII. The partially reduced fibrinogens formed gels in the presence of factor XIIIa. However, thiols such as DTT significantly increase the rate of gel formation and protein incorporation into fibrin(ogen) gel structures (Blombäck et al., 1985; Procyk et al., 1985). Thiols have a direct effect on factor XIII activity (Lorand & Jacobsen, 1958). However, the present work shows that the presence of low amounts of DTT during the cross-linking reaction will lead to the cleavage of at least three to five fibrinogen disulfide bonds, raising the possibility that fibrinogen thiol groups may participate in disulfide exchange reactions with thiols/disulfides on factor XIII. This type of interaction may possibly facilitate factor XIII–fibrinogen associations in solution and enhance the rate of gel formation.

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