Enzymatic Modifications of Human Plasma Fibronectin in Relation to Opsonizing Activity†

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ABSTRACT: Plasma fibronectin is one of the largest plasma proteins (M_r ~440 000), comprising two approximately equal polypeptide chains which are held together by a disulfide linkage near the C-terminal end of the molecule. The binding of gelatinized latex beads to liver slices as well as the internalization of these particles by macrophages, in the presence of heparin, is greatly enhanced by fibronectin. The question as to whether the entire covalent structure of fibronectin was necessary for opsonizing activity was approached by limited proteolytic degradations of the molecule. Patterns of controlled digestion with trypsin, cathepsin D, *Staphylococcus aureus* protease, and plasmin all indicate that the minimal unit necessary for retention of opsonic activity is some large (M_r 200 000 and 190 000) single-chain entity. Treatment with plasmin proved to be the most reliable procedure for generating the active split product which could be readily separated from the inactive, disulfide-containing C-terminal fragment. Incorporation of dansylcadaverine into plasma fibronectin (3.5 mol/mol of protein) by fibronoligase (coagulation factor $XIII_a$) did not affect the opsonic activity of the protein.

Fibronectin occurs on the surfaces of various cells (Furcht et al., 1978; Stenman & Vaheri, 1978; Mosher, 1980) and as a component of some basement membranes (Hedman et al., 1978; Bray, 1978). In addition, it is also present in blood plasma at a significant concentration (Blumenstock et al., 1977a,b; Mosher & Williams, 1978). Plasma fibronectin, a remarkably conserved protein throughout the vertebrate phylum (Weissmann et al., 1979), has a molecular weight of about 440 000, comprising two nearly equal polypeptide chains (Wagner & Hynes, 1979; Mosesson & Amrani, 1980; Sekiguchi et al., 1981) linked together by a disulfide bridge near the C-termini. There are indications that fibronectin may become covalently attached to fibrin in the course of normal blood coagulation through the functioning of the fibrin stabilizing (factor XIII) system (Mosher, 1975, 1976) and that this may contribute to the strengthening of the clot structure (Kamykowski et al., 1981). Other findings suggest that plasma fibronectin serves as an opsonin for the purpose of host defense, promoting the removal of foreign particulate matter from the circulation (Saba et al., 1978; Molnar et al., 1979). Covalently linked gelatin–latex beads, labeled with ^{125}I, proved to be especially useful for measuring the phagocytosis-enhancing activity of fibronectin in conjunction with both liver slices and isolated macrophages (Molnar et al., 1979; Check et al., 1979; Gudewicz et al., 1980; van de Water et al., 1981). We have utilized these assay systems to examine the effects of the enzymatic modifications of the plasma fibronectin molecule in regard to altering its opsonic activity. The present work deals with dansylcadaverine substitutions at the factor $XIII_a$ reactive sites and with the degradation of plasma fibronectin by various proteases. A preliminary report was presented previously (Molnar et al., 1979b).

Materials and Methods

The following enzymes were obtained from commercial sources: cathepsin D (Sigma Chemical Corp.); *Staphylococcus aureus* protease (Miles Labs); tosylphenylalanyl chloromethyl ketone (TPCK) treated trypsin (Worthington Biochemical Corp.). Urokinase and human α-thrombin, respectively, were gifts from Abbott Laboratories and from J. W. Fenton II of the New York State Department of Health, Albany, NY. Pepstatin was obtained through the courtesy of the U.S.–Japan Cancer Committee. Hirudin and phenylmethanesulfonyl fluoride were purchased from Sigma Chemical Co.; Trasylol was from Mobay Chemical Corp., FBA Pharmaceuticals. Gelatin was from Nutritional Biochemical Corp.; carboxylated latex beads (450 nm in diameter) were from Dow Chemical Co. The coupling of gelatin to the beads and the subsequent radioiodination were carried out by previously described methods (Molnar et al., 1979a). Dansylcadaverine (Lorand et al., 1968) was obtained as the hemifumarate salt through the courtesy of A. B. KABI, Stockholm. Sepharose 4B and Sephadex G-75 and G-100 gels were purchased from Pharmacia.

Plasminogen was isolated from human plasma by affinity chromatography on lysine–Sepharose (Deutsch & Mertz, 1971). Plasmin was prepared just prior to use by incubating 0.3–0.4 mg of plasminogen with 100 units of urokinase for 15 min at room temperature in 0.06 mL of solution containing 0.05 M lysine and 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) at pH 8.

Fibrin stabilizing factor (factor XIII) was kindly prepared by T. J. Janus from outdated human plasma on the basis of published procedures (Lorand & Gotoh, 1970; Curtis & Lorand, 1975). Activation of the zymogen was carried out on the day of the experiment by incubating for 30 min at room temperature 1.2 mg/mL factor XIII with 1.5 NIH units of thrombin in 0.05 M Tris-HCl–0.1 M NaCl at pH 7.5. The thrombin-modified zymogen was kept on ice before use. Fibronectin was isolated from human plasma by gradient elution chromatography from DEAE-cellulose as a byproduct of purifying factor XIII (Molnar et al., 1979a).

Dansylcadaverine was incorporated into fibronectin by incubating a 2 mM sample of the amine with 1 mg/mL fibronectin and with 22 μg/mL thrombin-modified factor XIII for 2 h at 37 °C, in the presence of 3 mM $CaCl_2$ in a solution containing 0.1 M NaCl and 0.05 M Tris-HCl of pH 7.5. Following the reaction, the material was dialyzed exhaustively at 4 °C against a 0.05 M Tris-HCl buffer, pH 7.5, with 1 mM ethylenediaminetetraacetic acid (EDTA). Throughout the

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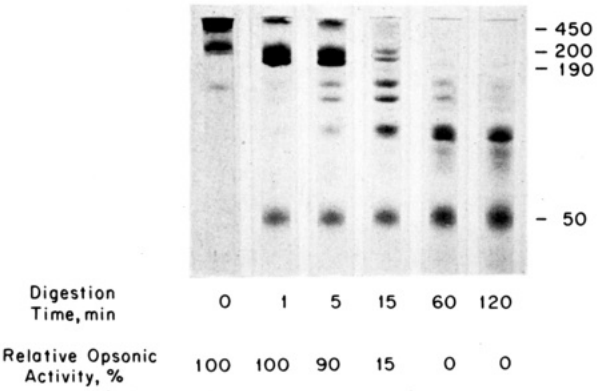

FIGURE 1: Digestion pattern of fibronectin with 0.5% (w/w) TPCK–trypsin, analyzed by polyacrylamide gel (3.8%) electrophoresis in the presence of 0.1% SDS and 6 M urea. Molecular weight designations ($\times 10^{-3}$) are shown on the right. Relative opsonizing activity, remaining in the digests after the indicated time periods, was measured by the liver slice assay and was expressed as the percentage activity of the native fibronectin material.

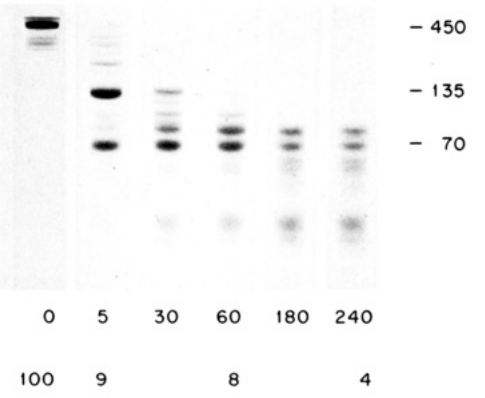

FIGURE 2: Digestion of fibronectin with a high concentration of cathepsin D (2% w/w). Analytical procedures and notations are as in Figure 1.

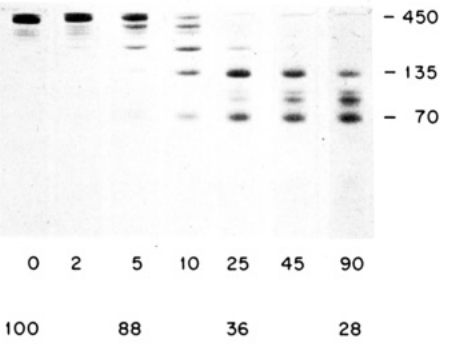

FIGURE 3: Digestion of fibronectin with a low concentration of cathepsin D (0.23% w/w). For procedures and notations, see Figure 1.

experiment, care was taken to protect the dansylcadaverine and its protein derivative from exposure to light. Dansylcadaverine incorporation was measured by fluorescence (Lorand et al., 1968), following digestion of the protein sample with 2% (w/w) trypsin overnight at 37 °C.

Gel electrophoretic examination of proteolytic digestion products of fibronectin was performed by the procedure of Weber & Osborn (1969) with 3.8% polyacrylamide, in the presence of 6 M urea and 0.1% sodium dodecyl sulfate (SDS), using a protein load of 25 μg per gel. Samples were denatured in 1% sodium dodecyl sulfate and 6 M freshly deionized urea and, when reduction was required, were also treated with 40 mM dithiothreitol.

Opsonic activities of the native plasma fibronectin and of the various enzymatic digests were measured with the use of ^{125}I-labeled gelatin-coated latex particles either for the fibronectin-dependent uptake by liver slices (Molnar et al., 1979a) or for phagocytosis by peritoneal macrophages (Gudewicz et al., 1980). In order to ensure a better uniformity of liver slices (100 ± 20 mg in weight), the former procedure was slightly modified by using a stainless-steel puncher to obtain disks of the tissue 1 cm in diameter. Protein determinations were carried out by the procedure of Lowry et al. (1951).

Results

An important aim of this work was to define the limits of digestion of the plasma fibronectin molecule with proteolytic enzymes which would still be compatible with retention of opsonic activity. Limited degradation procedures with trypsin, cathepsin D, *S. aureus* protease, and human plasmin gave interesting results when the fragmentation patterns obtained by acrylamide gel electrophoresis were compared with measurements of residual opsonic activity (liver slice assay; Molnar et al., 1979). Figures 1–5 pertain to the examination of the digestion products in 0.1% SDS and 6 M urea by electrophoresis in 3.8% polyacrylamide, without the addition of reducing agents. Specific conditions for the degradation of fibronectin by the different enzymes were as given below.

In the experiment shown in Figure 1, 2.9 mg/mL fibronectin was treated at 37 °C with 15 μg/mL TPCK–trypsin in 0.05 M Tris-HCl buffer, pH 7.5, for periods up to 2 h. At the times indicated, aliquots were withdrawn, and Trasylol was added in 5-fold molar excess over trypsin to terminate the digestion. Under these conditions, in spite of the extensive degradation seen on the electrophoretic profile, essentially full opsonic activity was maintained for up to 5 min of digestion. This correlated with the formation of large fragmentation products in the 200K and 190K molecular weight range. Further digestion, which caused the disappearance of these two species, led to a rapid loss of opsonic activity.

Digestion with cathepsin D was carried out at two concentrations of the enzyme (60 μg/mL in Figure 2 and 8 μg/mL in Figure 3). The reaction mixtures with 3 mg/mL fibronectin, in a solution of 0.05 M sodium citrate containing 0.4 mM phenylmethanesulfonyl fluoride, were incubated at 37 °C. At the times indicated in the figure legends, aliquots were withdrawn, and the reaction was terminated by the addition of a 6-fold molar excess of pepstatin (over cathepsin D) in 0.2 M Tris of pH 9, resulting in a final pH of 7.2 and a Tris concentration of 0.04 M. As seen in Figure 2, with the use of the higher concentration of cathepsin D, less than 10% of the original opsonic activity remained even at the earliest time point (5 min), when two major bands with apparent molecular weights of 135K and 70K were seen on gel electrophoresis in roughly equal proportion. However, with the lower concentration of cathepsin D (Figure 3), some 88% of the opsonic activity was preserved up to 5-min duration, i.e., before perceptible amounts of the 135K and 70K fragments were formed.

The digestion of fibronectin (2.7 mg/mL) with *S. aureus* protease (14 μg/mL) was carried out at 37 °C in 0.1 M ammonium bicarbonate of pH 7.8 and was terminated by the addition of phenylmethanesulfonyl fluoride to a concentration of 0.4 mM. Treatment with the enzyme, which acts quite selectively on endo-glutamyl peptides (Drapeau, 1976), allowed preservation of about 82% of the opsonic activity for 10 min and 70% for 60 min. It can be seen on the electrophoretic profiles in Figure 4 that, as before, retention of activity was

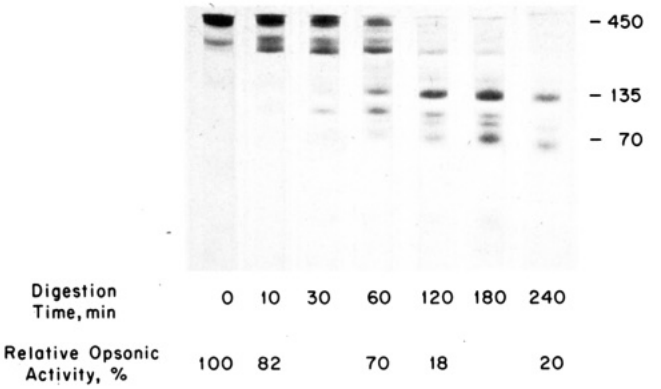

FIGURE 4: Effect of *S. aureus* protease (0.5% w/w) on fibronectin. For analytical details, see Figure 1.

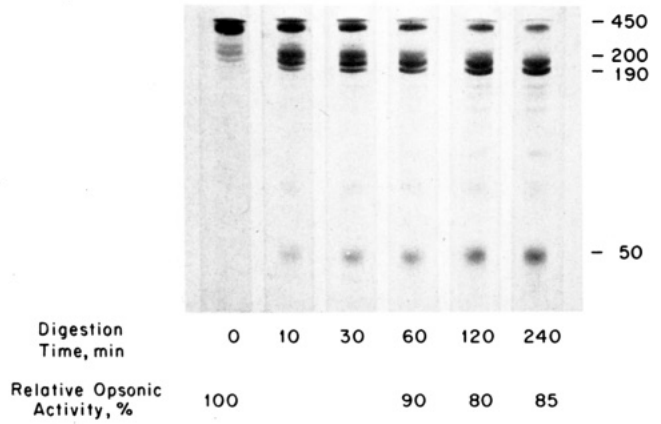

FIGURE 5: Plasmin (2% w/w) digestion patterns for fibronectin. Analytical procedures and notations are as in Figure 1.

associated with the presence of fragments larger than 135 K in molecular weight. When this and smaller sized products began to accumulate, opsonic activity became significantly lower.

In contrast to the above procedures, plasmin digestion offered the best means for a controlled degradation of the fibronectin molecule, with good retention of opsonizing activity for longer periods of time. By treatment of fibronectin (1 mg/mL) wiith human plasmin (20 μg/mL) at 37 °C in a medium containing 0.05 M Tris-HCl (pH 7.5) and 0.1 M NaCl, and termination of the reaction by the addition of a 5-fold molar excess of Trasylol (over plasmin), some 85–90% of the activity of the protein was retained in the mixture even after 4 h of digestion (Figure 5). Electrophoresis at this time, in the absence of a reducing agent, showed very little of the original 450K protein remaining, and the band pattern was dominated by three fragments representing molecular weights of about 200K, 190K, and 50K, respectively. A number of bands of low intensity (one of them corresponding to plasmin) were visible between the 190K and 50K species.

The digestion pattern with plasmin appeared to be very reproducible and lent itself readily for further analysis. Figure 6 shows electrophoretic profiles for the starting material and for the 2-h digest with and without reduction by 40 mM dithiothreitol. As expected, upon reduction, the original fibronectin gave rise to a strong band in the 200K weight region. With the 2-h digest, yet another change became obvious in that the 50K fragment disappeared and a new band emerged at about 30K. These observations would be compatible with the idea that the 50K plasmin degradation product comprised the disulfide-linked chain fragments from the carboxy-terminal region of fibronectin.

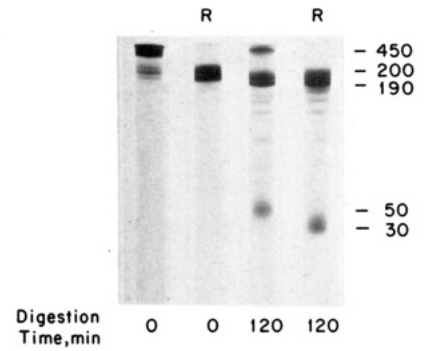

FIGURE 6: Comparison of SDS–polyacrylamide gel electrophoresis (PAGE) profiles of the 2-h plasmin (2% w/w) digest of fibronectin with the native protein (i.e., 0 min of digestion). Except for the addition of 40 mM dithiothreitol prior to electrophoresis as a reducing agent to the samples marked R, analytical procedures were identical with those given in Figure 1.

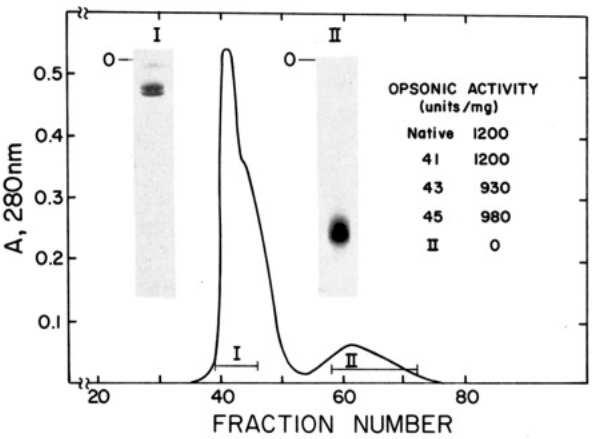

FIGURE 7: Isolation of the opsonically active fragments from the 2-h plasmin (2% w/w) digest of fibronectin, using a Sephadex G-75 gel filtration column. For experimental details, see the text. On the basis of the absorbancy profile (A_{280nm}), two protein pools (I and II) were separated as marked by horizontal bars. It could be shown by SDS–PAGE (inserts) that the pool I material contained the 190K and 200K fragments and that pool II comprised the 50K product. Measurements of opsonic activity with the liver slice assay (given as units per milligram of protein) revealed that fraction 41 in the pool I material had as high a specific activity as the native fibronectin molecule. No opsonic activity was associated with the 50K material of pool II.

Given the rather simple fragmentation pattern of fibronectin with plasmin and the good retention of opsonizing activity in the digestion mixture, it was possible to apply gel filtration chromatography to purify the active component. Fibronectin (3 mg/mL) in 0.05 M Tris-HCl and 0.1 M NaCl was treated at 37 °C and pH 7.5 with 60 μg/mL of plasmin. Following 2 h of incubation, a 4-fold molar excess of Trasylol (over plasmin) was admixed, and the 5-mL sample was passed through a lysine–Sepharose column (0.7 × 8 cm) to remove plasmin. After concentration in a dialysis bag immersed in Sephadex G-75, the material was applied to a Sephadex G-100 column (2.5 × 95 cm) which was equilibrated with 0.05 M Tris-HCl and 0.1 M NaCl at pH 7.5. Elution was carried out with the same buffer at a flow rate of 22 mL/h, and 4-mL fractions were collected. Figure 7 shows the elution profile, as measured by the absorbance at 280 nm. Fractions indicated by horizontal bars were combined into two pools (I and II) and were concentrated by ultrafiltration, by using Amicon membrane PM-30 for pool I and UM-10 for pool II. The SDS electrophoretic profiles showed that the pool I material contained the 200K and 190K fragments, whereas pool II represented the 50K molecular weight plasmin degradation

product of fibronectin. Essentially no opsonic activity was associated with the latter. By contrast, full specific activity could be recovered from the peak fraction of pool I.

The effect of plasmin digestion on the opsonic activity of fibronectin was also evaluated in the macrophage assay system, which provides a measure for the active internalization of the test particles (Gudewicz et al., 1980) rather than for simple binding to the surface of liver slices (Molnar et al., 1979a). Digestion of fibronectin (five different samples) for 4 h at 37 °C with plasmin (2% w/w) showed a complete retention of activity in the macrophage system as well.

N-(5-Aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide (i.e., dansylcadaverine) is one of the best synthetic amine substrates for coagulation factor XIII, activated by thrombin and Ca^{2+} (Lorand et al., 1968). This fluorescent amine could be shown to become incorporated in a site-specific manner into reactive γ-glutamine residues of a number of proteins which otherwise would be involved in forming γ-glutamyl-ϵ-lysine cross-links. When treated with dansylcadaverine in the presence of the thrombin-modified human factor XIII and Ca^{2+}, as given under Materials and Methods, fibronectin incorporated 3.5 mol of dansylcadaverine per 440000 g of protein. Significantly, the specific opsonic activity of fibronectin was unaffected by this treatment. Using liver slices, we found an opsonizing activity of 1500 units/mg for the starting protein preparation and 1470 units/mg for the dansylcadaverine-labeled material.

Digestion of the dansylcadaverine-labeled fibronectin with 2% (w/w) plasmin yielded fragments corresponding to those in Figures 5 and 6. When viewed under UV illumination [Mineralight, short wavelength; for methodology, see Lorand et al. (1972)], the nonreduced gels showed fluorescent bands at the 200K, 190K, and 50K molecular weight regions, whereas in reduced gels the latter fragment was absent and was replaced by a fluorescent band which moved close to the 30K marker.

Discussion

Plasma fibronectin is a large protein (M_r ~440000) comprising two constituent chains which are almost identical and are connected near the C-terminal region by a disulfide bridge [for recent reviews, see Mosesson & Amrani (1980) and Mosher (1980)]. Electron microscopic observations indicate an overall contour length of 120–160 nm for the molecule, and the two chains may be arranged either in an extended configuration where each polypeptide spans half the length of the strand (Erickson et al., 1981) or in a V-shaped structure where the two chains meet at an angle of about 70° (Engel et al., 1981). The strands themselves appear to be thin (2 nm in diameter) and flexible, without any recognizable globular features. The latter point is of interest because it is known that the fibronectin molecule comprises multiple binding domains. In the absence of globular regions, the binding sites must be spread along consecutive stretches of the chains. This notion is amply supported by the fact that, following limited digestion with proteases, collagen binding (Balian et al., 1979; Ruoslahti et al., 1979; Wagner & Hynes, 1979, 1980; Furie et al., 1980), actin binding (Keski-Oja & Yamada, 1981), heparin binding (Yamada et al., 1980; Hayashi & Yamada, 1981), DNA binding (Pande & Shively, 1982), fibrin binding (Iwanaga, 1978; Mosher et al., 1979; Sekiguchi & Hakamori, 1980; Sekiguchi et al., 1981), and cell-surface binding (Ruoslahti & Hayman, 1979; Hahn & Yamada, 1979) fragments could be isolated. In addition, using coagulation factor $XIII_a$ for transamidation, one could generate covalently linked hybrid structures of fibronectin in vitro with fibrin (Mosher, 1975), *S. aureus* (Mosher & Proctor, 1980), and collagen derivatives (Mosher et al., 1979). Thus, it is suggested that the factor $XIII_a$ reactive domain corresponds to a 27K fragment at the N-terminus of the chain, followed by a 45K gelatin (collagen) binding region, then a 120K cell attachment, and, finally, a 40K heparin binding region [see Ruoslahti et al. (1982)] close to the interchain disulfide bridge. Interestingly, internal sequence homologies characterize each binding domain (Petersen et al., 1983).

Among the known biological functions of the fibronectin molecule, promotion of phagocytosis appears to be the most complex. It has recently become possible to quantitate this activity (Saba & DiLuzio, 1965; Saba et al., 1966; Allen et al., 1973; Blumenstock et al., 1977, 1978). In the present study, we used latex beads to which gelatin was covalently attached and which were then made radioactive by labeling with ^{125}I (Molnar et al., 1979a). These stable and highly reproducible test particles are taken up by liver slices and by isolated macrophages (Gudewicz et al., 1980; Doran et al., 1980; Van DeWater et al., 1981), although internalization occurs only in the latter system. Inasmuch as heparin acts as a cofactor in both systems, in terms of opsonizing activity (Filkins & DiLuzio, 1966; Molnar et al., 1979a; Gudewicz et al., 1980), one may assume that, in addition to the collagen (i.e., gelatin) and the cell-surface binding regions of the molecule, the heparin binding domain of fibronectin is also needed for this activity.

The experimental results presented in this paper show that opsonization by fibronectin does not require the entire, covalently bonded doublet structure of the molecule. Some of the proteases (trypsin and plasmin), under certain conditions, caused the removal of a 50K fragment without any loss of opsonic activity (see Figures 1 and 5). Upon reduction with dithiothreitol, the released fragment moved in SDS gels with an approximate molecular weight of about 30K (Figure 6). Furthermore, in the case of dansylcadaverine-labeled fibronectin, both the 50K and its reduced derivative (~30K) fragments showed the presence of the fluorescent label.

The minimal opsonic unit for fibronectin under the experimental conditions employed appears to be a single-stranded half-molecule with a molecular weight greater than 135000. Actually, the digests in which the 135K and a 70K fragment were prominent (see Figure 3, 25–90 min, and Figure 4, 120–240 min) showed only about 20–30% activity. Full retention of opsonizing activity can be claimed only in those instances where polypeptides of approximately 200K and 190K in chain weight were present (see Figure 1, 1 and 5 min; Figure 3, 5 min; Figure 4, 10 and 60 min; and Figure 5, 1 up to 240 min). Though one cannot exclude the possibility that these split half-strands (200K and 190K) might not associate into a bifunctional system in solution even in the absence of the connecting disulfide segment, the electron microscopic evidence put forth by Erickson et al. (1981) regarding the plasmin digestion products of fibronectin would seem to suggest that a single thickness (i.e., 2 nm) prevails in the structures of half-length as well. It is interesting to note that proteolytic fragments of this size range also seem to display chemotactic and phagocytosis-enhancing activities toward monocytes (Norris et al., 1983; Czop et al., 1982).

Employing gel filtration, these larger products could be purified from the plasmin digest (Figure 7), and the isolated material showed essentially a complete retention of opsonizing potency. Albeit, following plasmin digestion, the system would become monofunctional as opposed to the original disulfide-linked bifunctional structure, all of the binding domains

considered to be essential for the expression of opsonic activity; i.e., the gelatin or collagen, cell-surface and heparin binding areas, would obviously still exist in a linked, colinear manner. Relevant to these studies are the observations of Ehrlich et al. (1981) that extensive digestion of plasma fibronectin by plasmin caused a loss of opsonic activity and the generation of fragments which reduced the clearance rates of gelatinized colloid particles in rats. A recent abstract by Rourke et al. (1983), from the same laboratory, however, reports considerable retention of opsonic activity in the 190 000–200 000 molecular weight fragment, confirming our findings.

The results with the plasmin digest of dansylcadaverine-substituted fibronectin, into which 3.5 mol of the amine was incorporated by factor $XIII_a$, clearly show that the molecule contains two different sets of factor $XIII_a$ reactive γ-glutamine sites. According to literature reports, one of these is located in the 50K segment, which is thought to represent the disulfide-linked C-terminal region portion (Jilek & Hörmann, 1977), and the other is contained in the N-terminal region of the large 200K and 190K fragments (Mosher et al., 1980; Akiyama et al., 1981; Petersen et al., 1983).

Registry No. Plasmin, 9001-90-5; trypsin, 9002-07-7.

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Characterization of the Membrane Binding Domain of γ-Glutamyltranspeptidase by Specific Labeling Techniques†

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ABSTRACT: The amphipathic form of γ-glutamyltranspeptidase was labeled either by reductive methylation of primary amino groups or by galactose oxidase/NaB^3H_4 labeling of galactose residues. The labeled enzyme was asymmetrically reconstituted into unilamellar phosphatidylcholine vesicles and subjected to partial papain proteolysis, and the resulting products were resolved by Sepharose 4B chromatography. Chromatography of the vesicle-associated material on Sephadex LH-60 yields an 8700 molecular weight peptide which is labeled by both techniques. This peptide, therefore, contains lysine residues and/or the NH_2-terminus of the large subunit and a galactose-containing oligosaccharide side chain. This peptide appears to be identical with peptide I which is labeled by 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine [Frielle, T., Brunner, J., & Curthoys, N. P. (1982) *J. Biol. Chem. 257*, 14979–14982]. A second hydrophobic peptide (peptide II) which is also labeled by the membrane-permeable, photoactivatable probe is not significantly labeled either by reductive methylation or by galactose oxidase/NaB^3H_4 labeling. Sephadex G-25 chromatography of the ^{3}H-labeled hydrophilic peptides released from the vesicles by papain proteolysis yields a [^{3}H]galactose-labeled peptide of 2600 molecular weight (peptide III) and a 1300 molecular weight peptide labeled by reductive methylation (peptide IV). Peptide I, but not peptide IV, partitions into a series of primary aliphatic alcohols and can be reconstituted into vesicles. The hydrophilic peptides are probably derived either from a peripheral sequence of the membrane binding domain or from the region which connects the hydrophilic domain of the large subunit with the membrane binding domain.

Rat renal γ-glutamyltranspeptidase is an amphipathic integral membrane glycoprotein that is asymmetrically associated with the brush border membrane of the proximal tubule (Tsao & Curthoys, 1980). The enzyme possesses a limited hydrophobic domain that is responsible for its membrane association (Curthoys & Hughey, 1979). The catalytic activity is contained within a separate hydrophilic domain that is highly glycosylated and positioned away from the membrane surface by a sequence of amino acids that is sensitive to papain. As a result, the γ-glutamyltranspeptidase purified following solubilization by limited papain proteolysis is soluble in aqueous buffers and has a molecular weight for 69 000 (Hughey & Curthoys, 1976). In contrast, purification following solubilization with Triton X-100 yields an amphipathic enzyme that is soluble only at concentrations of detergent above the critical micelle concentration. After correction for the mass of bound detergent, the molecular weight of the Triton X-100 purified enzyme was estimated to be 87 000 (Hughey & Curthoys, 1976). Only the amphipathic form of the enzyme can be asymmetrically reconstituted into unilamellar phosphatidylcholine vesicles (Hughey et al., 1979).

γ-Glutamyltranspeptidase is composed of two nonidentical subunits. The molecular weight of the smaller subunit, which contains the γ-glutamyl binding site (Tate & Meister, 1977), is unaltered by papain treatment. However, the large subunit of the Triton-purified enzyme is 21 000 daltons greater than that of the papain-purified enzyme (Horiuchi et al., 1978). The isolated small subunits of the two forms of enzyme both contain NH_2-terminal threonine residues, whereas the NH_2-terminal residues of the large subunits are nonidentical (Tsuji et al., 1980). Furthermore, the reaction of the reconstituted enzyme with the membrane-soluble, photactivatable reagent 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID)[1] results in the labeling of only a limited segment of the large subunit (Frielle et al., 1982). Thus, the NH_2-terminal segment of the large subunit apparently contains the hydrophobic membrane binding domain of γ-glutamyltranspeptidase.

Amino acid sequence analyses indicate that the membrane binding domains of NADPH–cytochrome P-450 reductase (Black & Coon, 1982) and the intestinal brush border membrane enzymes aminopeptidase N (Feracci et al., 1982) and the sucrase-isomaltase complex (Spiess et al., 1982) possess NH_2-terminal hydrophilic sequences which contain one to three lysine residues. The NH_2-terminal segment of the sucrase–isomaltase complex also contains an oligosaccharide side chain which is linked to a threonine residue at position 11 (Frank et al., 1978). In each case, the hydrophilic sequence is contiguous with a hydrophobic sequence which is integrated into the lipid bilayer. It has been proposed that the charged res-

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[1] Abbreviations: [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PγGT, γ-glutamyltranspeptidase purified following papain proteolysis; V_R, retention volume; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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