Dimeric Half-Molecules of Human Fibrinogen Are Joined through Disulfide Bonds in an Antiparallel Orientation†

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ABSTRACT: Human fibrinogen is a dimer composed of two identical halves. Each dimeric half contains three peptide chains (α, β, and γ) linked by disulfide bonds. The two half-molecules are joined by three disulfide bonds, one between the two α-chains (residue α-28) and two between the two γ-chains (residues γ-8 and γ-9). In the absence of any definitive experimental evidence, it has been presumed that the joined halves were aligned in a parallel orientation similar to the situation found in immunoglobulins. We have now determined that the two γ-chains—hence, the dimeric halves—are connected in an antiparallel manner. A tryptic peptide containing γ-chain residues 6–14 was isolated as a disulfide-linked dimer from CNBr-treated fragment E. Synthetic peptides corresponding to this sequence were prepared, from which parallel and antiparallel dimers were constructed. During the syntheses, cysteine thiol groups were protected as *p*-methoxybenzyl and acetamidomethyl sulfides; the peptides were dimerized by selective deprotection and disulfide bond formation. First, the *p*-methoxybenzyl groups were removed by liquid hydrogen fluoride and the newly exposed thiols oxidized in the presence of potassium ferricyanide. Then the monocystine compound was converted to the double-cystine product by iodolytic cleavage of the acetamidomethyl group with concomitant disulfide bond formation. This selectivity was used to prepare peptide dimers which modeled both parallel and antiparallel arrangements. The antiparallel-oriented synthetic peptide was indistinguishable from the native tryptic peptide as judged by elution from reverse-phase high-performance liquid chromatography and circular dichroism spectroscopy. The parallel-oriented synthetic peptide differed from the native material by both criteria.

Human fibrinogen is made up the three pairs of nonidentical polypeptide chains. They are linked through an assortment of disulfide bonds to form a dimeric molecule, $(\alpha,\beta,\gamma)_2$ with a molecular weight of 340 000. Organization of the six chains into a more or less linear trinodular structure constitutes the currently accepted structural model of human fibrinogen (Doolittle, 1973, 1981). The two half-molecules are joined by three disulfide bonds in a small central domain region. The latter is flanked by two identical and slightly larger regions. The central domain contains, along with the amino termini of all 6 chains, 11 of the 29 disulfide bridges found in human fibrinogen (Blomback, 1970). Of the 11 bonds, 8 are involved in various interchain covalent associations within each half-molecule. The remaining three disulfide bonds unite the two halves to form the dimeric protein. The half-cystine at position 28 in the α-chain is linked with the same residue in the other α-chain. Similarly, γ-chain cysteines at positions 8 and 9 are paired with their counterparts to form a vicinal double-cystine connection. The arrangement of these disulfide-linked pairs was elucidated through amino acid sequence studies on cystine-containing peptides isolated from tryptic digests of the N-terminal disulfide knot (N-DSK), a fragment derived from whole human fibrinogen following treatment with cyanogen bromide (Blomback et al., 1968). This fragment is composed of most of the central domain moiety of the trinodular structure; as such, it contains the region where the half-molecules are joined. After thorough characterization of the N-DSK, Blomback (1970) proposed an arrangement of the disulfide bridges in the N-terminal "knot". In particular, the two subunits were joined through loci on the α-chain and the γ-chain, as mentioned above, in a manner that implied a 2-fold axis of symmetry, and, in this depiction, the axis of rotation ran through the three symmetric S–S bonds. The amino termini of the subunits were aligned in a parallel manner in order to accommodate this sort of symmetry. This alignment, particularly with respect to the two γ-chain cystine bonds, was similar to the arrangement of the disulfide-linked heavy chains in immunoglobulins (Gall et al., 1968; Edelman et al., 1969). The presence of two neighboring disulfide bonds effectively locks the directional orientation of the peptide chains in the N-DSK—and by extension the rest of the protein molecule—in a parallel alignment. Over the past dozen years then, the schematic representation of fibrinogen has been based on a parallel orientation of the half-molecules. Moreover, both the conversion to fibrin and subsequent polymerization have been explained in terms of such a structure. The orientation has never been demonstrated experimentally, however.

We report here that the orientation of the γ-chains about the two adjacent disulfide bonds at positions γ-8 and γ-9 is antiparallel. To this end, the native disulfide-linked segment of the γ-chain containing residues 6–14 was isolated from a tryptic hydrolysate of cyanogen bromide treated fragment E. The latter structure was derived from a plasmin digest of whole human fibrinogen and, like the N-DSK, contains most of the central domain moiety of the molecule (Marder, 1971). Actually, the two fragments are approximately 80% mutually overlapping (Takagi & Doolittle, 1975). The native tryptic peptide was compared with synthetic peptides assembled in parallel and antiparallel forms. In this regard, the appropriate γ-chain sequence was synthesized as a monomeric nonapeptide. Incorporation of differentially protected cysteine residues into the synthetic regimen provided the selectivity needed to form disulfide-linked dimeric peptides that modeled parallel and antiparallel alignments. The peptide isolated from fragment E, the antiparallel model peptide, and the parallel model peptide were all compared by analytical reverse-phase high-performance liquid chromatography (HPLC)[1] and circular

† From the Department of Chemistry, University of California, San Diego, La Jolla, California 92093. *Received November 9, 1982.* The investigation was supported by a grant from the National Institutes of Health (HL 18576). This paper is based on a Ph.D. dissertation submitted by P.D.H. to the University of California, San Diego.

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0006-2960/83/0422-2049$01.50/0

dichroism spectroscopy.

Experimental Procedures

Materials. Human fibrinogen was isolated from plasma obtained from the San Diego Blood Bank by cold ethanol precipitation as described previously (Doolittle et al., 1967). Fragment E was prepared by plasmin digestion of fibrinogen followed by chromatography over DEAE-cellulose (Nussenzweig et al., 1961; Doolittle et al., 1977). Human plasmin was obtained from Kabi and dissolved in 50% glycerol (10 units/mL) or prepared in situ from human plasminogen (National Red Cross) activated by streptokinase (Sigma) as described previously (Weinstein & Doolittle, 1972). The following were purchased from the indicated vendor: TPCK-trypsin (Worthington), cyanogen bromide (Matheson Coleman and Bell, MCB), Boc-amino acids (Vega and Bachem), dicyclohexylcarbodiimide (Aldrich). [^{14}C]Leucine was obtained from ICN and diluted with cold L-leucine to a specific activity of 0.1 mCi/mmol. *S*-(Acetamidomethyl)-L-cysteine was synthesized as described by Veber et al. (1972). Boc derivatives of *S*-(acetamidomethyl)-L-cysteine and L-[^{14}C]leucine were prepared according to Itoh et al. (1975).

Routine Methods. Amino acid analyses were performed on a Spinco Model 119 analyzer. All samples were hydrolyzed in 5.7 N HCl at 108 °C for 24 h in evacuated sealed tubes. Amino-terminal analyses were performed by the Dns-Cl procedure, essentially as described by Gray (1972). Performic acid oxidation was accomplished by a modification of the procedure described by Hirs (1967). An equal volume of freshly prepared performic acid was added to a solution of peptide (10 mg/mL) solubilized in formic acid (>98%, MCB) at 0 °C. The oxidative scission of the disulfide and the conversion to cysteic acid were effected during the course of a 1-h period at room temperature. The reaction mixture was diluted 50–100-fold with water and lyophilized over NaOH pellets. The lyophilizate was taken up in water for analytical HPLC or in 5.7 N HCl for amino acid analysis. HPLC was performed on a Beckman Model 332 liquid chromatograph equipped with a 5-μm Ultrasphere-ODS reverse-phase column (4.5 × 250 mm, Altex). Solvent A was 0.05% F_3AcOH/H_2O, and solvent B was acetonitrile (HPLC grade, MCB).

Results

Isolation of Dimeric γ-Chain Peptides. The disulfide-linked dimeric peptide containing γ-chain residues 6–14 was isolated from a tryptic hydrolysate of CNBr-treated fragment E. The initial step involved cleavage with CNBr. Typically, freeze-dried fragment E (55 mg) was dissolved in 70% formic acid at a concentration of 7–9 mg/mL. CNBr was added to a concentration of 15 mg/mL and the reaction allowed to continue for 13–18 h at room temperature. After this time, the sample was diluted 10-fold with cold water and lyophilized. The freeze-dried mixture of fragments was dissolved in 5.5 mL of 0.1 M NH_4HCO_3, pH 8.0, and digested with 2.75 mg of TPCK-treated trypsin at 37 °C for 16–18 h. The weight ratio of enzyme to substrate was 1:20. The digest was applied directly to a column of Sephadex G-50 (2.5 × 100 cm) equilibrated with 0.05 M NH_4HCO_3, pH 8.0. The peptides were eluted with the same buffer at a flow rate of 40 mL/h and collected as 4.5-mL fractions. Three peaks were observed (Figure 1). Fractions corresponding to pools I–III were combined and freeze-dried, and aliquots were subjected to amino acid analysis. Cysteine-containing peptides were present in each pool. A portion of each was subjected to paper electrophoresis. Approximately 300 nmol was applied to the middle of 3 × 16.5 in. paper (Schleicher & Schuell, 2043-B) and electrophoresed in pyridine/acetic acid buffer, pH 6.4, at 300 V for 3.5 h. All acidic peptides were eluted with 0.01 N NH_4OH, lyophilized, and screened for the presence of cysteine by amino acid analysis. In the case of pool II, a major peptide band that stained positively for arginine (Yamada & Itano, 1966) and migrated approximately half the distance traveled by the reference glutamic acid was eluted. The eluate was further purified by reverse-phase HPLC (Figure 2). The most prominent peptide eluted at 24 min in a 35-min elution program that ranged from 25 to 50% acetonitrile. Amino acid composition and NH_2-terminal amino acid data were consistent with the structure of the expected peptide (Table I).

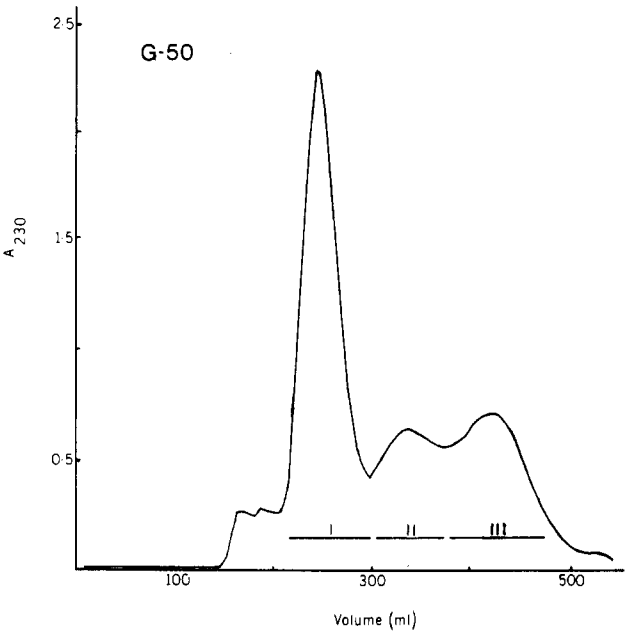


FIGURE 1: Gel filtration of tryptic peptides on Sephadex G-50. Fragment E (55 mg, 1 μmol) was treated with CNBr, freeze-dried, and digested with trypsin. The digestion was applied to a column (2.5 × 100 cm) equilibrated with 0.05 M NH_4HCO_3, pH 8.0; the peptides were eluted with the same buffer. Pool II was found to contain the dimeric γ-chain peptide (residues 6–14).

Table I: Amino Acid Composition and Amino-Terminal Residue Determination

amino acid	γ-chain[a] tryptic peptide	synthetic anti-parallel peptide	synthetic parallel peptide	theoretical value
Asx	3.10	3.25	3.00	3
Glx	1.20	1.20	1.10	1
Cys	1.90	1.90	2.00	2
Ile	0.94	0.75	0.81	1
Leu	0.96	0.85	0.95	1
Arg	1.00	1.10	1.08	1
NH_2-terminal[b] amino acid	Asx	Asx	Asx	Asx

[a] Values are expressed as moles per mole of peptide and represent an average of three determinations. Samples were hydrolyzed in evacuated sealed tubes for 24 h at 108 °C, in 5.7 N HCl. [b] Determined with the dansyl chloride method (Gray, 1972).

[1] Abbreviations: CNBr, cyanogen bromide; Dns-Cl, 5-(dimethylamino)naphthalene-1-sulfonyl chloride; DMF, dimethylformamide; DCCD, dicyclohexylcarbodiimide; Boc, *tert*-butoxycarbonyl; F_3AcOH, trifluoroacetic acid; Tos, *p*-toluenesulfonyl; OBzl, benzyloxy; MBzl, *p*-methoxybenzyl; Acm, acetamidomethyl; HF, hydrogen fluoride; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography.

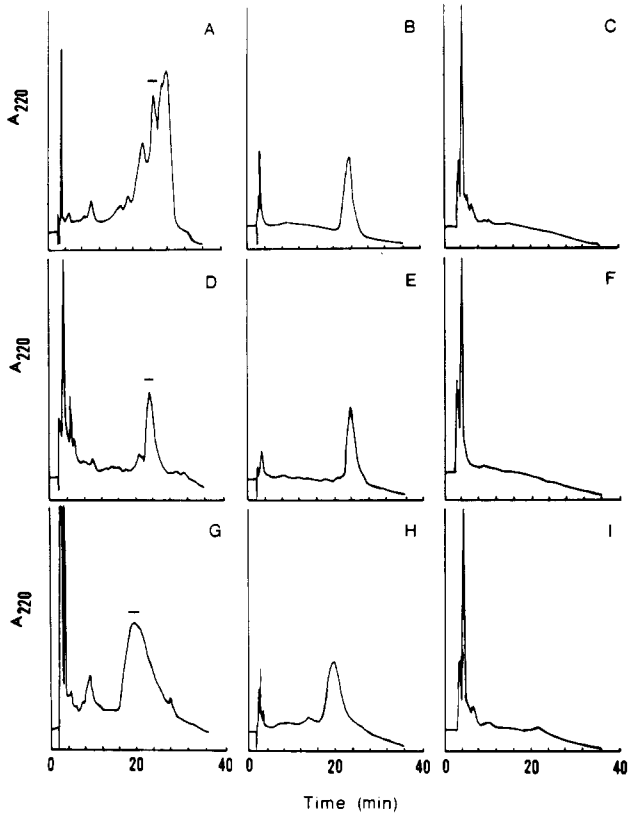
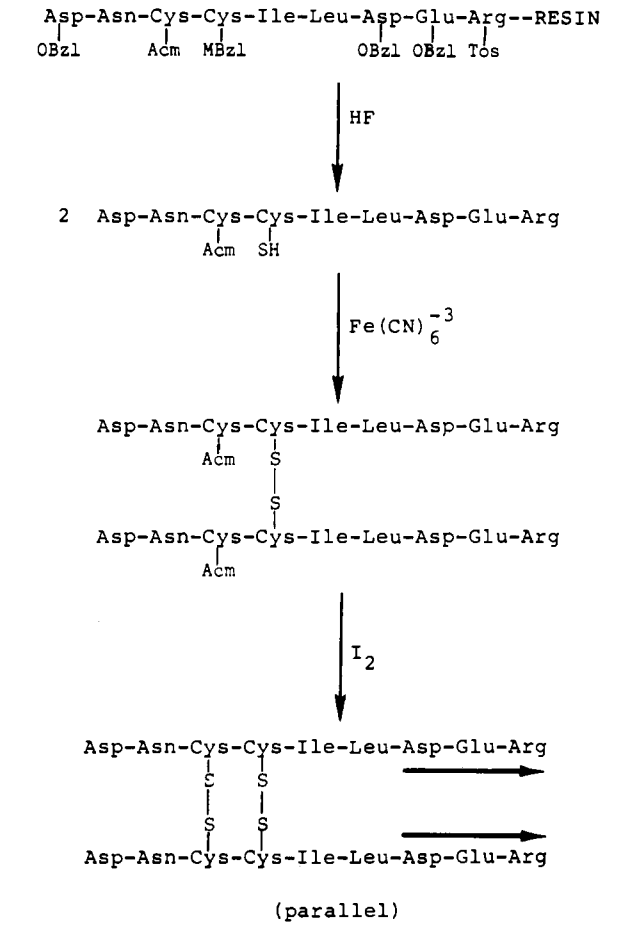


FIGURE 2: Analytical HPLC elution profiles. Panels from left to right show chromatograms of material eluted from pH 6.4 paper electrophoresis, rechromatography of peptides purified from pH 6.4 eluates by reverse-phase HPLC, and chromatograms of material after performic acid oxidation. Elution profiles in panels A–C correspond to a synthetic antiparallel peptide, panels D–F correspond to a γ-chain tryptic peptide, and panels G–I correspond to a synthetic parallel peptide. The bars shown in profiles A, D, and G indicate material collected from each eluate that was used for subsequent rechromatography, performic acid oxidation, and CD spectroscopy. In all cases, the peptide material (20–50 nmol) was eluted from a reverse-phase column (4 × 250 mm, 5-μm Ultrasphere-ODS, Altex) by using 0.05% trifluoroacetic acid and a linear gradient of acetonitrile from 25 to 50% over 35 min.

Based on 55 mg (1 μmol) of fragment E, a yield of 125 ± 25 nmol (12.5%) of pure peptide was typically obtained. This material was lyophilized and stored under vacuum.

Synthesis of the Parallel Model Peptide. The synthesis used to prepare this cystine-containing peptide depended on the specific formaiton of disulfide bonds. This was accomplished by protecting the thiol groups as two different thioethers. Selective introduction of cystine bonds was effected by removing each protecting group under different conditions (Scheme I). A nonapeptide corresponding to γ-chain residues 6–14 was synthesized according to established Merrifield solid-phase synthetic procedures (Stewart & Young, 1969; Erickson & Merrifield, 1976). The first amino acid, Boc-N^g-tosyl-L-arginine, was esterified to chloromethylated polystyrene resin (Bio-Rad, 1% cross-linked, 1.34 mequiv/g) by using a recently described procedure employing potassium fluoride and DMF (Horiki et al., 1978). Briefly, chloromethylated polymer (5 g, 1 equiv) and anhydrous potassium fluoride (0.8 g, 3 equiv) were added to a solution of Boc-N^g-tosyl-L-arginine (2.5 g, 1.5 equiv) in 30 mL of Sequanol-grade DMF (Pierce). The reaction mixture was stirred at 50 °C in an oil bath for 40 h. After the mixture was washed and dried, an aliquot (3–5 mg) of the resin was hydrolyzed in 50% propionic acid/HCl for 4 h at 125 °C in an evacuated sealed tube. The degree of substitution was determined to be

Scheme I



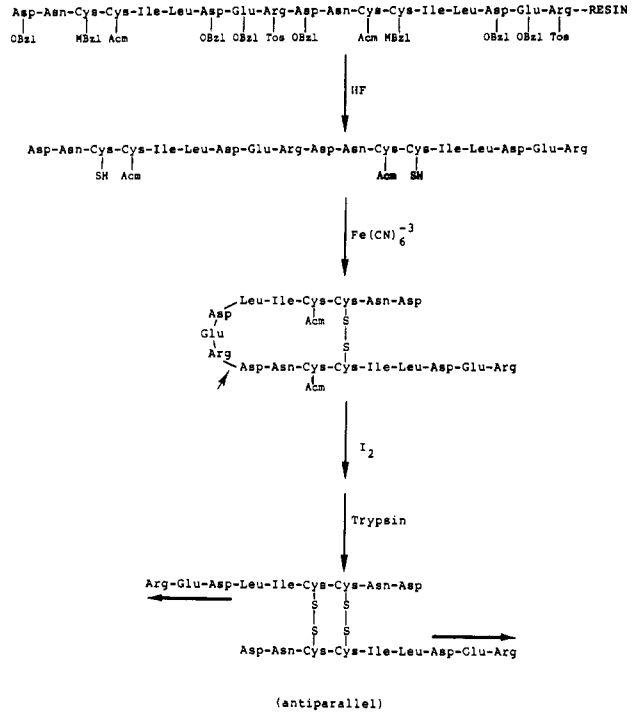
0.65 mmol/g of resin. Subsequent amino acids were double coupled in appropriate solvents through a DCCD-mediated reaction. Boc-L-[^{14}C]leucine was added for the initial coupling and followed by cold Boc-L-leucine in the second reaction. Boc-L-asparagine was added as a *p*-nitrophenyl ester. Aspartic acid and glutamic acid were protected as β- and γ-benzyl esters, respectively. In the case of cysteine, the thiol groups were protected as *p*-methoxybenzyl (MBzl) and acetamidomethyl (Acm) thioethers. The Boc group was removed by treatment with 50% F_3AcOH in dichloromethane for 30 min followed by neutralization with 10% triethylamine in chloroform. The resin was washed with appropriate solvents before and after each deprotection and neutralization step. Boc removal and completeness of coupling were monitored by a ninhydrin color test (Kaiser et al., 1970). The completed nonapeptide was cleaved from the resin with simultaneous removal of side-chain protecting groups by treatment with anhydrous HF. Typically, 2 g of peptide resin was treated with anhydrous HF (10 mL/g) in the presence of a 5-fold molar excess of anisole. The reaction time was 40 min; the temperature was kept between 0 and 4 °C by an ice/H_2O bath. After HF was removed by water aspiration, the resin was washed with two portions (30 mL) of anhydrous ethyl ether to remove anisole. The peptide was washed from the resin with 10% aqueous acetic acid (four 30-mL portions); the combined washings were immediately lyophilized and freeze-dried from demineralized water twice more. The nonapeptide was deprotected completely by this treatment except for the Acm group on cysteine which is not removable by HF. Oxidation of the exposed thiol groups to the monocystine compound proceeded directly (Scheme I). The formation of the first disulfide bond was accomplished by using potassium ferri-

cyanide as the oxidant (Hope et al., 1962). In general, the peptide (30–40 mg) was dissolved in 0.15 M NH_4OAc, pH 6.9 (10 mg/mL); the concentration of thiol was determined with 5,5′-dithiobis(2-nitrobenzoic acid) as described by Habeeb (1972). The peptide solution was added dropwise over a 15-min period to a stirred solution containing an excess (10% over the thiol concentration) of potassium ferricyanide (9.8 mg/mL 0.15 M NH_4OAc, pH 6.9). The pale yellow reaction mixture was stirred for an additional 45 min at room temperature. Throughout the oxidation, the pH was monitored and adjusted to pH 6.9 by the addition of 1.5 N NH_4OH. After 1 h, the solution was acidified with 0.5 volume of glacial acetic acid, e.g., 2–4 mL, and stirred with 0.5 g of AG3X4A resin (Bio-Rad) for an additional 10 min. The weak anion exchanger removed ferro- and ferricyanide. The resin was filtered off, yielding a clear filtrate that was lyophilized.

The second disulfide bond was formed when the acetamidomethyl group was removed iodolytically (Kamber et al., 1980; J. Rivier, personal comunication). This procedure was carried out by dissolving the monocystine compound (15 mg) in 8 mL of a solution of DMF (Sequanal, Pierce) and F_3AcOH (8:1). The peptide solution was cooled in an ice/H_2O bath and purged briefly with nitrogen. A 10-fold molar excess of I_2 (MCB), dissolved in 1 mL of the same mixed solvent, was added; the burgundy-colored reaction mixture was removed from the ice/H_2O bath and stirred for 10 min under nitrogen at room temperature. Termination of the oxidation was accomplished by reducing excess iodine with ascorbic acid (24.6 mg) dissolved in 2 mL of 0.1 M sodium citrate, pH 5.5. Upon addition of the reducing solution, the reaction mixture cleared. It was diluted 5-fold with water and lyophilized. The salty residue resulting was taken up in a small volume (5 mL) of 0.05 M NH_4HCO_3, pH 8.0, and chromatographed over the same Sephadex G-50 column used to fractionate the tryptic digest of CNBr-treated fragment E. Fractions eluting in the region of pool II were combined and freeze-dried (Figure 1). This material was purified by the combination of paper electrophoresis at pH 6.4 and reverse-phase HPLC as described above for the native γ-chain tryptic peptide. HPLC of the synthetic material following paper electrophoresis showed a broad peak, the central portion of which was isolated and used for subsequent comparative experiments (Figure 2). Amino acid composition and amino-terminal amino acid data were consistent with expected values (Table I). The overall yield starting from 35 mg of nonapeptide was 7.8 mg (22%) of fully oxidized parallel dimer. The lyophilized peptide was stored under vacuum.

Synthesis of the Antiparallel Model Peptide. The antiparallel model peptide was prepared from an octadecapeptide that was essentially a linear dimer of the γ-chain sequence containing residues 6–14. Introduction of the first disulfide bond was accomplished by an intramolecular cyclization to form the monocystine compound; the Acm group was removed iodolytically as described for the parallel synthesis. Treatment of the fully oxidized peptide with trypsin resulted in the antiparallel structure (Scheme II). The octadecapeptide needed for preparation of the antiparallel model was made by duplicating the nonapeptide structure already on the resin. The synthesis of the additional peptide unit was accomplished as previously described except that the order of addition of the differentially protected cysteine residues was reversed. After cleavage and deprotection in anhydrous HF, the peptide was cyclized by oxidizing the thiol groups with potassium ferricyanide. As an example, the linear dimer (66 mg) was dissolved in 0.5% aqueous acetic acid (300 mL) and pumped at

Scheme II



a rate of 15 mL/h into a solution of potassium ferricyanide (22.5 mg) in 300 mL of 0.1 M NH_4HCO_3, pH 8.0, over a 24-h period. Dilute reaction conditions (0.1 mM) and an acidic reservoir for the unoxidized material were employed to minimize intermolecular polymerization and encourage intramolecular disulfide bond formation. The reaction mixture was acidified with 5 mL of glacial acetic acid followed by removal of ferro- and ferricyanide with AG3X4A anion exchanger (1.5 g). The resin was removed by filtration and the clear filtrate lyophilized. The monocystine compound was taken up in a minimal volume of 0.05 M NH_4HCO_3, pH 8.0, and chromatographed over Sephadex G-50 (2.5 × 100 cm). Separation of the cyclized material from the high molecular weight polymer was accomplished, and fractions eluting in the same vicinity as the native γ-chain peptide were pooled and lyophilized. The yield of the cyclized monocystine compound was 25 mg (38%). Formation of the second disulfide bond was accomplished by oxidation with iodine as described. The peptide with both disulfide bonds formed was gel filtered and pooled as outlined for the parallel model. The overall yield relative to the unoxidized octadecapeptide was 4.8 mg (7.3%). Tryptic cleavage of the Arg–Asp bond resulted in the antiparallel model peptide. The peptide was dissolved in 0.1 M NH_4HCO_3 (1 mL), pH 8.0, and 0.1 mg of TPCK–trypsin was added. The cleavage continued for 6 h at 37 °C, after which the sample was lyophilized. The weight ratio of enzyme to peptide was 1:48. As in the other isolations described above, the material was purfied by paper electrophoresis at pH 6.4 and reverse-phase HPLC. Unlike the native peptide and the synthetic parallel preparations, the HPLC step revealed the presence of several closely eluting peaks (Figure 2). One of the peaks exhibited the same retention time as the native peptide and was collected on a preparative scale by repeated HPLC runs. A portion of this material was subjected to amino acid analysis and NH_2-terminal analysis (Table I). The peptide was stored lyophilized and under vacuum.

Comparison of Synthetic and Native Peptides. (*a*) *Analytical HPLC.* Purified synthetic peptide in the antiparallel orientation chromatographed identically on reverse-phase

HPLC with native γ-chain peptide. Elution profiles from analytical HPLC runs on the peptides purified as described above are shown in Figure 2. The elution time for the parallel model peptide was 4 min earlier than the elution times for the antiparallel model peptide and the peptide isolated from fragment E. The latter two peptides eluted at the same time. A portion of each of the three peptides was oxidized by performic acid. Such treatment should produce a nonapeptide containing two cysteic acid residues. Moreover, the newly derived material should be the same from each peptide irrespective of parallel or antiparallel orientation. In each of the three cases, the peptide peak eluting between 20 and 25 min disappeared and was replaced by a sharp, early eluting spike (Figure 2). This result was consistent with the generation of a smaller, more polar peptide. Amino acid analysis of the early eluting peptide from each oxidation was characterized by the absence of cysteine and the appearance of cysteic acid. There was no change in the relative amounts of the other amino acids.

These results show that a difference between parallel and antiparallel preparations can be discerned by reverse-phase HPLC and indicate that the peptide chains of the native dimeric peptide are oriented in an antiparallel manner.

(*b*) *Circular Dichroism Spectroscopy*. The interaction of disulfide bonds with circularly polarized light in the near-UV region can provide useful information about conformation (Adler et al., 1974; Kahn, 1979). CD bands characteristic of the S–S bond occur between 250 and 270 nm. The magnitude and direction of the absorption in this region are a function of the disulfide bond, i.e., absolute geometry of the C–S–S–C dihedral angle. Accordingly, the three peptides were examined by CD spectroscopy between 230 and 330 nm. CD spectra were recorded on a Jasco Model J-10 spectropolarimeter. The peptides were dissolved in 0.1 N HCl at concentrations ranging from 0.15 to 0.3 mM. The temperature was maintained at 22 °C by means of an external temperature bath. The instrument was calibrated with a 0.1% aqueous solution of *d*-10-camphorsulfonic acid (MCB) and L-cystine (Sigma) at 0.5 mg/mL in 0.1 N HCl (Adler et al., 1974; Takagi & Itoh, 1972).

The patterns of the CD spectra recorded for the antiparallel model peptide and the native γ-chain peptide were virtually identical (Figure 3). Each was characterized by broad maxima at 254 nm preceded by a shoulder around 265 nm and a slight negative band at 237 nm. The spectra of the parallel preparation were significantly different on two counts: a much more pronounced negative band in the vicinity of 232 nm and a skewed, somewhat less broad, positive absorption at 254 nm.

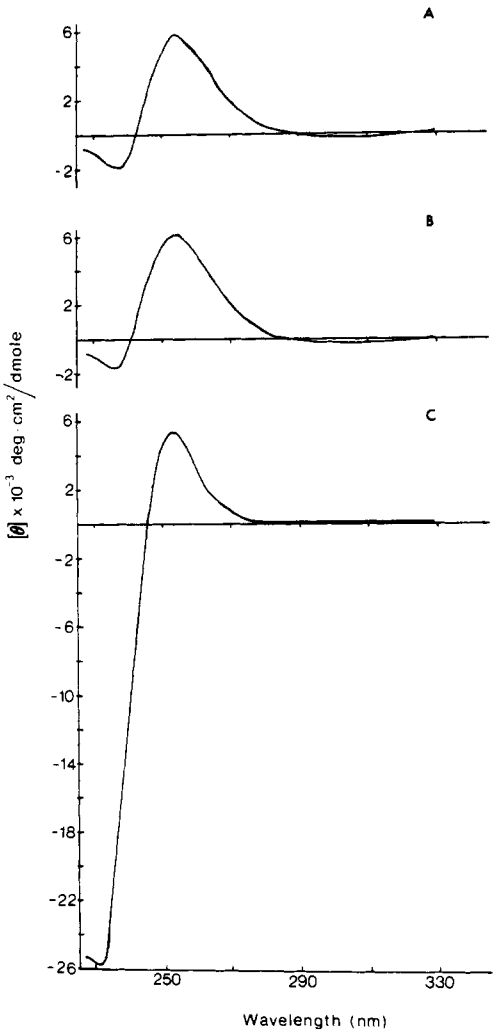


FIGURE 3: Circular dichroism spectra of (A) a dimeric γ-chain peptide isolated from fragment E, (B) a synthetic antiparallel model peptide, and (C) a synthetic parallel model peptide. Samples were dissolved in 0.1 N HCl at concentrations between 0.15 and 0.30 mM and scanned between 330 and 230 nm at 22 °C. [θ] was computed as the molar ellipticity.

Discussion

Two adjacent disulfide bonds, involving cysteine residues at positions 8 and 9, join the γ-chains in the central domain region of human fibrinogen to form the dimeric protein. Theoretically, the two chains can be aligned relative to one another in a parallel manner, i.e., common amino- to carboxyl-terminus direction, or in an antiparallel manner, i.e., opposing amino- to carboxyl-terminus direction. In order to determine which orientation exists in human fibrinogen, we isolated an unreduced tryptic peptide containing residues 6–14 of the γ-chain from fragment E. This peptide was compared with synthetic peptides of the same sequence that modeled both parallel and antiparallel orientations. The synthetic peptide dimeric compounds were prepared explicitly by selective deprotection and oxidation of cysteine thiol groups. In the parallel case, disulfide bonds linked positions γ-8 with γ-8 and γ-9 with γ-9 (Scheme I). Conversely, the successive oxidation of cysteine residues γ-8 and γ-9 yielded the antiparallel structure (Scheme II).

Experiments comparing the native dimeric γ-chain tryptic peptide with the two synthetic peptides revealed that the synthetic antiparallel model peptide was indistinguishable from the γ-chain peptide by the criteria employed. In particular, elution times from analytical reverse-phase HPLC runs on purified material were the same for the antiparallel peptide and the native peptide; the synthetic parallel model peptide eluted at a different (earlier) time (Figure 2). This difference was not artificial, as shown by oxidative scission of the disulfide bonds with performic acid. Under the conditions used, this treatment should produce two nonapeptide half-molecules that contain cysteic acid and no cysteine. As expected, a smaller and considerably more polar peptide was observed by reverse-phase HPLC, but more importantly, following oxidation the same elution profile was observed for each of the three peptides irrespective of orientation—parallel or antiparallel—and origin—synthetic or naturally occurring.

An addtional comparison was made by taking advantage of the disulfide bond as a chromophore in the near-UV region. Disulfide bonds can exist with a right- or left-hand screw sense and thus are optically active—uniquely so with circularly polarized light (Beychok, 1965). Circular dichroism spec-

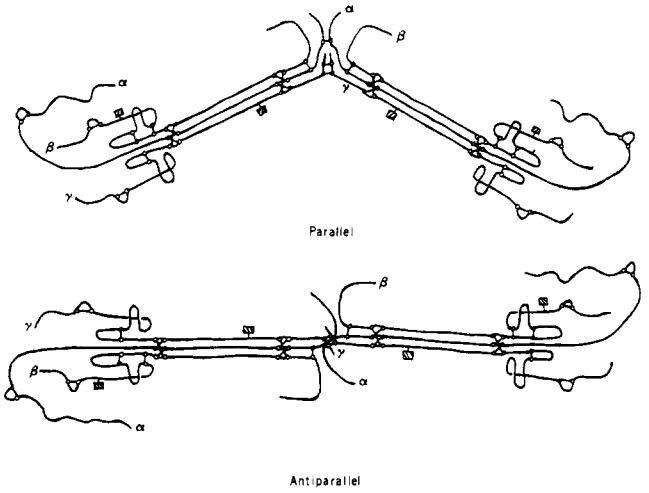


FIGURE 4: Proposed arrangement of the six polypeptide chains of human fibrinogen in parallel and antiparallel alignments about the adjacent γ-chain disulfide bonds involving residues γ-8 and γ-9. The dyad axis, through these same bonds, is in the plane of the paper for the parallel figure and is projecting normal to the plane of the paper for the antiparallel figure. Shown also are the relative locations of the remaining 27 disulfide bonds and the carbohydrate clusters (hatched squares). It should be stressed that these are two-dimensional representations and that the three-dimensional arrangement of chains apart from the junction of the two half-molecules may be quite different.

troscopy was used to compare the synthetic peptides modeling parallel and antiparallel structures with the naturally occurring peptide. The scans presented in Figure 3 demonstrate virtually superimposable spectra recorded for the antiparallel model peptide and the γ-chain tryptic peptide dimer. This observation also indicates that the relative orientation of the two γ-chains in the native dimeric peptide is antiparallel. The magnitude and positive nature of the ellipticity at 254 nm suggest a right-hand screw sense and a dihedral angle around the S–S bond in the vicinity of +90° (Kahn, 1979). Incorporating these observations, we assembled space-filling models of the region about the vicinal cysteine residues and found that the two peptide chains are readily accommodated alongside one another when aligned in an antiparallel manner. Under these circumstances, they are joined by disulfide bonds having a right-hand screw sense and a dihedral angle of approximately 90° around the S–S bonds.

The consequences of an antiparallel orientation of the γ-chains in the central domain can be extended to include the entire human fibrinogen molecule. According to the currently accepted schematic depiction, fibrinogen is a trinodular structure. There is a central region containing six amino termini—two from each of the α-, β-, and γ-chains, including the fibrinopeptides A and B—where the half-molecules are joined. This middle nodule is flanked by two identical and slightly larger domains which are tethered to the central portion by equivalent segments from each chain thought to be in the form of "coiled coils" (Doolittle et al., 1978). Taken together, the three regions are arranged in a linear manner to yield a trinodular structure. This model was first proposed by Hall & Slayter (1959) on the basis of electron micrographs of bovine fibrinogen visualized by platinum shadow casting and has endured, comparing favorably with data amassed from the study of fibrinogen by other techniques [for reviews, see Doolittle (1973, 1981)]. Recently, electron microscopy studies of human and bovine fibrinogen visualized by shadowing and negative stain procedures have appeared which confirm the observations of Hall and Slayter (Fowler & Erickson, 1979; Williams, 1981; Mosesson et al., 1981). In all these reports, the majority of molecules are seen as linear trinodular structures. Moreover, the micrographs published by Williams (1981) show fibrinogen molecules whose overall shape is best described as being similar to an integral sign—an observation consistent with an antiparallel alignment, as dipicted in Figure 4.

In large measure, the conceptual understanding of fibrin formation and subsequent polymerization events leading to an insoluble gel is unchanged in light of a fibrinogen molecule with half-molecules joined in an antiparallel manner. There are a number of details that are affected, however, beginning with the initial events in the process. With regard to the removal of the fibrinopeptides by thrombin, it must be noted that the locations of the fibrinopeptides A and B relative to each other are fundamentally different in the two alignments (Figure 4). Beyond that, the "knobs" so exposed by this limited proteolysis, which interact with "holes" in the terminal domains of adjacent fibrin molecules, will also have different orientations. The differences will be reflected in subsequent associations.

The initial fibrin dimer–oligomer will still elongate in a bidirectional staggered overlapping manner two molecules thick to form an intermediate polymer as diagrammed previously (Doolittle, 1973, 1981). The intermediate polymer stage is characterized not only by a half-molecule overlap but also by an end to end alignment of terminal domains contributed by adjacent fibrin monomers. Abutment in this manner allows for stabilization of the polymer by an enzyme-catalyzed formation of intermolecular covalent bonds. Specifically, the carboxy-terminal portions of γ-chains from neighboring molecules are covalently linked through two ε-(γ-glutamyl)lysine bonds introduced by the action of factor $XIII_a$. The orientation of two γ-chains so joined is antiparallel (Chen & Doolittle, 1971). With respect to the α-chains and factor $XIII_a$-mediated cross-linking, the relative location of glutamyl acceptor sites and putative lysine donor sites in the carboxy-terminal region may necessitate an antiparallel orientation of the chains (Cottrell et al., 1979). These two aspects of fibrin polymerization—γ-chain cross-linking at the intermediate polymer stage and α-chain cross-linking to form multimeric arrays—are major structural features that involve antiparallel molecular juxtaposition. That the fibrinogen molecule is itself dimerized in an antiparallel manner is a result consistent with accepted structure–function properties of the molecule.

Acknowledgments

We are grateful to Marcia Riley for preparing human fibrinogen and to Dennis Trovato for operation of the amino acid analyzer. We thank Jean Rivier and Harry Anderson of the Peptide Biology Lab at The Salk Institute for use of their HF apparatus and helpful discussions about iodolytic cleavage of the acetamidomethyl group.

References

Adler, A. J., Greenfield, N. J., & Fasman, G. D. (1974) *Methods Enzymol. 28*, 675.

Beychok, S. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 999.

Blomback, B. (1970) *Symp. Zool. Soc. London No. 2*, 167.

Blomback, B., Blomback, M., Henschen, A., Hessel, B., Iwanaga, S., & Woods, K. R. (1968) *Nature (London) 218*, 130.

Chen, R., & Doolittle, R. F. (1971) *Biochemistry 10*, 4486.

Cottrell, B. A., Strong, D. D., Watt, K. W. K., & Doolittle, R. F. (1979) *Biochemistry 18*, 5405.

Doolittle, R. F. (1973) *Adv. Protein Chem. 27*, 1–109.

Doolittle, R. F. (1981) in *Haemostasis and Thrombosis* (Bloom, A. L., & Thomas, D. P., Eds.) Chapter 11, pp 163–191, Churchill Livingstone, London.
Doolittle, R. F., Schubert, D., & Schwartz, S. R. (1967) *Arch. Biochem. Biophys. 118*, 456.
Doolittle, R. F., Cassman, K. G., Cottrell, B. A., Friezner, S. J., & Takagi, T. (1977) *Biochemistry 16*, 1710.
Doolittle, R. F., Goldbaum, D. M., & Doolittle, L. R. (1978) *J. Mol. Biol. 120*, 311.
Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlich, P., Rutishauser, U., & Waxdel, M. J. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 78.
Erickson, B. W., & Merrifield, R. B. (1976) *Proteins (3rd Ed.) 2*, 256.
Fowler, W. E., & Erickson, H. P. (1979) *J. Mol. Biol. 134*, 241.
Gall, W. E., Cunningham, B. A., Waxdel, M. J., Konigsberg, W. H., & Edelman, G. M. (1968) *Biochemistry 7*, 1973.
Gray, W. R. (1972) *Methods Enzymol. 25*, 11.
Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 247.
Hall, C., & Slayter, H. (1959) *J. Biophys. Biochem. Cytol. 5*, 11.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 59.
Hope, D. B., Murti, V. V. S., & Du Vigneaud, V. (1962) *J. Biol. Chem. 237*, 1563.
Horiki, K., Igano, K., & Inouye, K. (1978) *Chem. Lett.*, 165–168.
Itoh, N., Nagiwana, D., & Kamiya, T. (1975) *Tetrahedron Lett.*, 4393.
Kahn, P. (1979) *Methods Enzymol. 61*, 339.
Kaiser, E., Colescott, R. L., Bassinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595.
Kamber, B., Hartman, A., Eisler, K., Riniker, B., Rink, H., Sieber, P., & Rittel, W. (1980) *Helv. Chim. Acta 63*, 899.
Marder, V. J. (1971) *Scand. J. Haematol., Suppl. No. 13*, 21.
Mosesson, M. W., Hainfeld, J., Wall, J., & Haschemeyer, R. H. (1981) *J. Mol. Biol. 153*, 695.
Nussenzweig, V., Seligman, M., Pelmont, J., & Grabar, P. (1961) *Ann. Inst. Pasteur, Paris 100*, 377.
Stewart, J. M., & Young, J. D. (1969) *Solid Phase Peptide Synthesis*, W. H. Freeman, San Francisco, CA.
Takagi, T., & Itoh, N. (1972) *Biochim. Biophys. Acta 257*, 11.
Takagi, T., & Doolittle, R. F. (1975) *Biochemistry 14*, 940.
Veber, D. F., Milkowski, J. D., Varga, S. L., Denkewalter, R. G., & Hirschman, R. (1972) *J. Am. Chem. Soc. 94*, 5456.
Weinstein, M. J., & Doolittle, R. F. (1972) *Biochim. Biophys. Acta 258*, 577.
Williams, R. C. (1981) *J. Mol. Biol. 150*, 399.
Yamada, S., & Itano, H. A. (1966) *Biochim. Biophys. Acta 130*, 538.

Conversion of Spin State and Protein Structure in *Rhodospirillum rubrum* Ferric Cytochrome *c'* Coupled with Dissociation of Dimer†

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ABSTRACT: Correlation between the spin states of *Rhodospirillum rubrum* cytochrome *c'* and its protein structure was studied by employing proton magnetic resonance, ultracentrifugation, and low-angle laser light scattering. ^{1}H NMR studies showed that the cytochrome *c'* is in high-spin states at up to pD 11 and in low-spin states at pD higher than 12 or in the presence of 2-propanol or sodium dodecyl sulfate ($NaDodSO_4$) at neutral pD. The protein signals in the 360-MHz ^{1}H NMR spectrum are widely spread up to pD 11 but show a coalesced pattern in the strongly alkaline pD region (>12) or in the presence of 2-propanol or $NaDodSO_4$. Therefore, it was concluded that the protein structure is rigid in the high-spin states and relaxed in the low-spin states. The molecular weight of the cytochrome *c'* was examined by sedimentation equilibrium and low-angle laser light scattering methods under various conditions. It was deduced that the cytochrome *c'* in the high-spin state has dimer form, whereas it takes monomer form in the low-spin state. This indicates that there is a correlation between spin states and the monomer–dimer conversion. A relaxed structure which is triggered by the dissociation of the dimer form into monomers could be primarily responsible for the low-spin state. The biological significance of the correlation is discussed in connection with the membrane-bound type of *R. rubrum* cytochrome *c'*.

Cytochrome *c'* constitutes a class of electron transport proteins which are widely distributed in phototrophic and aerobic bacteria (Bartsch, 1978). The heme is covalently bound to cysteine residues, as in other *c*-type cytochromes, but the properties of the proteins are quite different. The visible absorption spectrum of cytochrome *c'* at neutral pH resembles that of myoglobin rather than that of cytochrome *c*. Three and two spectroscopically distinguishable forms were observed in the oxidized and reduced states, respectively, by the use of electronic absorption (Horio & Kamen, 1961; Imai et al., 1969a), circular dichroism (Imai et al., 1969b; Rawlings et al., 1977), resonance Raman (Strekas & Spiro, 1974; Kitagawa et al., 1977), and nuclear magnetic resonance (Emptage et al., 1981; La Mar et al., 1981) spectroscopies. In the case of the oxidized state, type I characterized by the Soret band at 390 nm appears at acidic and neutral pH. The spin state of this form has been controversial. Originally it was assigned to a high-spin state (Horio & Kamen, 1961; Moss et al., 1968; Ehrenberg & Kamen, 1965; Tasaki et al., 1967) but afterwards interpreted as a quantum mechanical admixture of the intermediate-spin ($S = {}^3/_2$) and high spin ($S = {}^5/_2$) states on

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0006-2960/83/0422-2055$01.50/0