Identification of Protein-disulfide Isomerase Activity in Fibronectin*

Kurt J. Langenbach‡ and Jane Sottile§

From the Department of Physiology and Cell Biology, Albany Medical College, Albany, New York 12208

Assembly and degradation of fibronectin-containing extracellular matrices are dynamic processes that are up-regulated during wound healing, embryogenesis, and metastasis. Although several of the early steps leading to fibronectin deposition have been identified, the mechanisms leading to the accumulation of fibronectin in disulfide-stabilized multimers are largely unknown. Disulfide-stabilized fibronectin multimers are thought to arise through intra- or intermolecular disulfide exchange. Several proteins involved in disulfide exchange reactions contain the sequence Cys-X-X-Cys in their active sites, including thioredoxin and protein-disulfide isomerase. The twelfth type I module of fibronectin (I12) contains a Cys-X-X-Cys motif, suggesting that fibronectin may have the intrinsic ability to catalyze disulfide bond rearrangement. Using an established protein refolding assay, we demonstrate here that fibronectin has protein-disulfide isomerase activity and that this activity is localized to the carboxyl-terminal type I module I12. I12 was as active on an equal molar basis as intact fibronectin, indicating that most of the protein-disulfide isomerase activity of fibronectin is localized to I12. Moreover, the protein-disulfide isomerase activity of fibronectin appears to be partially cryptic since limited proteolysis of I12 increased its isomerase activity and dramatically enhanced the rate of RNase refolding. This is the first demonstration that fibronectin contains protein-disulfide isomerase activity and suggests that cross-linking of fibronectin in the extracellular matrix may be catalyzed by a disulfide isomerase activity contained within the fibronectin molecule.

Multimeric fibronectin is a major constituent of extracellular matrices found throughout the body and plays a role in a wide variety of biological events, including maintenance of endothelial cell integrity, platelet adhesion, and cell migration during blood vessel repair (1–3). Fibronectin circulates at high concentrations in plasma as a soluble dimeric molecule and exists in an insoluble multimeric form in the extracellular matrix of loose connective tissue, granulation tissue, and basement membranes (1–5). This insoluble multimeric form of fibronectin is thought to be the primary functional form of the molecule (1, 3–5), mediating adhesive and migratory events associated with wound repair, neovascularization, and embryonic development.

Deposition of fibronectin into the extracellular matrix is a cell-mediated, multistep process that involves the binding of soluble fibronectin to specific sites on the surface of substrate-attached cells that have been termed matrix assembly sites (6). Subsequent homophilic binding interactions between fibronectin molecules lead to the deposition of high molecular mass, disulfide-stabilized multimers into the extracellular matrix (7–11). Fibronectin that is deposited in tissues in vivo and in the matrix of cells cultured in vitro is in the form of high molecular mass multimers (1, 12–14). This multimeric fibronectin can be converted to monomeric fibronectin upon treatment with disulfide-reducing agents (12–14), suggesting that fibronectin in the extracellular matrix is stabilized by disulfide cross-linking. It has been postulated that this cross-linking event occurs by a disulfide exchange mechanism involving type I or II modules in the 70-kDa amino terminus of the molecule (15). However, the regions involved in disulfide cross-linking of fibronectin in the extracellular matrix have not been identified (16). In addition, the mechanisms of this cross-linking are unknown.

A number of proteins involved in disulfide exchange reactions, including protein-disulfide isomerase and thioredoxin (17–20), contain the sequence Cys-X-X-Cys in their active sites. The fifth and sixth cysteines in the twelfth type I module of fibronectin (I12) are arranged in a similar sequence, Cys-Asp-Asn-Cys (21), suggesting that fibronectin may contain a disulfide isomerase activity. To determine whether fibronectin contains an intrinsic protein-disulfide isomerase activity, we assayed fibronectin as well as proteolytic and recombinant fragments of fibronectin for their ability to reactivate reduced and denatured RNase. Using an established protein refolding assay (22, 23), we found that fibronectin contains a protein-disulfide isomerase- and thioredoxin-like activity and that this activity is localized to I12. This is the first demonstration that fibronectin contains protein-disulfide isomerase activity and suggests that fibronectin may catalyze disulfide bond rearrangement during its incorporation into the extracellular matrix.

EXPERIMENTAL PROCEDURES

Materials—Gelatin-Sepharose, Sephadex G-25, and SP Sephadex C-25 were obtained from Amersham Pharmacia Biotech. Protein-disulfide isomerase (107 kDa) (EC 5.3.4.1) and thioredoxin (11.7 kDa) were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). The remaining chemicals were purchased from Sigma unless otherwise indicated.

Fibronectin and Fibronectin Fragments—Human fibronectin was purified from a fibronectin and fibrinogen-rich by-product of factor VIII production as described (24). The 160/180-kDa proteolytic fragment of fibronectin was generated by limited trypsin digestion of intact fibronectin essentially as described (25). The 40-kDa (cathepsin and trypsin) gelatin-binding and 70-kDa (cathepsin) amino-terminal fragments of fibronectin were the generous gifts of Drs. Paula McKeown-Longo and Denise Hocking (Albany Medical College, Albany, NY) and were made as described (6, 26). The 110- and 19-kDa thermolysin fragments of fibronectin were the generous gifts of Dr. Ken Ingham (American Red Cross, Bethesda, MD) and were prepared as described (27–29). A schematic diagram of various fibronectin fragments used in this study is shown in Fig. 3 (inset).

* This work was supported in part by Grant 950318 from the American Heart Association and Grant HL50549 from the National Institutes of Health (to J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported by National Institutes of Health Predoctoral Training Grant 7T32-HL07194.
§ To whom correspondence should be addressed: Dept. of Physiology and Cell Biology (A-134), Neil Hellman Medical Research Bldg., Albany Medical College, 47 New Scotland Ave., Albany, NY 12208. Tel.: 518-262-6689; Fax: 518-262-5669; E-mail: jsottile@ccgateway.amc.edu.

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Generation of I12/pVL1392/His9—The baculovirus expression vector pVL1392 (Invitrogen, Carlsbad, CA) was modified so that genes cloned into the multiple cloning site would be produced with six histidines at the carboxyl terminus of the protein. The complementary oligonucleotides 5' -CCCAATTATGATCG-3' and 5' -GGATCTCATCATCCATCTCAAG-3' were used to ligate pVL1392 that was previously digested with EcoRI and BamHI. I12 was amplified with the sense primer 5' -CCCAATTATGATCTCATCTCCATCTCAAG-3' and the antisense primer 5' -CCCAATTATGATCGCACCCGAGCTCTGCGGAGC-3' using PCR with a template 300. The EcoRI restriction enzyme site at the 5'-end (underlined letters), and the antisense primer has an EcoRI restriction enzyme site at the 5'-end (underlined letters). The base in boldface (C) was added to the antisense primer following the EcoRI site to maintain the correct reading frame. Polymersase chain reaction-amplified DNA was subcloned into the baculovirus vector pVL1392/His9. Cloning I12 upstream of the EcoRI site resulted in the addition of Gly-Ile-Leu before the six histidine tag. Amplified DNA contains sequences coding for the fibronectin signal sequence and the I12 coding sequence. DNA was sequenced (31) to ensure that no DNA mutations were introduced during polymersase chain reaction amplification and to verify the sequence of the modified pVL1392 vector.

Baculovirus Expression—I12/pVL1392/His9 was cotransfected into insect cells with Baculogold DNA (Pharmingen, San Diego, CA) following the manufacturer's instructions. Recombinant viruses were prepared using established methods (32, 33). SF21 insect cells were grown under serum-free conditions using SF900-II (Life Technologies, Inc.). Conditioned medium containing recombinant I12 was applied to an SP column equilibrated with 0.1% acetic acid. Peak samples were pooled, and the pH was adjusted to 8.6, at room temperature. RNase was then purified on a Sephadex G-25 column with Serva blue G (Serva, Paramus, NJ) or silver nitrate (35).

Production and Reactivation of Reduced and Denatured RNase—RNase was reduced and denatured essentially as described by Pigjet and Schuster (22). Briefly, RNase A (30 mg) was incubated overnight in 6 M guanidine HCl containing 0.15 M diithiothreitol and 0.1 M Tris, pH 8, at room temperature. RNase was then purified on a Sephadex G-25 column equilibrated with 0.1% acetic acid. Peak samples were pooled, concentrated, and then eluted with 350 mM NaCl and 100 mM Tris, pH 6.2. Purified I12 was dialyzed into phosphate-buffered saline or into 30 M NaCl, 1 mM EDTA, and 0.1 M Tris prior to use in the RNase refolding assay. Purity of proteins was assessed using a discontinuous Tricine SDS-polyacrylamide gel electrophoresis system according to the method of Schagger and von Jagow (34) and visualized with either 0.025% Serva blue G (Serva, Paranaus, NJ) or silver nitrate (35).

RESULTS

Fibronectin Contains Protein-disulfide Isomerase Activity—Soluble protomeric fibronectin is believed to be stabilized in the extracellular matrix as a result of disulfide cross-linking. Fibronectin contains a Cys-XX-Cys motif in I12 (21). This motif has been identified as the active-site sequence in other proteins that exhibit disulfide isomerase activity (17-20). To determine whether fibronectin has disulfide isomerase activity, we used an established protein refolding assay. This assay has been widely used to assess the isomerase activity of thioredoxin (22, 23, 38), protein-disulfide isomerase (38), and other proteins (23, 39) and measures the ability of proteins to catalyze the refolding of reduced and denatured RNase (36). During the process of RNase refolding, non-native as well as native disulfide bonds form (22, 40-43). Therefore, both disulfide oxidation as well as isomerization reactions must occur to achieve the native folded state.

Intact fibronectin at a concentration of 4 MμM enhanced the refolding of RNase at a rate similar to that of 1 MμM protein-disulfide isomerase (Fig. 1) and 30 MμM thioredoxin (data not shown). A control protein, ovalbumin, which contains disulfide bonds as well as free sulphydryl residues, but lacks the Cys-XX-Cys motif, showed little activity in this assay (Fig. 1). The ability of fibronectin to catalyze RNase refolding was concentration-dependent. Intact fibronectin at a concentration of 3 MμM had ~84% of the activity of 4 MμM fibronectin; 2 MμM fibronectin had ~74% of the activity (data not shown). To directly compare the ability of fibronectin, protein-disulfide isomerase, and thioredoxin to catalyze the refolding of reduced and denatured RNase, kcat and Kcat/Km values were determined. The ability of fibronectin to catalyze RNase refolding followed typical Michaelis-Menten kinetics (Fig. 2B, inset). The reactions catalyzed by protein-disulfide isomerase (Fig. 2A, inset) and thioredoxin (data not shown) also followed typical Michaelis-Menten kinetics. Lineeweaver-Burk plots (Fig. 2 and data not shown) were used to determine the Vmax, Km, and kcat/Km values for each of these proteins (Table 1). As shown in Table 1, fibronectin is ~9-fold more active than thioredoxin and 9-fold less active than protein-disulfide isomerase in catalyzing RNase refolding.

To localize the disulfide isomerase activity within fibronectin, proteolytic fragments of fibronectin encompassing the entire fibronectin molecule (Fig. 3, inset) were assayed for activity. Fragments of fibronectin that demonstrated the most activity in this assay were the 160/180- and 19-kDa fragments, which contain the carboxyl-terminal type II procollagen modules IIα1-I12 (Fig. 3). The 40- and 70-kDa fragments, which contain proangiogenic modules, but do not contain the Cys-XX-Cys motif, had activity only slightly higher than the control protein, ovalbumin (Fig. 3). The central 110-kDa cell-binding fragment of fibronectin, which contains one free sulphydryl residue, but no disulfide bonds (21), had little activity in this assay (Fig. 3). These data indicate that the protein-disulfide isomerase activity of fibronectin is localized in the two carboxyl-terminal fragments of fibronectin.
bronectin is localized to the 19-kDa fragment that contains I_{10-12}.

I_{12} Demonstrates Disulfide Isomerase Activity in the RNase Refolding Assay—I_{12} is located within the 19-kDa fragment and contains the Cys-X-X-Cys motif. Therefore, to determine whether the disulfide isomerase activity of the 19-kDa fragment could be further localized to I_{12}, recombinant I_{12} was generated using a baculovirus expression system and tested in the RNase refolding assay. As shown in Fig. 4, I_{12} at a concentration of 4 μM was more active than 4 μM intact fibronectin and as active as 1 μM protein-disulfide isomerase. These data suggest that most or all of fibronectin’s protein-disulfide isomerase activity is localized to I_{12}.

Regulation of the Protein-disulfide Isomerase Activity of Fibronectin—Deposition of fibronectin into the extracellular matrix is a highly regulated process (44–48). If fibronectin’s protein-disulfide isomerase activity is involved in cross-linking of fibronectin in the extracellular matrix, then it would be expected that its protein-disulfide isomerase activity would also be tightly regulated. One mechanism by which this activity may be regulated is through conformation-induced activation. In such a model, isomerase activity would be partially masked in the native soluble fibronectin molecule and enhanced by conformational changes induced upon binding of fibronectin to cell surfaces. If fibronectin’s protein-disulfide isomerase activity is partially cryptic, limited proteolysis might enhance its protein-disulfide isomerase activity by generating smaller fragments whose conformations are distinct from those in the intact molecule. Others have shown that proteolytic fragments of proteins can have enhanced or novel activities when compared with native proteins (49, 50). For example, the 40-kDa gelatin-binding fragment and the amino- and carboxyl-terminal fibrin-binding fragments of fibronectin have enhanced chemotactic activity when compared with the activity of intact fibronectin (49, 50). In addition, proteolytic fragments of plasminogen (51) and collagen XVIII (52) have anti-angiogenic properties not associated with the native molecules.

To determine whether fibronectin’s protein-disulfide isomerase activity could be increased by limited proteolysis, the 19-kDa fragment of fibronectin (containing I_{10-12}) was digested with chymotrypsin and assayed for its ability to catalyze RNase refolding. Analysis of the digested and undigested 19-kDa fragment by Tricine/SDS-polyacrylamide gel electrophoresis indicated that digestion of the 19-kDa fragment by chymotrypsin resulted in generation of fragments with apparent molecular
masses of ~14, 12, and 10 kDa (data not shown). The chymotrypsin-generated fragments of the 19-kDa fragment at a concentration of 1 μM had activity similar to 4 μM intact 19-kDa fragment as measured by the RNase refolding assay (Fig. 5A). Moreover, chymotrypsin proteolysis of the 19-kDa fragment (4 μM) resulted in a dramatic increase in the rate of RNase refolding (Fig. 5A and Table II). Table II compares the time required for RNase to reach 50% of maximal refolding in the presence of various proteins or fibronectin fragments. Whereas the intact 19-kDa fragment required 66 h to refold 50% of the RNase, proteolytic 19-kDa fragments required only 18 h. In comparison, RNase treated with 4 μM protein-disulfide isomerase reached 50% of its native refolded state in 28 h, whereas RNase treated with 30 μM thioredoxin required 63 h. In the uncatalyzed reaction, >100 h was required. The increase in protein-disulfide isomerase activity after limited proteolysis was specific to the 19-kDa fragment since the ability of the 40-kDa gelatin-binding fragment of fibronectin to refold RNase did not change substantially upon limited digestion with chymotrypsin (Fig. 5B and Table II). Chymotrypsin digestion of the 40-kDa fragment generated fragments of 31, 28, 27, 16, and 11 kDa (data not shown). These data indicate that limited proteolysis of the 19-kDa fragment increases its protein-disulfide isomerase activity, most likely due to generation of a fragment whose conformation favorably exposes its protein-disulfide isomerase activity.

### Table I

| Protein-disulfide isomerase | k<sub>cat</sub> | K<sub>m</sub> | k<sub>cat</sub>K<sub>m</sub> | V<sub>max</sub> | k<sub>cat</sub>/V<sub>max</sub> |
|----------------------------|----------------|------------|-----------------|-------------|-----------------|
| Fibronectin                | 0.0017         | 62.5       | 27.2            |             |                 |
| Thioredoxin                | 0.0002         | 66.5       | 2.9             |             |                 |
| Protein-disulfide isomerase| 0.0067         | 27.7       | 241.9           |             |                 |

Protein-disulfide isomerase activity of I<sub>12</sub>. Shown are the kinetics of refolding of reduced and denatured RNase in the presence of 4 μM I<sub>12</sub> ( ), 4 μM fibronectin (FN; ▲), 1 μM protein-disulfide isomerase (PDI; ■), or 4 μM ovalbumin (●) or in an uncatalyzed reaction (●). Refolding of reduced and denatured RNase was performed as described under “Experimental Procedures.” RNase activity is expressed as percent of total RNase activity. The data represent one of three similar experiments.
FIG. 5. Chymotrypsin treatment of the 19-kDa fragment increases its isomerase activity. The 19- and 40-kDa fragments were digested with 0.1 mg/ml α-chymotrypsin as described under “Experimental Procedures.” A, refolding of reduced and denatured RNase was performed in the presence of 4 μM (C) or 1 μM (D) chymotrypsin-digested 19-kDa fragment, 4 μM undigested 19-kDa fragment (●), 4 μM fibronectin (FN, ), 1 μM protein-disulfide isomerase (PDI, ), or 4 μM ovalbumin (○) or in an uncatalyzed reaction (▲). B, refolding of reduced and denatured RNase was performed in the presence of 4 μM digested (▲) or undigested (●) 40-kDa fragment, 1 μM protein-disulfide isomerase (■), or 4 μM ovalbumin (○) or in an uncatalyzed reaction (▲). The addition of phenylmethylsulfonyl fluoride and α-chymotrypsin to the uncatalyzed reaction did not alter the RNase refolding rate (data not shown). RNase activity is expressed as percent of total RNase activity. The data shown represent one of five similar experiments.

TABLE II
Chymotrypsin treatment of the 19-kDa fragment decreases the time required to reach 50% native refolding

| Proteins          | Hours |
|-------------------|-------|
| Uncatalyzed       | >100  |
| 4 μM digested 19-kDa | 17.7 ± 4.9 |
| 2 μM digested 19-kDa | 54.7 ± 19.0 |
| 1 μM digested 19-kDa | 76.4 ± 8.4 |
| 0.25 μM digested 19-kDa | 107.0 ± 9.9 |
| 4 μM 19-kDa       | 65.8 ± 6.3 |
| 4 μM 40-kDa       | 90.8 ± 12.7 |
| 4 μM digested 40-kDa | 70.8 ± 12.9 |
| 4 μM intact fibronectin | 79.6 ± 6.2 |
| 1 μM PDI          | 48.5 ± 2.6 |
| 4 μM PDI          | 27.5 ± 2.9 |
| 30 μM thioredoxin | 63.3 ± 9.9 |

a PDI, protein-disulfide isomerase.
b The range of duplicate samples.

DISCUSSION

In this report, we have demonstrated that the extracellular matrix protein fibronectin contains an intrinsic protein-disulfide isomerase activity. This is the first demonstration of protein-disulfide isomerase activity in fibronectin and suggests that fibronectin may catalyze disulfide bond rearrangement during its incorporation into the extracellular matrix. Other covalent cross-linking events may also lead to fibronectin multimerization under certain circumstances (53, 54). For example, fibronectin can be covalently cross-linked in the extracellular matrix via ε-(γ-glutamyl)lysyl bonds by activated factor XIII (53, 54). It has also been suggested that fibronectin multimers may be stabilized by noncovalent interactions since attempts to identify cross-linked regions of fibronectin have not been successful (16). However, the ability of multimeric extracellular matrix fibronectin to be converted to monomeric fibronectin by treatment with disulfide-reducing agents (13, 14) suggests that extracellular matrix fibronectin is stabilized predominantly by inter- or intramolecular disulfide exchange.

Our data support a model whereby incorporation of soluble fibronectin into the extracellular matrix involves a disulfide exchange mechanism catalyzed by an isomerase activity located within the fibronectin molecule. Disulfide-stabilized, multimeric fibronectin has been reported to be a functionally distinct form of fibronectin that has enhanced adhesive properties, is active in suppressing cell migration and tumor formation (55, 56), and mediates enhanced binding of bacteria to host tissue (57). Recent evidence also suggests that the extracellular matrix multimeric form of fibronectin has growth-promoting properties not possessed by protomeric fibronectin (58) and that the effects of matrix fibronectin on cell growth are dependent on its exact molecular configuration (59). It has also been shown that treatment of cells with a fragment derived from the first type III module of fibronectin (III1C) can lead to inhibition of fibronectin deposition or disruption of a preexisting fibronectin matrix and also results in inhibition of cell growth (60, 61). Thus, the identification of protein-disulfide isomerase activity within the fibronectin molecule is important not only for elucidating the biochemical mechanisms that regulate fibronectin multimerization, but also in defining the functional consequences of this multimerization.

In the RNase refolding assay, 4 μM fibronectin had disulfide isomerase activity similar to that of 1 μM protein-disulfide isomerase or 30 μM thioredoxin (Figs. 1 and 2). Analysis of kcat/Km values indicated that fibronectin is a 9-fold more active than thioredoxin and 9-fold less active than protein-disulfide isomerase in catalyzing RNase refolding. The rates determined in this study for protein-disulfide isomerase are lower than those previously reported (19, 62, 63). However, in those previous reports, RNase refolding was performed in the presence of reduced and oxidized glutathione at pH 8, conditions substantially different from those used in our assays. Thus, these differences likely account for the differences in rates reported.
The RNase refolding assay used in this and other studies predominately measures the ability of proteins to catalyze dithiol oxidation reactions (17). However, refolding of reduced and denatured RNase to a native state involves formation (oxidation) and rearrangement (isomerization) of non-native disulfide bonds until the final folded state is achieved (22, 40–42), a process that is thought to be driven by the search for the most stable protein conformation (17). The assay buffer employed in this study does not require the addition of an external oxidative agent or the prior “activation” of protein catalysts by the addition of a reducing agent (64, 65) and thus is likely to more closely approximate the environment outside the cells where fibronectin deposition into the extracellular matrix occurs.

Our data indicate that fibronectin’s protein-disulfide isomerase activity is localized predominantly to the last type I module, I12 (Fig. 4), which contains the Cys-X-X-Cys motif (21). I12 was more active on a molar basis than intact fibronectin, indicating that most or all of fibronectin’s isomerase activity is localized to I12. Localization of the protein-disulfide isomerase activity of fibronectin to I12 suggests the possibility that the Cys-X-X-Cys motif of I12 may be important in catalyzing disulfide cross-linking of fibronectin in the extracellular matrix since the Cys-X-X-Cys motif in protein-disulfide isomerase and thioredoxin is the active-site sequence required for catalyzing disulfide bond isomerization (17–20). The Cys-X-X-Cys motif in von Willebrand factor has also been shown to be critical for the activity of von Willebrand factor to form disulfide-stabilized multimers (66).

We have previously shown that fibronectin containing mutations in I12 or lacking I12 can become incorporated into the extracellular matrix of cells containing a pre-established matrix (30). However, if the protein-disulfide isomerase activity of I12 is critical for the formation of disulfide-stabilized fibronectin multimers, it is possible that the non-mutant fibronectin present in the assay provided this activity. In support of this, we and others have shown that fibronectin deletion mutants lacking the Arg-Gly-Asp sequence (67, 68) or lacking a large internal portion (III1–I12) (69) become incorporated into the extracellular matrix of cells containing a pre-established fibronectin matrix (67, 69), but are not incorporated into the extracellular matrix of cells lacking a pre-established fibronectin matrix (68, 69).

The cryptic nature of fibronectin’s protein-disulfide isomerase activity is consistent with the highly regulated nature of fibronectin matrix assembly (44–48). Our data indicate that the protein-disulfide isomerase activity of fibronectin can be increased by limited proteolysis, a treatment that likely results in changes in protein conformation. Conformational changes have also been detected in fibronectin following binding of fibronectin to surfaces (50, 70) or following alterations in pH and ionic strength (71, 72). Conformational alterations in fibronectin have been shown to lead to exposure of cryptic binding sites (9, 10). Conformational alteration of IIII generates a binding site for the 70-kDa amino terminus of fibronectin (10). Similarly, conformational alteration of III10 leads to exposure of a cryptic binding site for III1 (9). Fibronectin that is incubated with a fragment of III (III1–I12) (55) or with conformationally altered III10 (9) forms disulfide-stabilized multimers, perhaps due to conformational changes in fibronectin induced by interaction with III1, or with altered III10. These data suggest a model in which the interaction of fibronectin with cell surfaces triggers a series of conformational changes leading to exposure of fibronectin-fibronectin interactive sites (9, 10, 55, 68, 73, 74) and activation of fibronectin’s disulfide isomerase activity that may be important for fibronectin fibril formation. Future studies will be directed toward defining the interactions that regulate fibronectin’s protein-disulfide isomerase activity and determining whether this activity mediates fibronectin cross-linking during fibronectin matrix assembly.

Acknowledgments—We thank Drs. Ken Ingham, Paula McKeown-Longo, and Denise Hocking for providing fibronectin fragments; Drs. Leo Reichert and Patricia Grando for help with the RNase refolding assays; Drs. John Jeffrey, David Mann, Dudley Strickland, and Tom Anderson for help with the enzyme kinetics; Drs. Denise Hocking, John Jeffrey, Susan LaFlamme, and Paula McKeown-Longo for critically reading this manuscript; and Dr. Peter Vincent for assistance with the reading this manuscript; and Dr. Peter Vincent for assistance with the reading this manuscript; and Dr. Peter Vincent for assistance with the reading this manuscript; and Dr. Peter Vincent for assistance with the reading this manuscript; and Dr. Peter Vincent for assistance with the reading this manuscript; and Dr. Peter Vincent for assistance with the reading this manuscript; and Dr. Peter Vincent for assistance with the reading this manuscript; and Dr. Peter Vincent for assistance with the reading this manuscript.
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