Fibronectin exists in a soluble form in body fluids and as a fibrillar component of the extracellular matrix. Matrix fibronectin associates as large complexes in SDS unless a reducing reagent is also present. This observation suggests that complex formation is due to interprotomeric disulfides that form by thiol-disulfide exchange. To localize the presumptive new disulfides, we labeled protomeric fibronectin by the chloramine-T method or with 125I-Bolton-Hunter reagent, incorporated 125I-fibronectin into the matrix of cultured fibroblasts, and subjected matrix fibronectin to acid or cyanogen bromide digestion. When cyanogen bromide digests of matrix 125I-fibronectin and protomeric 125I-fibronectin labeled with Bolton-Hunter reagent were analyzed by two-dimensional polyacrylamide gel electrophoresis in SDS, with the first dimension being nonreducing and the second reducing, we were not able to identify any fragments of matrix fibronectin that migrated as high molecular weight complexes in the first dimension. Limited acid digestion of matrix 125I-fibronectin also dissociated the majority of the high molecular weight complexes. Since we could account for all of the parts of fibronectin that contain cysteine or cystine, we conclude that matrix fibronectin is not stabilized by interprotomeric disulfides. We propose, instead, that stabilization is mediated by noncovalent protein-protein interactions that are sensitive to reduction, cyanogen bromide digestion, or limited acid digestion.

Fibronectin exists in two forms, soluble and insoluble. Soluble protomeric fibronectin is found at substantial concentrations in plasma and other body fluids and in the conditioned media of cultured cells. Insoluble fibronectin is a fibrillar component of the extracellular matrix (1–3). The insoluble form of fibronectin mediates cell attachment, provides a substrate for cell migration during embryogenesis and wound healing, and thus is considered to be the primary functional form of the protein (2, 4–7). Absence of fibronectin is lethal to mouse embryos (8). In vitro and in vivo studies have shown that matrix fibronectin consists of both endogenously synthesized cellular fibronectin and circulating plasma fibronectin (3, 9, 10).

Unlike many other matrix molecules with conspicuous self-assembly characteristics, purified plasma fibronectin forms insoluble precipitates in vitro only under special conditions of uncertain physiological significance (11–17). In fibroblast cultures, fibronectin fibrils are found on the surface of or between cells (1). Deposited matrix fibronectin is in the form of multimers that resist dissociation with SDS unless sulfhydryl-containing reagents are also present (18–23). This observation suggests that the multimers are stabilized by interprotomeric disulfides. Because blockage of the free sulfhydryls of fibronectin does not impede subsequent formation of SDS-stable multimers, it was further hypothesized that the interprotomeric disulfides form by thiol-disulfide exchange rather than by oxidation of free sulfhydryls (4, 23). Transglutaminase-mediated cross-linking by activated blood coagulation factor XII also promotes insolubilization (24, 25).

Identification of the new disulfides should be as simple as comparing polyacrylamide gel electrophoresis (PAGE) patterns in SDS of fibronectin fragments analyzed without or with reducing reagent. Fragments of 125I-labeled protomer and multimer should be the same after reduction, whereas without reduction, two fragments should migrate as a complex in the digest of multimer but not in the digest of protomer. Using such reasoning, our laboratory has argued that multimerization of 125I-fibronectin involves disulfides in the 70-kDa cathepsin D NH2-terminal fragment (23). We found it to be impossible to advance beyond our 1984 study (23) using proteases and began to question the existence of interprotomeric disulfides. In the present paper, we report the negative results of a more rigorous search for interprotomeric disulfides using acid and cyanogen bromide digestion. Because we could identify all of the parts of fibronectin that contain disulfides, these experiments indicate that interprotomeric disulfides do not form during stabilization of fibronectin and are compatible with the formation of strong noncovalent protein-protein interactions that resist disruption with SDS.

MATERIALS AND METHODS

Labeling of Fibronectin—Fibronectin was iodinated on tyrosines by the chloramine-T method (26) or on lysines using Bolton-Hunter reagent (iodinated 3-(4-hydroxyphenyl)propionic acid N-hydroxy succinimide ester) (ICN, Costa Mesa, CA). For the lysine labeling, 200 μl of 10 mg/ml fibronectin in 0.1 M borate buffer, pH 8.5, was placed in a reaction flask containing the dried 125I-Bolton-Hunter reagent at 0°C and rocked for 1 h. Glycine in borate buffer was added to the reaction mixture (final concentration, 66 μM) to stop the reaction. Iodinated fibronectin was separated from glycine and unreacted reagent by passage through a Sephadex G-25 column (8-ml bed volume). Bovine serum albumin and phenylmethylsulfonyl fluoride (Sigma) were added to the labeled protein to final concentrations of 0.2% and 1 mM, respectively. The mixture was dialyzed against phosphate-buffered saline, pH 7.4, and stored in small aliquots at –70°C until used. The labeled protein was detected by SDS-PAGE without or with reduction followed by autoradiography (data not shown) and had a specific activity of 77 Ci/mg fibronectin.

The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; NEM, N-ethylmaleimide; DSF, disulfide-containing fragment.
Cyanogen Bromide Digestion of Protemeric and Stabilized Matrix Fibronectin—Human neonatal foreskin fibroblast TJ 6F cells were cultured as described (24). To isolate matrix-bound 125I-fibronectin, confluent cells in 10-cm tissue culture plates were incubated with 2 × 10^6 cpm/ml Bolton-Hunter reagent-labeled 125I-fibronectin and 1 μg/ml unlabeled fibronectin in culture medium containing 100 units/ml penicillin, 50 μg/ml streptomycin sulfate. After 48 h at 37°C, the cells were washed three times with medium and incubated for an additional 4 h at 37°C. The cell layers were washed three times with 10 mM Tris, 150 mM sodium chloride, pH 7.4, and treated with 1% deoxycholate (Sigma) in 20 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 2 mM N-ethylmaleimide (NEM) on ice for 10 min. The deoxycholate-insoluble material was poured off the plate, and the deoxycholate-insoluble material left behind in the plate was washed quickly with cold water containing 2 mM EDTA and 2 mM NEM. Unlabeled protemeric fibronectin, 400 μg was added to the plate, and the mix was scraped in 1% SDS, 1 mM EDTA, and 1 mM NEM at 80°C. The scraped material was dialyzed against 0.1% SDS containing 1 mM EDTA and 1 mM NEM, followed by lyophilization. Unlabeled protemeric fibronectin was also mixed with protemeric 125I-fibronectin, and that mixture was treated with SDS and lyophilized alongside matrix 125I-fibronectin. The lyophilized pellets were suspended in parallel in 200 μl of 70% formic acid and digested with 120 μl of 5 M cyanogen bromide (27) in acetonitrile (Aldrich) at room temperature in the dark for 24 h in an atmosphere of N2. The reactions were quenched by dilution with water, and the reaction mixtures were lyophilized. The pellets were resuspended in nonreducing SDS sample buffer and analyzed by non-reducing/reducing two-dimensional SDS-PAGE.

Acid Digestion of Stabilized Matrix Fibronectin—TJ 6F cells were incubated with 125I-fibronectin labeled by the chloramine-T method. Cells were treated with deoxycholate, and deoxycholate-insoluble matrix was isolated with hot SDS as described above. Formic acid was added to a final concentration of 70%, and digestion was carried out at 37°C (28) for the designated time lengths. Digested samples were lyophilized, resuspended in SDS sample buffer, and analyzed by SDS-PAGE.

SDS-PAGE—One- and two-dimensional nonreducing/reducing SDS-PAGE analyses were carried out as described previously (23). Lanes containing separated nonreduced samples, including both the stacking and separating gels, were cut out and soaked in 60 μl Tris, pH 6.8, containing 2% SDS and 10% β-mercaptoethanol for 30 min at 60°C. The gel slice was put on top of the stacking gel of the second dimension and sealed with 1% agarose in 60 μl Tris, pH 6.8, containing 1% SDS. After gel electrophoresis, the gel was stained with Coomassie Blue, destained with 10% acetic acid, and soaked in 40% methanol, 5% glycerol, and 10% acetic acid for 3 h prior to drying in a gel dryer using a gradient heating program. Autoradiography was done by phosphor-imaging or by exposing Kodak X-Omat AR film to the dried gels.

In some experiments, proteins were transferred to nitrocellulose paper and analyzed by immunoblotting using the 9D2, IST-2, and IST-7 monoclonal antibodies (29, 30), or rabbit antibodies to the NH2-terminal 70-kDa cathepsin D fragment.

RESULTS AND DISCUSSION

Fig. 1 shows the location of Met-Xaa and Asp-Pro peptide bonds in the A and B subunits of human plasma fibronectin that should be sensitive to cyanogen bromide and acid cleavage, respectively (27, 28, 31). Fig. 2 tabulates the predicted sizes of cyanogen bromide and acid digestion fragments under nonreducing and reducing conditions and identifies fragments that contain carbohydrates and therefore that have larger sizes than predicted from the amino acid sequence. Plasma fibronectin is assumed to be composed of one subunit with a V region and one subunit with no V region (2, 31, 32).

In preliminary experiments, we reproduced the patterns that Gold et al. (33) obtained when nonreduced and reduced cyanogen bromide digests of unlabeled fibronectin were analyzed by one-dimensional SDS-PAGE. 125I-Fibronectin labeled by our standard chloramine-T technique (26) fragmented less completely than unlabeled fibronectin did, presumably because of partial oxidation of methionines. Fibronectin labeled with Bolton-Hunter reagent, in contrast, fragmented as completely as unlabeled fibronectin did. In addition, the ratio of radioactivity to protein was fairly constant among fragments when the protein staining and autoradiographic patterns were compared (not shown); such a result is predicted by the distribution of lysines in fibronectin (Fig. 2).

Fibronectin labeled with Bolton-Hunter reagent bound to cells and became insolubilized with the same kinetics as fibronectin labeled by the chloramine-T technique (data not shown). After the 48-h incubation with cell layers and the 4-h chase, >70% 125I-fibronectin was present in SDS-stable multimers when analyzed by SDS-PAGE without reduction. In order to destroy cellular acid proteases completely before subjecting cellular extracts to cyanogen bromide digestion in formic acid, a protocol was developed in which the cells were rapidly lysed with 1% deoxycholate in the presence of protease inhibitors, and deoxycholate-insoluble material was treated with warm 1% SDS before digestion. NEM was also added to block the two free sulfhydyls (Fig. 1) and prevent artifactual formation of disulfide-linked multimers (11, 23).

Three disulfide-containing fragments (DSF-1, DSF-2, and DSF-3) account for all of the disulfide-containing parts of fibronectin (Figs. 2 and 3). If disulfide exchange occurs, one of these fragments should be involved. DSF-1 extends from the beginning of module I-1 to the end of module II-2, DSF-2 extends from the beginning of module I-1 to the middle of module III-4, and DSF-3 extends from III-12 through the interchain disulfides to the middle of the V region of the other subunit (Figs. 1 and 2). Acid cleavage in addition to cyanogen bromide digestion would result in smaller versions of DSF-2 and DSF-3. DSF-1 under reducing conditions should give rise to fragments of 10, 13.2, and somewhat greater than 13.4 kDa and smaller fragments; reduced DSF-2 should contain a major fragment of somewhat greater than 46 kDa; and reduced DSF-3 should contain a fragment of about 39 kDa from the
subunit lacking the V region (B subunit), a fragment of somewhat greater than 17 kDa from the subunit with the V region (A subunit), and smaller peptides.

By analysis of two-dimensional gels, we could identify all three DSFs. The small reduced fragments of DSF-1 fell off the diagonal after reduction as expected. A 60-kDa fragment of reduced DSF-2 was recognized by monoclonal antibody 9D2 to the III-1 module (Fig. 3, actual blot not shown). A 39-kDa fragment of reduced DSF-3 reacted with monoclonal antibodies to modules III-12–III-14 constituting the COOH-terminal heparin-binding region (IST-2) (30) and to the III-15 sequence (IST-7) (30), whereas a 17-kDa fragment reacted only with IST-7 antibody to the III-15 sequence (Fig. 3, actual blot not shown). The two-dimensional protein staining and immunoblotting patterns were more complicated than shown in Fig. 3 because of incomplete cleavage giving rise to larger DSFs and acid cleavage at Asp-Pro bonds giving rise to smaller DSFs of DSF-2 and DSF-3.

Autoradiographic patterns of cyanogen bromide digestion of protomers and multimers shown in Fig. 4 were chosen because

![Fig. 2. Tabulation of cyanogen bromide (A) and acid (B) fragments of human plasma fibronectin. Met-Xaa (M/X) and Asp-Pro (D/P) peptide bonds are described by residue numbers and module location. Residues are numbered beginning with the NH2-terminal pyroglutamate of human fibronectin (31). The type III modules invariantly present in the two fibronectin subunits are numbered 1–15 (2). Fragments generated are described by number of residues (N) and size (kDa), with a (+) to indicate the need of correction for carbohydrate modification. Lysine content (K) in the reduced form of the fragment is also shown. The disulfide-bonded covalent structures of fragments without reduction (DSFs) are given. Fragments that are generated due to the acidic condition of the cyanogen bromide digestion are marked with *. The fibronectin subunit that contains the V region is denoted A chain. The subunit that lacks the V region is denoted B chain.](image)

![Fig. 3. Sketch of nonreducing/reducing two-dimensional SDS-PAGE of cyanogen bromide digestion of protomeric fibronectin. Arrows point to expected migration of DSF-1, -2, and -3 in the first (nonreduced) dimension as in Fig. 4. The major spot detected by 9D2 is stippled. The major spot detected only by IST-7 is single cross-hatched. The major spot detected by IST-2 and IST-7 is double cross-hatched.](image)
the intensities of exposure were similar and pertinent spots were well separated. The patterns are representative of seven independent comparisons of digest patterns of protomeric and matrix fibronectin from two different preparations of labeled protomeric fibronectin. We were not able to identify any fragments that migrated reproducibly as higher molecular weight complexes in the first nonreducing dimension of matrix $^{125}$I-fibronectin digest. Some differences were noted among individual matched autoradiograms, e.g. the vertical spikes above the diagonal in Fig. 4B that are not present in Fig. 4A and the intensities of some spots including DSF-2 and DSF-3 that seem to be weaker in some of the matrix digests. However, these differences were not reproducible. Since we could account for all of the parts of fibronectin that contained disulfides as in DSF-1, DSF-2, and DSF-3, and none of these fragments were lost or linked in the digest of matrix, we can only conclude that insolubilization of fibronectin by the cells is not mediated by formation of specific disulfides between fibronectin protomers. These results, albeit with less sensitivity, are also evidence against the possibilities that disulfide exchange occurs between fibronectin and other (unlabeled) matrix molecules or that disulfide exchange is highly degenerate and involves many fragments so that no one fragment disappears or no one disulfide-linked pair of fragments appears strongly enough to be detected.

The evidence against interprotomeric disulfides from the two-dimensional gels is best for DSF-1, which appeared as intense spots in autoradiograms of cyanogen bromide-digested
ND Digest
1h 4h 24h

Fig. 5. Acid digestion of matrix 125I-fibronectin. Phosphorimages of acid digest of matrix 125I-fibronectin labeled by the chloramine-T method are shown. Isolated matrix 125I-fibronectin was incubated in 70% formic acid at 37°C for designated time lengths. ND, matrix 125I-fibronectin that was incubated at 37°C for 24 h in the absence of acid. Samples were separated by SDS-PAGE with a 9% separating gel without reducing reagent. 125I-Methylated molecular size markers (myo-cin, approximately 200 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; and carbonic anhydrase, 30 kDa) migrated as indicated. Arrows point to the top of the stacking gel and the interface between the stacking and separating gels.

protomeric and matrix fibronectin. DSF-2 and DSF-3 fragments were less intense because of lighter labeling and the presence of the acid cleavage sites that generated additional fragments (Figs. 1 and 2). In order to analyze the DSF-2 region better, we digested matrix 125I-fibronectin with formic acid alone (Fig. 5). After 24 h, 94-, 63-, and 31-kDa fragments were present, and only a minor portion migrated as high molecular weight complexes under nonreducing conditions (Fig. 5). By immunoblotting with 9D2 (to the III-1 module), the 94- and 31-kDa fragments were positive, whereas the 94-, 63-, and 31-kDa fragments reacted with polyclonal antibodies to the NH2-terminal 70-kDa region (I-1-I-9 modules). Thus, the major fragments released by acid digestion represent cleavage at the Asp-Pro sequences between I-8 and I-9 (Asp-525) and between III-2 and III-3 (Asp-785), with incomplete cleavage at Asp-525 yielding the 94-kDa band. These results are more convincing evidence that the DSF-2 region is not involved in interprotomeric disulfides in fibronectin matrix.

 Fragments from the DSF-3 region also labeled less intensely in the cyanogen bromide digests (Fig. 4) and were not readily identified in the acid digests (Fig. 5). The lack of evidence that these fragments were disulfide-linked other than through the interchain disulfides that hold the dimer together (Fig. 4) is in accordance with the finding that deletion of modules I-10–I-12 in the DSF-3 region results in dimeric molecules capable of forming disulfide-stable complexes (34).

SDS-stable fibronectin multimers did not dissociate with short treatments with 70% formic acid (Fig. 5) or, in other experiments, with several denaturing reagents other than SDS including 0.2 M potassium thiocyanate, 8 M urea, and 6 M guanidine (results not shown). We also tested 1,10-phenanthroline because of the possibility that fibronectin-matrix complexes might be stabilized by Zn2+ binding to vicinal histidinal residues in I-7 and I-8 (2, 31) and could show no dissociation (result not shown).

The acid cleavage sites are adjacent to the I-9/III-1 module pair that our laboratory has implicated in fibronectin-fibronectin interactions on the basis of antibody and fragment inhibition studies (29). I-9 is a stable module that is independently folded (35). III-1 is unique among type III modules for having a stable core structure, even after removal of β strands that are expected to be essential for folding (36). Module III-1 has received considerable interest by virtue of its interaction with other parts of fibronectin (15, 37, 38). Studies of the interactions of recombinant III-1 with a deletion set of recombinant 70-kDa fragment constructs indicate that III-1 binds to the I-4/I-5 module pair. NMR spectroscopy of the recombinant I-4/I-5 module pair reveals that a tryptophan unique to I-4 interacts with an arginine unique to I-5; this interaction is a striking feature of the hydrophobic interface that docks the two modules together into a fixed structure (39). One can speculate that a conformational change takes place whereby the tryptophan of I-4, instead of anchoring the disulfide interaction of I-4 with I-5, interacts in trans with I-9 or III-1. Such a scenario would explain the susceptibility of fibronectin multimers to dissociation by disulfide bond reduction, which would destabilize I-4, I-5, and I-9, and by acid cleavage, which might destabilize I-9 and III-1. This scenario would also account for the observation that partial cathepsin D digests of matrix fibronectin, the NH2-terminal 70-kDa fragment of fibronectin comprised of I-1–I-9 bands in an SDS-stable manner to high molecular weight material (23). Another scenario is that fibronectin assembly is an example of “three-dimensional domain swapping” in which oligomers form by virtue of formation of an interprotomeric interface that is identical to an intraprotomeric interface between domains of the protomer (40). Against this scenario, however, is the fact that SDS-stable interactions have not been identified between parts of protomeric fibronectin.

Can the strength of noncovalent association of fibronectin multimers in aqueous solution resist dissociation with SDS and the other protein denaturants? We have considered two examples of such stable interactions in other proteins. The binding of bovine serum albumin to streptavidin causes the subunits of tetrameric streptavidin to be associated so tightly as to withstand dissociation with SDS and heat (41). The large negative free energy of association is thought to be due to van der Waals' forces dispersion effects in the nearly ideal preformed cavity that streptavidin presents to bovine serum albumin (42). Mutation of a critical tryptophan that helps to form this cavity and that contributes to the hydrophobic subunit interface results in streptavidin that binds bovine serum albumin but that dissociates when treated with SDS and heat (42). Some ternary complexes of the α and β subunits of class II major histocompatibility complex glycoproteins and antigenic or class II-associated invariable chain peptides are stable in SDS. This stability varies considerably with the peptides even though the peptides bind to a common groove in a common conformation (43, 44). Binding of the peptide is stabilized by multiple hydrogen bonds and by contacts with pockets in the binding groove (44, 45). Some SDS-stable, peptide class II glycoprotein complexes dissociate in the presence of reducing agent (46), presumably due to disruption of the disulfide bridge in the class II β1 domain (47). Thus, the notion of protein-protein interactions that are stable in SDS and are broken in SDS plus reducing agent has a well studied precedent. Both of these examples of SDS-stable interactions are characterized by proteins associating to form a site for strong binding of a third moiety. By analogy, multimerization of fibronectin may occur by a scenario that involves ternary complexes, e.g. the I-4/I-5 and I-9/III-1 module pairs and a third site in polymerizing fibronectin or an associated protein.

2 J. Sottile and D. Mosher, submitted for publication.
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