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One Free Sulfhydryl Group of Plasma Fibronectin Becomes Titratable upon Binding of the Protein to Solid Substrates†

C. Narasimhan,‡ Ching-San Lai,*,‡ Arthur Haas,§ and James McCarthy∥

National Biomedical ESR Center and Department of Radiology and Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226, and Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455

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ABSTRACT: The accessibility in human plasma fibronectin of the two free sulfhydryl groups per chain to sulfhydryl reagents 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and a maleimide derivative has been examined. For soluble fibronectin, the free sulfhydryl groups are not accessible to DTNB unless urea or guanidine hydrochloride is added [Smith et al. (1982) *J. Biol. Chem. 257*, 5831-5838]. Upon binding to polystyrene beads, 0.87 ± 0.05 sulfhydryl group per chain becomes titratable to DTNB. Experiments using fibronectin fragments demonstrate that this newly exposed sulfhydryl group is located in a Type III homologous unit between the DNA-binding and the cell-binding domains. The results suggest that, upon adsorption to solid substrates, plasma fibronectin undergoes a conformational change, thereby exposing one buried sulfhydryl group. These findings have implications regarding the "surface activation" of adhesion activities of fibronectin.

Plasma fibronectin (Fn) is a glycoprotein present in blood plasma, consisting of two subunits of about 250 kDa each linked together by two disulfide bridges near the carboxyl termini (McDonagh, 1985). The protein plays important roles in cell adhesion, wound healing, and phagocytosis. Most of these functions are expressed when Fn is attached to a surface, either solid substrates in vitro or basement membranes in vivo. For example, plasma Fn and its fragments adsorbed to polystyrene beads have been shown to promote the adhesion of fibroblast cells (McAbee & Grinnell, 1983; Schwarz & Juliano, 1984).

Human plasma Fn contains two free sulfhydryl groups per chain: one located in a Type III homologous unit between the DNA-binding and cell-binding domains (Skorstengaard et al., 1985) (designated as SH-1) and the other in the fibrin-binding domain near the carboxyl terminus (Garcia-Pardo et al., 1985) (designated as SH-2) (see Figure 2).

Previous studies have shown that the two free sulfhydryl groups in soluble Fn are not accessible to sulfhydryl reagents such as DTNB or maleimide derivatives in the absence of chaotropic agents (Smith et al., 1982; Lai & Tooney, 1984). ESR spin-label studies indicated that these two sulfhydryl groups of plasma Fn are in a cleft about 10.5 Å in length (Lai et al., 1984).

In this paper, we demonstrate that, upon binding of plasma Fn to polystyrene beads, SH-1 is exposed and becomes titratable by DTNB, while SH-2 remains buried, suggesting that plasma Fn undergoes a conformational change upon adsorption to polystyrene beads. The proximity of SH-1 to the cell adhesion domains of Fn (Skorstengaard et al., 1985) suggests that this regional change in conformation may have importance in the expression of adhesion activities of the molecule.

Materials and Methods

Materials. Tris(hydroxymethyl)aminomethane (Tris) and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma (St. Louis, MO). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and guanidine hydrochloride were purchased from Pierce (Rockford, IL). Aquacide III was obtained from Calbiochem (San Diego, CA). Polystyrene latex beads with amino groups on the surface were purchased from Polysciences (Warrington, PA); beads with diameter 0.1 or 0.5 μm were used throughout the study. According to the manufacturer's estimation, the bead suspension contains 2.5% solids. The bead concentration used in this study was calculated on the basis of this information.

Plasma fibronectin (Fn) was isolated from freshly frozen human plasma by using gelatin-Sepharose 4B affinity chromatography (Engvall & Ruoslahti, 1977). It was essentially pure as determined by 5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The 75-kDa and the C-terminal 31-kDa fragments of Fn were purified as described previously (McCarthy, 1986).

Purification of Tryptic Fragments of Fn. The fragments 34 kDa, 185 kDa, and 215 kDa were purified according to the method of Sekiguchi and Hakamori (1983), except that the separation of the 185-kDa fragment from the 215-kDa fragment was carried out at 22 °C by high-performance liquid chromatography using the Pharmacia (Uppsala, Sweden) FPLC (fast protein liquid chromatography) system equipped with a GP-250 gradient programmer. Briefly, 0.5 mL of the fragment mixture (0.6 mg/mL) in buffer A (50 mM Tris and

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*To whom correspondence should be addressed.
‡National Biomedical ESR Center.
§Department of Radiology and Biochemistry.
∥Department of Laboratory Medicine and Pathology.

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0.5 mM EDTA, pH 7.6) was injected onto an HR 5/10 Mono Q anion-exchange column equilibrated in buffer A. Fragments were eluted by NaCl gradient (12.5 mM/min) at a flow rate of 1 mL/min. The fractions containing the pure 185 kDa and the pure 215 kDa were pooled separately, dialyzed against 20 mM Tris and 0.15 M NaCl, pH 7.4 (TBS), and then concentrated with Aquacide III. The concentrations of plasma Fn and isolated fragments were estimated on the basis of the extinction coefficients of 1.28 (intact Fn; Mosesson et al., 1975), 1.0 (75 kDa), 1.22 (185 kDa), 1.21 (215 kDa), and 1.25 (34 kDa) mg mL^{-1} cm^{-1}, respectively; the extinction coefficients of the fragments were estimated in this study. The concentrations of the fragments also were determined by using the Bio-Rad dye binding assay with known concentrations of bovine serum albumin as a standard. For isolated fragments, the concentrations estimated by these two methods differed by about 10%.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–PAGE was performed according to the method of Laemmli (1970) with 5, 8, or 12% gels, depending upon the size of the fragment analyzed. Samples were reduced with 2% mercaptoethanol prior to loading on the gel. The apparent molecular weights of the fragments were estimated by using the following proteins as standards: myosin, 200 000; *E coli* β-galactosidase, 116 250; rabbit muscle phosphorylase *b*, 97 400; bovine serum albumin (BSA), 66 000; hen egg white ovalbumin, 42 699; bovine carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; hen egg white lysozyme, 14 400.

Preparation of Fn-Coated Beads. Fn-coated beads were prepared by the method of McAbee and Grinnell (1983) with a slight modification. About 9.1×10^{12} beads were incubated with 0.45 mL of Fn in TBS (0.5 mg/mL final concentration) for 10 min in a 37 °C water bath with gentle shaking. After incubation, the mixture was washed with TBS by centrifugation using an Eppendorf centrifuge. The amount of Fn bound to the beads was estimated by subtracting the total amount added from the amount recovered in the supernatant. Under these experimental conditions, about 73% of added Fn was found to bind to beads; this corresponds to a value of 343 ng/cm^2 (the amount of bound Fn per square centimeter of the bead surface), which is below 618 ng/cm^2 at saturation (McAbee & Grinnell, 1983). No aggregation of Fn-coated beads was observed during the course of experiments as examined under a microscope. Fn fragment coated beads were prepared in a similar manner. Except for the 185-kDa fragment, all other fragments used in this study were found to bind at least as tightly as intact Fn to polystyrene beads (not shown). The binding affinity of the 185-kDa fragment to the beads was about 70% of that of intact Fn; estimation was made by comparing the amounts of Fn and the fragment (in moles) bound to the same number of beads.

Determination of Free Sulfhydryl Contents. Free sulfhydryl contents of native Fn and its fragments were determined by Ellman's method (Ellman, 1959). (a) Soluble Fn: to 0.8 mL of Fn or fragments (about 0.5 mg/mL) was added 0.2 mL of DTNB (4 mg/mL). Immediately after the reaction mixture was diluted to 2 mL with either TBS or 8 M guanidine hydrochloride, the absorbance at 412 nm was monitored continuously at 22 °C. The amount of dinitrophenol anion produced corresponding to the amount of sulfhydryl groups was quantitated by using the extinction coefficient of 1.36×10^4 at 412 nm. (b) Protein-coated beads: Fn- or fragment-coated beads (9.1×10^{12} beads) prepared as described above were suspended in 1.7 mL of TBS alone or TBS containing 4 M guanidine hydrochloride to which was added 0.3 mL of DTNB

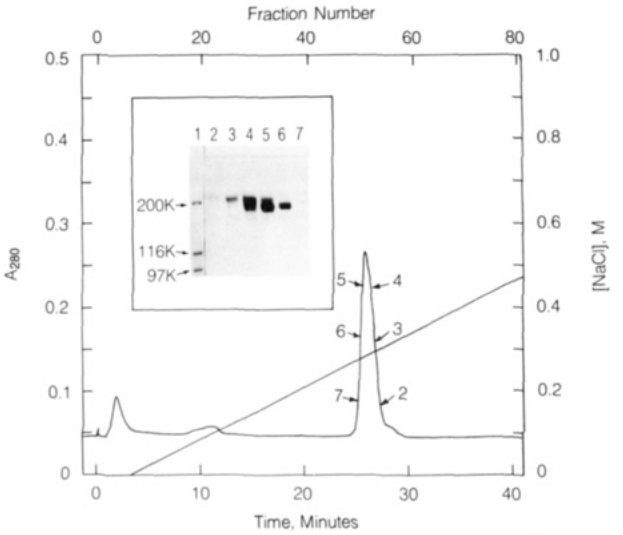

FIGURE 1: FPLC elution profile of a mixture of the 185- and 215-kDa fragments of plasma Fn. Experimental conditions were as described under Materials and Methods. (Inset) 6% SDS–PAGE gel profile corresponding to the peak fractions in the FPLC tracing: lane 1, protein standards; lane 2, fraction 55; lane 3, fraction 54; lane 4, fraction 53; lane 5, fraction 52; lane 6, fraction 51; lane 7, fraction 50.

(4 mg/mL). After incubation at 22 °C for 40 min with gentle mixing, the reaction mixture was separated by centrifugation. The titratable sulfhydryl groups in the protein was calculated on the basis of the amount of dinitrophenol anion generated in the supernatant and the amount of the protein bound to the beads. Polystyrene beads alone did not react with DTNB.

RESULTS

Preparation of 185- and 215-kDa Fragments Using FPLC. Figure 1 shows the typical fast protein liquid chromatographic (FPLC) elution profile of a mixture of the 185- and 215-kDa fragments. The two fragments partially overlap in their elution positions within the NaCl gradient, which is consistent with the small net charge difference between these two fragments (McDonagh, 1985). However, some resolution of the fragments is evident from the trailing shoulder. As shown in the SDS gel (inset of Figure 1), leading and trailing fractions from the peak (fractions 51 and 54) contained the pure 185-kDa fragment and 215-kDa fragment, respectively. To our knowledge, this is the first time that the FPLC technique has been used for the preparation of Fn fragments. Previously, Sekiguchi and Hakamori (1983) used fibrin affinity column chromatography to separate these two fragments on the basis of their differential fibrin-binding activity. The advantage of the FPLC procedure over the fibrin affinity column chromatography for separation of these two fragments is that the former requires no urea or other chaotropic agent so that the local envrionments of the free sulfhydryl groups present in the fragments are less likely to be perturbed. Slight variation in the elution profile from different Fn preparations was noted, which may be attributed to the charge differences resulting from the microheterogeneity of carbohydrate moieties in plasma Fn. The results obtained by analytical isoelectric focusing showed multiple bands between pH 5 and 6 (not shown), which is consistent with marked charge heterogeneity.

DTNB Titration Experiments. The results of DTNB titrations for free sulfhydryl groups in Fn and its fragments, both in solution and on beads, are summarized in Table I. For soluble Fn, the free sulfhydryl groups were not accessible to DTNB as shown previously (Smith et al., 1982; Lai et al., 1984). Upon addition of 4 M guanidine hydrochloride, we

Table I: Free Sulfhydryl Group Contents of Fn and Its Fragments Estimated by DTNB Titration

samples	-SH per chain	
	no guanidine	4 M guanidine
Fn	0.0	1.79 ± 0.24[a]
Fn + beads[b]	0.87 ± 0.05	1.82 ± 0.18
31 kDa	0.0	0.84 ± 0.01
31 kDa + beads	0.0	1.10 ± 0.05
34 kDa	0.0	1.24 ± 0.16
34 kDa + beads	0.0	1.08 ± 0.03
75 kDa	0.0	0.94 ± 0.02
75 kDa + beads	0.81 ± 0.03	0.81 ± 0.03
185 kDa	0.0	0.90 ± 0.02
185 kDa + beads	0.99 ± 0.08	0.99 ± 0.11
215 kDa	0.0	1.73 ± 0.06
215 kDa + beads	0.92 ± 0.11	1.86 ± 0.09

[a] Mean ± SD, two or more independent measurements. [b] 0.1-μm beads; 0.5-μm beads were used for all other experiments.

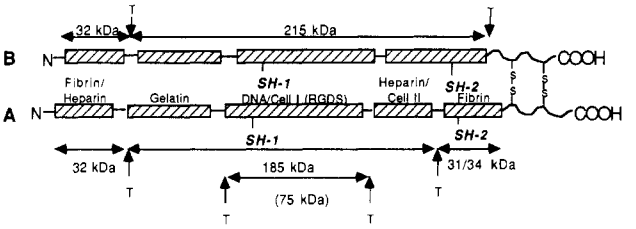

FIGURE 2: Schematic diagram depicting the trypsin cleavage sites (T) on the Fn molecule that gave rise to the fragments used in this study. The binding domains of Fn also are indicated (McDonagh, 1985; McCarthy et al., 1986).

estimate that about 1.79 ± 0.24 sulfhydryl groups per chain become accessible (see Table I), which is in accord with previous data (Smith et al., 1982; Lai et al., 1984). Interestingly, when Fn was bound to the beads, about 0.87 ± 0.05 sulfhydryl group per chain became titratable by DTNB in the absence of guanidine hydrochloride. This indicates that surface binding induces a conformational change in the Fn molecule, resulting in the exposure of one buried sulfhydryl group. Similar results were obtained when a spin-labeled maleimide derivative was used to modify the newly exposed sulfhydryl group (Narasimhan and Lai, unpublished observation).

The question arises whether SH-1 or SH-2 in the protein (see Figure 2) is exposed upon surface binding. It has been shown previously that the adsorption of plasma Fn to the hydrophobic surfaces is irreversible (McAbee & Grinnell, 1983; Schwarz & Juliano, 1984; Grinnell & Feld, 1981; Iwamoto et al., 1985; Jonsson et al., 1982). This agrees with our inability to recover the bound protein from the surface of polystyrene beads using a variety of detergents and denaturants including SDS, urea, and guanidine hydrochloride. Such tight binding precluded the most direct assignment of the position of the newly exposed sulfhydryl group by using a radiolabeled sulfhydryl reagent followed by fragment identification. Thus, to answer the above question, we have prepared Fn fragments that contain either SH-1 or SH-2 or both and determined their accessibility to DTNB reagent, both in solution and on beads. These Fn fragments include the 215-, 185-, 75-, 34-, and 31-kDa fragments; the origins of these fragments and their sulfhydryl contents are shown schematically in Figure 2.

The DTNB titration profile of the 215-kDa fragment behaves similarly to that of the intact Fn molecule. For example, DTNB titration showed that, in the absence of guanidine hydrochloride, the fragment in solution has no titratable sulfhydryl group. In the presence of 4 M guanidine hydrochloride, about 1.86 ± 0.09 sulfhydryl groups were shown to react with DTNB. When adsorbed onto the beads, 0.92 ± 0.11 sulfhydryl per chain became titratable (Table I).

To determine whether SH-2 located in the C-terminal fibrin-binding domain (see Figure 2) is exposed when Fn is adsorbed to the beads, we have prepared polystyrene beads coated with either the 31- or 34-kDa fragment. As shown in Table I, SH-2 contained in these fragments was not accessible to DTNB unless 4 M guanidine hydrochloride was added, regardless of whether the reaction was carried out in solution or on the beads. The results suggest that SH-1 rather than SH-2 is the newly exposed sulfhydryl group. This conclusion is supported by the DTNB titration profiles for the 75- and 185-kDa fragments, each of which contains only SH-1 (see Figure 2 and Table I). In solution the free sulfhydryl group of these fragments reacted with DTNB only when guanidine hydrochloride was present. In contrast, when bound to beads, 0.81 ± 0.03 and 0.99 ± 0.08 sulfhydryl group became exposed on the 75- and 185-kDa fragments, respectively (see Table I). Therefore, the buried SH-1 on the 75- or 185-kDa fragment became exposed when bound to polystyrene beads. Thus, the appearance of one DTNB-titratable free sulfhydryl group in plasma Fn upon adsorption of the protein to the beads results from exposure of SH-1.

Discussion

In this study, we demonstrate the differential exposure of one free sulfhydryl group in plasma Fn upon binding of the protein to polystyrene beads. The reason why only SH-1 becomes exposed might relate to the difference in the location of the two SH sites within the primary structure of plasma Fn (Skorstengaard et al., 1986; Kornblihtt et al., 1985). Although SH-1 and SH-2 are both situated in Type III homologous units, SH-1 is located in an interdomainal region between the DNA-binding and cell-binding domains, whereas SH-2 is within the fibrin-binding domain. The DNA-binding, cell-binding, and fibrin-binding domains are resistant to further proteolytic digestion (Skorstengaard et al., 1985), suggesting that they are stable and tightly folded structures. On the other hand, the interdomainal region, such as the Type III homologous sequence containing SH-1, is more sensitive to proteolytic attack and is probably more flexible (Skorstengaard et al., 1985). This may explain why, upon surface binding, SH-1 located in an interdomainal region is exposed, whereas SH-2 located in a binding domain is not. The magnitude of the conformational change induced by the surface association was estimated to be at least 4 × 6 Å, the dimension of DTNB molecule, although the upper limit is not known at present. Our recent ESR spin-label studies reveal that the spectrum of the labeled SH-1 site in plasma Fn changes from a strongly immobilized component into a weakly immobilized component upon binding of the labeled protein onto the beads, indicating that SH-1 becomes very flexible when the protein is bound to the surface (Narasimhan and Lai, manuscript in preparation).

It is also worthy of note that all Fn fragments prepared in this study, including the 215-, 185-, 75-, 34-, and 31-kDa fragments, are shown to bind to polystyrene beads, indicating that various regions of the Fn molecule are in direct contact with solid substrates. In addition, the results in Table I show that in solution free sulfhydryl groups present in the fragments are not titratable by DTNB unless guanidine hydrochloride is added, which is consistent with the notion that isolated binding domains of Fn retain their compactness and tight folding (Skorstengaard et al., 1985).

Soluble plasma Fn binds to fibroblast cells in suspension with a moderate affinity (Akiyama & Yamada, 1985). However, a 50-fold increase in affinity was observed when Fn was first

coated on the surface of polystyrene beads (McAbee & Grinnell, 1983). On the basis of these observations, it has been suggested that "surface activation" of plasma Fn is required for binding to the cell, although the nature of this hypothetical "activation" of Fn is not yet known (Akiyama & Yamada, 1987). In their studies of binding of Fn fragments to fibroblast cells, Akiyama and Yamada (1985) have observed that although the 75-kDa fragment binds as strongly as intact Fn to the cell surface, the affinity of the 11.5-kDa fragment to the cell is too low to be measured (Akiyama & Yamada, 1985). To account for this, they postulated that regions outside of the 11.5-kDa fragment, but within the 75-kDa fragment, are required for maximal cell surface binding. The present observations demonstrate that binding of the 75-kDa fragment to the beads induces a change in the fragment conformation as indicated by the change in exposure of SH-1 to bulk solvent. Although the exact conformational change of the fragment induced upon adsorption to the surface is not yet known, it is possible that, through intramolecular interactions, such a change in the fragment conformation may in turn affect the Arg-Gly-Asp sequence containing 11.5-kDa fragment to adopt a proper conformation for cell surface receptor interaction (Akiyama & Yamada, 1987; Ruoslahti & Pierschbacher, 1987). It is of interest to note that this region of Fn has been shown to promote tumor cell haptotaxis (McCarthy et al., 1985), implying that the ability of substratum-bound Fn to promote migration by haptotaxis may also relate to surface-induced conformational changes of the molecule.

The function of free sulfhydryl groups in Fn is still not known. We have shown previously that the free sulfhydryl groups in Fn are not required for mediation of initial cell attachment and spreading (Lai & Tooney, 1984). However, the possibility that SH-1 serves as a second putative cell binding site within the 75-kDa fragment through disulfide formations with other cell surface molecules at later stages after initial cell attachment warrants further investigation.

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