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Thrombospondin Is a Substrate for Blood Coagulation Factor XIIIa†

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ABSTRACT: Thrombospondin (TSP) is released from α granules of activated platelets, binds to platelet surfaces, and copolymerizes with fibrin. In the present experiments, we investigated the action of factor XIIIa (plasma transglutaminase) on TSP. Factor XIIIa catalyzed incorporation of [^{14}C]putrescine into soluble TSP and ligation of TSP to itself and to fibrin intermediates. Proteolytic digestion of [^{14}C]putrescine-labeled TSP with trypsin or thrombin yielded a labeled disulfide-bonded core of 90 or 120–130 kilodalton (kDa) subunits, labeled fragments of less than 10 kDa, and an unlabeled 30-kDa heparin-binding fragment, indicating the presence of multiple factor XIIIa reactive glutaminyl residues located in several domains of the molecule. TSP became ligated in fibrin clots formed from amidinated fibrinogen, i.e., fibrin that could not contribute lysyl residues to factor IIIa catalyzed cross-links. The disulfide-bonded core of TSP formed upon thrombin digestion copolymerized with fibrin as efficiently as intact TSP. However, a lower proportion of the disulfide-bonded core became ligated. These results indicate that TSP, both in clots and in solution, contributes glutaminyl and lysyl residues to factor XIIIa catalyzed ligation. Cross-linking may be important in stabilizing interactions among TSP, fibrinogen, or fibrin and other molecules in hemostatic plugs.

Thrombospodin (TSP) is a major protein secreted from the α granules of activated platelets (Hagen, 1975). It is present in plasma at a concentration of only 20–100 ng/mL, but its concentration in serum is about 20 μg/mL due to platelet release (Saglio & Slayter, 1982; Dawes et al., 1983; Mosher et al., 1983). TSP is also secreted by a variety of cells in culture (McPherson et al., 1981; Sage et al., 1981; Mosher et al., 1982; Raugi et al., 1982; Jaffe et al., 1983).

TSP may be involved in several aspects of blood coagulation.

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Upon secretion from platelets, it binds to the platelet surface and may participate in platelet aggregation, possibly by stabilizing fibrinogen binding to platelets (Jaffe et al., 1982; Gartner et al., 1984; Leung, 1984; Dixit et al., 1985; Haverstick et al., 1985). TSP binds to fibrinogen (Leung & Nachman, 1982; Lahav et al., 1984), probably to the α chain (Tuszynski et al., 1985). TSP becomes an intrinsic part of the fibrin meshwork that forms around the platelet plug and influences the structure of the fibrin network (Bale et al., 1985; Bale & Mosher, 1986). TSP has also been shown to interact with other plasma proteins, including plasminogen (Silverstein et al., 1984), fibronectin (Lahav et al., 1982, 1984), and histidine-rich glycoprotein (Leung et al., 1984), and to possibly modulate plasminogen activation (Silverstein et al., 1985).

Factor XIIIa (plasma transglutaminase) is activated by thrombin and stabilizes the noncovalently bonded fibrin network by introduction of covalent linkages. It catalyzes an acyl transfer reaction in which a glutaminyl residue is the acyl donor, a lysyl residue is the acyl acceptor, and an ϵ-(γ-glutamyl)lysyl amide bond is the product (Folk & Finlayson, 1977). Factor XIIIa can also introduce primary amines into fibrinogen and fibrin (Lorand et al., 1972). Amine incorporation has been used to identify and study other substrates of factor XIIIa that contain reactive glutaminyl residues including fibronectin (Mosher, 1975, 1976), α-2-macroglobulin (Mosher, 1976), and α-2-plasmin inhibitor (Sakata & Aoki, 1980).

In fibrin, factor XIIIa rapidly forms the ϵ-(γ-glutamyl)lysyl linkage between two γ chains in adjacent monomers within a staggered overlapped fibrin protofibril (McKee et al., 1970). Polymers of α chains resulting from ligation of adjacent protofibrils within a larger fibrin fiber form at a slower rate (McKee et al., 1972). Factor XIIIa also has been shown to catalyze formation of cross-links between fibrin and other plasma proteins including fibronectin (Mosher, 1975) and α-2-plasmin inhibitor (Sakata & Aoki, 1980). Although these two proteins theoretically need only to contribute lysyl residues to the cross-link, in fact the cross-links seem to be between lysyl residues in fibrin and glutaminyl residues in fibronectin (Mosher & Johnson, 1983a) and α-2-plasmin inhibitor (Sakata & Aoki, 1982).

In these studies, we show that TSP is a substrate for factor XIIIa, describe several actions of factor XIIIa on TSP, and discuss the possible role of these reactions in blood coagulation.

Materials and Methods

Materials. Bio-Gel P-300, Affi-Gel heparin, and gel electrophoresis materials were from Bio-Rad (Richmond, CA). Na^{125}I, [^{14}C]putrescine, and En^{3}Hance for fluorography were purchased from New England Nuclear (Boston, MA). Materials for autoradiography were from Kodak (Rochester, NY). L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin was from Worthington (Freehold, NJ). Soybean trypsin inhibitor, bovine serum albumin, hirudin, and *p*-nitrophenyl *p*-guanidinobenzoate were from Sigma (St. Louis, MO).

Proteins. Human platelet TSP was purified as previously described (Bale et al., 1985). All buffers contained 0.1 mM calcium chloride. TSP concentration was calculated on the basis of a published extinction coefficient at 280 nm (Margossian et al., 1981) with correction for scattering at 320 nm. A representative TSP preparation containing 366 μg/mL of protein was analyzed for possible contamination by fibrinogen and fibronectin. A commercial assay for fibrin degradation products (Dade) showed that there was less than 0.8 μg/mL fibrinogen in the preparation. Fibronectin contamination was analyzed by an enzyme-linked immunoadsorbant assay and found to be less than 0.2 μg/mL. Purified TSP was iodinated with Na^{125}I by the Chloramine T method and repurified on Affi-Gel heparin as described previously (McKeown-Longo et al., 1984). Buffers contained 0.1 mM calcium chloride. No identifiable ^{125}I-labeled fibrinogen or fibronectin was detected when [^{125}I]TSP was analyzed by gel electrophoresis followed by autoradiography, even when the autoradiographs were overexposed (see lane a of Figure 2).

Human fibrinogen was purified from the cryoprecipitate by ammonium sulfate precipitation and chromatography on DEAE-cellulose (Mosher & Blout, 1973). Peak 1 fibrinogen was used for most experiments. It contained no factor XIII activity. Some experiments used peak 2 fibrinogen, which contained sufficient factor XIIIa activity to completely ligate fibrin formed from it. Fibrinogen concentration was determined by using a published extinction coefficient (Mihalyi, 1968) with correction for scattering at 320 nm. Clotability was measured spectrophotometrically (Roberts et al., 1974). Lysyl residues of fibrinogen were modified by reaction with 0.3 M ethyl acetimidate (Phillips & York, 1973). Clotability was unaffected by the modification.

Human plasma factor XIII was purified from fresh frozen plasma according to published procedures (Lorand & Gotoh, 1970; Kazema et al., 1976). It was stored at 4 °C in 0.05 M Tris-HCl, 0.15 M NaCl, and 1 mM EDTA, pH 7.5. Concentration was determined by using a published extinction coefficient (Schwartz et al., 1973).

Factor XIII, 1 mg/mL, was activated to factor XIIIa by incubation with thrombin and calcium chloride for 90 min (Lorand & Gotoh, 1970; Bale et al., 1982). In some experiments, thrombin was inhibited with hirudin at a 10-fold or greater excess by unitage.

Human α-thrombin was a generous gift from Dr. John Fenton II, New York State Department of Health, Albany, NY.

Gel Electrophoresis. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS–PAGE) was performed on slabs of 8% separating and 3.3% stacking gels by using a discontinuous buffer system (Laemmli, 1970). SDS–agarose gel electrophoresis was performed with a buffer system containing 0.1 M phosphate, pH 7.1, as previously described (Bale et al., 1982) on 2% slabs. The denaturing solvent for sample preparation contained 9 M urea and 3% SDS. This was mixed one part to five with protein solution; 10% β-mercaptoethanol (BME) was added if reduction of disulfide bonds was desired. Washed clots containing about 0.2 mg of fibrin were dissolved in 0.2 mL of denaturing solution containing 10% BME. Reduction of disulfide bonds was accomplished by heating the samples for 10 min at 90–95 °C. Sample size for electrophoresis was adjusted to equalize radioactivity in each lane of a slab gel. Proteins were visualized by staining with Coomassie Brilliant Blue. For visualization of ^{125}I-labeled proteins, slabs were dried and autoradiographed with XAR-Z film. The proportions of ^{125}I-labeled proteins in various bands within a single lane were quantified by excision of appropriate bands from the dried gel, followed by analysis for radioactivity. Bands containing [^{14}C]putrescine were identified by fluorography (Laskey & Mills, 1975).

Fibrin oligomers formed at pH 8.5, ionic strength 0.45, were used as size standards for agarose gel electrophoresis. Under these conditions, lateral aggregation of protofibrils was essentially eliminated, and a polymerizing mixture quenched shortly before the gel point provided oligomers of a wide range of sizes (Bale et al., 1982).

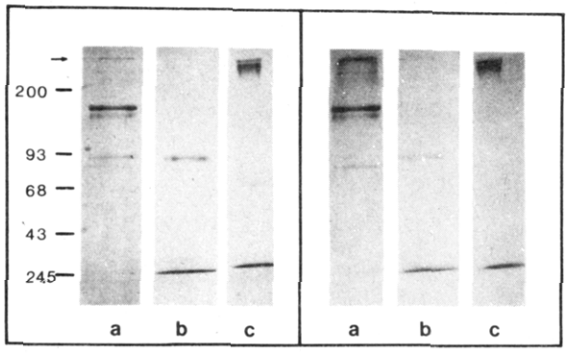

FIGURE 1: Incorporation of [^{14}C]putrescine into TSP. SDS–PAGE of TSP labeled with [^{14}C]putrescine before and after digestion with trypsin: protein staining (left panel) and fluorography (right panel). TSP, 230 μg/mL, was incubated with 9.6×10^{-5} M [^{14}C]putrescine, 133 μg/mL factor XIIIa, and 2 mM calcium chloride at 22 °C for 24 h. Thrombin, 0.13 units/mL, required for activation of factor XIII, was inhibited with hirudin, 2 units/mL. A portion of the labeled protein, 62 μg/mL, was digested with trypsin, 5 μg/mL, for 6 min at room temperature. Lane a, intact protein, reduced; lane b, digested protein, reduced; lane c, digested protein, unreduced. An arrow denotes the top of the separating gel. The dark lines near the bottom of the figure in lanes b and c represent radioactive protein with the front of the gel.

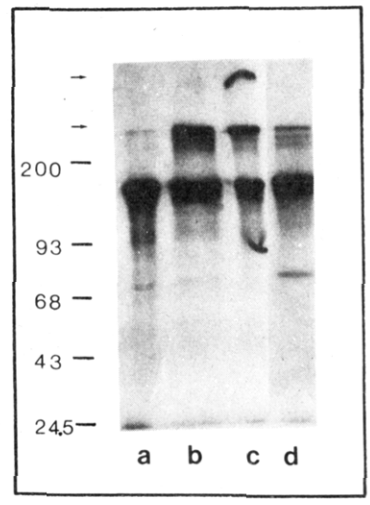

FIGURE 2: Ligation of TSP. Autoradiography of electrophoretograms of TSP ligated in the presence and absence of fibrin. TSP, 50 μg/mL, and ^{125}I-TSP, 1 μg/mL, were incubated with factor XIIIa, 50 μg/mL, thrombin, 0.05 units/mL, and calcium chloride, 2 mM, in the presence and absence of fibrinogen, 0.27 mg/mL, for 24 h. Lane a, TSP; lane b, TSP ligated in the absence of fibrin; lane c, extracted, washed, and solubilized clot; lane d, supernatant from the clot. Arrows denote the top of the stacking gel and the top of the separating gel.

RESULTS

Labeling of TSP with [^{14}C]Putrescine. To determine if TSP contains factor XIIIa reactive glutaminyl residues, TSP was incubated with [^{14}C]putrescine and calcium ion for 24 h in the presence and absence of factor XIIIa. Unincorporated putrescine was removed by gel filtration on Bio-Gel P-300, and fractions were analyzed for radioactivity and protein concentration. In three experiments, TSP incorporated 0.5–2.1 mol of putrescine/mol of TSP (M_r 450 000) in the presence of factor XIIIa, whereas <0.1–0.4 mol of putrescine/mol of TSP was bound in the absence of factor XIIIa. In order to show that incorporation of [^{14}C]putrescine in the presence of factor XIIIa was covalent, complexes were analyzed by SDS–PAGE (Figure 1). Coomassie Brilliant Blue staining detected minor bands near and at the top of the separating gel and at 145, 90, and 85 kDa as well as a major band corresponding to the 160-kDa subunit of TSP. Fluorography revealed major bands of radioactivity at the top of the separating gel and coincident with the intact subunit and minor bands corresponding to other bands identified by protein stain. These radioactive bands were absent when TSP incubated with [^{14}C]putrescine in the absence of factor XIIIa was analyzed. Bands at 145, 90, and 85 kDa probably correspond to degradation due to incompletely inhibited thrombin (Lawler et al., 1982). The bands near and at the top of the separating gel probably correspond to large molecular weight complexes of putrescine and TSP. As described below, TSP cross-links to itself. In addition, putrescine is a difunctional primary amine and could ligate two TSP molecules, as has been shown for amidinated casein, fibronectin, and fibrin (Schrode & Folk, 1978; Mosher et al., 1979). A qualitative comparison of the intensities (as detected by protein stain and fluorography) of the bands at the top of the separating gel and the position of the TSP subunit suggests that the large complexes are enriched in putrescine. Because of the multiple possibilities leading to the ligated radioactive material, the number of reactive glutaminyl residues in TSP could not be determined with any confidence. Due to limitations of quantities and concentrations of TSP, incorporation of other ^{14}C-labeled primary amines, including methylamine and glycine ethyl ester, proved experimentally difficult and did not provide us with additional information concerning the number of reactive glutaminyl residues in TSP.

[^{14}C]Putrescine-labeled TSP was digested with trypsin to identify domains of TSP containing reactive glutaminyl residues (Figure 1). In agreement with previous findings (Lawler & Slayter, 1981), protein staining of tryptic digests analyzed by SDS–PAGE identified fragments at 90 and 30 kDa and at the front. Without reduction, the 90-kDa subunits migrated as a disulfide-bonded complex. Fluorography indicated that [^{14}C]putrescine was incorporated into the disulfide-bonded tryptic core of 90-kDa subunits but not the 30-kDa fragment. Substantial label was also present at the front, indicating that one or more small fragments with reactive glutaminyl residues were removed during digestion. (Dark bands near the bottom of lanes b and c of Figure 1 indicate radioactive protein moving at the front.) Similar results were obtained in other trypsin digestion experiments and in experiments in which [^{14}C]putrescine-labeled TSP was digested with thrombin. In thrombin digests, a labeled disulfide-bonded core of subunits of approximately 120–130 kDa was found in autoradiographs after SDS–PAGE, whereas the 30-kDa fragment contained no label (results not shown). These results indicate that each of the three apparently equivalent polypeptide chains of TSP contains at least two reactive glutaminyl residues. Relative intensities of labeling indicate that most of the reactive residues in the 160-kDa subunit are removed as small fragments by the proteolytic digestion. The 270-kDa trypsin-resistant core that is responsible for interaction with polymerizing fibrin (Bale et al., 1985), however, also contains reactive glutaminyl residues.

TSP–TSP Ligation. We previously reported that TSP became ligated in fibrin clots in the presence of factor XIIIa whereas TSP in the clot supernatant remained largely unligated (Bale et al., 1985). In other experiments, however, when we incubated TSP alone with factor XIIIa, TSP–TSP ligation was noted. Figure 2 shows autoradiographic patterns after SDS–PAGE of reduced samples of starting (unligated) ^{125}I-TSP, ^{125}I-TSP incubated by itself with factor XIIIa, and the extracted fibrin network and supernatant of a clot formed in the presence of ^{125}I-TSP and factor XIIIa. Low thrombin

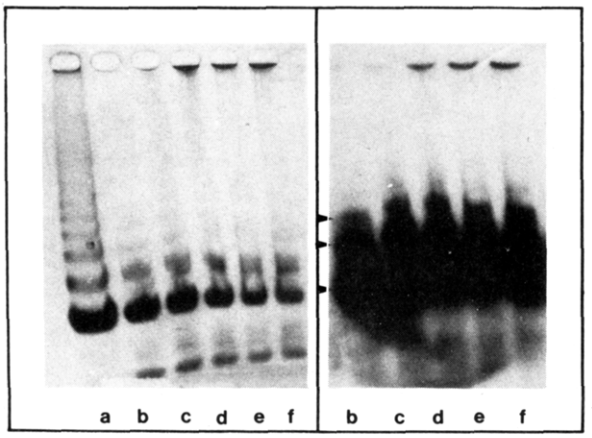

FIGURE 3: Ligation of TSP during fibrin polymerization. Agarose gel electrophoretic analysis of ligation of ^{125}I-TSP during fibrin polymerization prior to the gel point: protein staining (left panel) and autoradiography (right panel). Fibrinogen, 0.27 mg/mL, was clotted with thrombin, 0.008 units/mL, in the presence of factor XIIIa, 30 μg/mL, TSP, 30 μg/mL, ^{125}I-TSP, 2 μg/mL, and calcium chloride 1 mM. The clotting time (t_c) was 5.5 min. Aliquots were quenched at t/t_c = (b) 0.18, (c) 0.36, (d) 0.73, (e) 0.91, and (f) 1.0. Lane a shows fibrin oligomer standards (see Materials and Methods). The autoradiogram was overexposed to demonstrate all ligated ^{125}I-TSP. Arrows denote radioactive bands estimated to have molecular sizes of (from bottom to top) 460, 1150, and 1570 kDa when compared to the fibrin oligomer standards. Electrophoresis was in SDS on unreduced samples.

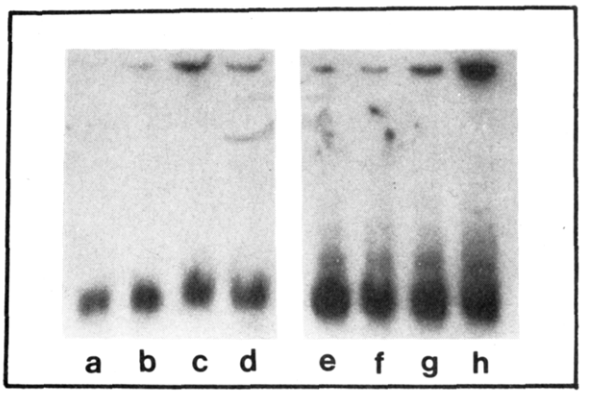

FIGURE 4: Ligation of TSP to fibrin oligomers. Agarose gel electrophoretic analysis of ligation of ^{125}I-TSP in mixtures in which thrombin was inhibited. Fibrinogen, 0.27 mg/mL, was clotted with thrombin, 0.01 units/mL, in the presence of factor XIIIa, 30 μg/mL, TSP, 50 μg/mL, ^{125}I-TSP, 2 μg/mL, and calcium chloride, 3 mM. The t_c was 9.5 min. At 4 (left panel) and 8 min (right panel), hirudin was added at 2 units/mL to inhibit the thrombin. Subsequent ligation of TSP was monitored by electrophoresis of the mixtures at 0.5 (lanes a and e), 1 (lanes b and f), 2 (lanes c and g), and 24 (lanes d and h) h. Electrophoresis was in SDS on unreduced samples.

concentrations and room temperature were used to minimize TSP digestion by thrombin. In the absence of fibrin, 28.5% of TSP was ligated into large covalent complexes. These results indicate that in addition to containing reactive glutaminyl residues as described above, TSP can contribute lysyl residues to the covalent bond catalyzed by factor XIIIa and hence be covalently polymerized in solution. In comparison, 43.5% of TSP incorporated into clots was ligated, whereas only 9.6% of TSP in the clot supernatant was ligated. Increased ligation of TSP in the presence of fibrin compared with TSP in solution indicates that TSP is either ligated to fibrin or is bound to fibrin in such a manner as to favor TSP–TSP ligation. Decreased ligation of TSP in supernatants of clots compared with solutions of TSP alone could be due to lower concentrations of TSP or factor XIIIa in solution due to partitioning of the two proteins in the clot (Bale et al., 1985; Folk & Chung, 1975; Greenberg & Shuman, 1982). It could also be due to the preference of factor XIIIa to act on fibrin as a substrate.

TSP Ligation during Fibrin Polymerization. We used agarose gel electrophoresis to gain insight into TSP ligation during fibrin polymerization. Figure 3 shows the results of agarose gel electrophoresis and autoradiography performed on unreduced samples of a polymerizing mixture of fibrinogen, ^{125}I-TSP, factor XIII, and thrombin at times prior to gelation. Protein staining shows that covalently linked oligomers of a variety of sizes are present, corresponding to protofibrils with ligated γ chains (Nelb et al., 1980). Species too large to penetrate the gel also are present, corresponding to laterally aggregated protofibrils covalently bonded by both γ and α chains. Since the quantity of labeled TSP present in the reaction mixture was small, protein staining only visualized fibrin. The corresponding autoradiograph was highly overexposed to detect all cross-linked species containing ^{125}I-TSP. In comparison to fibrin oligomers of various sizes, the bands containing labeled TSP had sizes of approximately 460, 1150, and 1570 kDa. Although these sizes are comparable with monomeric TSP and one and two TSP molecules bonded to fibrin dimers, it is not possible to rule out the possibility that TSP was cross-linked to itself due to limitations of size estimations. As the larger fibrin oligomers accumulated as assessed by protein staining material that did not penetrate the gel, TSP was found with this material in increasing amounts. Despite the overexposure of the autoradiogram, intermediate-sized TSP oligomers were not seen. Thus, the results suggest that covalent ligation of TSP occurs preferentially to the larger fibrin intermediates.

A second kind of experiment to show that TSP could be ligated to fibrin was carried out as follows. Thrombin was inhibited at stages when soluble fibrin oligomers were present but gelation had not occurred. It has been shown previously (Bale et al., 1982) that, under conditions of the experiment, ligation of fibrin is closely coupled to fibrin polymerization and that polymerization of the fibrinogen–fibrin mixture does not occur once thrombin is inhibited. Ligation of TSP, however, could continue. Figure 4 shows autoradiographs of agarose gel electrophoresis of samples in which thrombin was inhibited at 0.42 (left) or 0.84 (right) the clotting time. Over the subsequent 24 h, labeled TSP was progressively ligated. More ligation of ^{125}I-TSP took place in the sample that had the larger fibrin oligomers. These results provide further evidence that TSP can be covalently ligated to fibrin intermediates.

TSP Ligation with Amidinated Fibrin. To test if TSP could contribute both lysyl and glutaminyl residues to cross-links formed with fibrin, normal or amidinated fibrinogen was clotted in the presence of ^{125}I-TSP with or without factor XIII. The results of gel electrophoresis and autoradiography are shown in Figure 5. TSP was incorporated into clots of amidinated fibrin to the same extent as into clots of unmodified fibrin in the presence or absence of factor XIII (39–43 μg/mL). Amidinated fibrinogen clotted in the presence of factor XIIIa remained unligated, indicating that lysyl residues involved in formation of ligated γ-chain dimers and α-chain polymers had been blocked. The amount of TSP ligated in clots of underivatized and amidinated fibrin was the same (20 μg/mL). However, the size of the complexes into which TSP was ligated was smaller in the modified fibrin. The fact that TSP was found in larger complexes when ligation of fibrin was possible indicates that TSP is ligated to fibrin. The ligation of TSP observed in amidinated fibrin could be among fibrin-

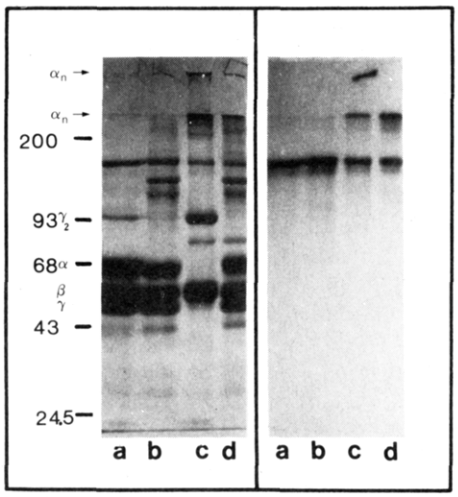

FIGURE 5: Ligation of TSP to amidinated fibrin. SDS–PAGE analysis of TSP incorporation and ligation into clots made from control and amidinated fibrinogen: protein staining (left panel) and autoradiography (right panel). Normal or amidinated fibrinogen, 0.27 mg/mL, was clotted with thrombin, 0.05 units/mL, in the presence of TSP, 50 μg/mL, ^{125}I-TSP, 0.7 μg/mL, calcium chloride, 3 mM, and factor XIII, 0 or 26 μg/mL. After 24 h, the clots were extracted, washed, and solubilized. Lane a, normal fibrin, XIII absent; lane b, amidinated fibrin, XIII absent; lane c, normal fibrin, XIII present; lane d, amidinated fibrin, XIII present. Bands corresponding to the various chains of fibrinogen and fibrin are noted. The arrows denote the tops of the stacking gel and the separating gel.

bound TSP molecules. However, inasmuch as the majority of ligated TSP could not enter the separating gel and the number of TSP molecules in the network was small compared with the number of fibrinogen molecules, it seems unlikely that a sufficient number of TSP molecules could be cross-linked into such high molecular weight complexes. Thus, it seems likely that TSP, in large part, was ligated to several α chains of amidinated fibrin, and to α-chain oligomers of unmodified fibrin.

Effect of Proteolytic Digestion of TSP on Ligation to Fibrin. Samples of ^{125}I-TSP digested to various degrees with thrombin in the presence of calcium ion were analyzed for their ability to become incorporated into and ligated to fibrin. Thrombin was chosen as the proteolytic agent to minimize the extent of proteolysis. In agreement with previous reports (Lawler & Slayter, 1981; Lawler et al., 1982, 1985), thrombin degraded TSP to disulfide-bonded fragments containing 140-kDa subunits and subsequently 120-kDa subunits. Samples of the digest were rapidly frozen at various time points, stored at −70 °C, and thawed immediately before addition to a solution of fibrinogen, factor XIII, and calcium ion. Sufficient thrombin was present in the thawed digestion mixture to induce clotting of the fibrinogen in about 1 min but not to further degrade ^{125}I-TSP. After 24 h, the clots were extracted, washed, and analyzed for total radioactivity and for distribution of radioactivity in various bands after SDS–PAGE. The extent of incorporation of degraded TSP was not diminished compared to intact TSP, but the extent of ligation was significantly reduced, as shown in Figure 6. Reduction in ligation correlated with loss of the 160-kDa band of intact TSP. Although the amount of intact TSP decreased to 5% of control, however, the extent of ligation decreased only to 25% of control. The fact that loss of reactive glutaminyl residue(s) in the small fragment(s) (see Figure 1) was paralleled by decreased ligation suggests that these residues contribute substantially to the ligation reaction with fibrin. The residual ligation may be mediated by the reactive glutaminyl residues in the thrombin resistant core.

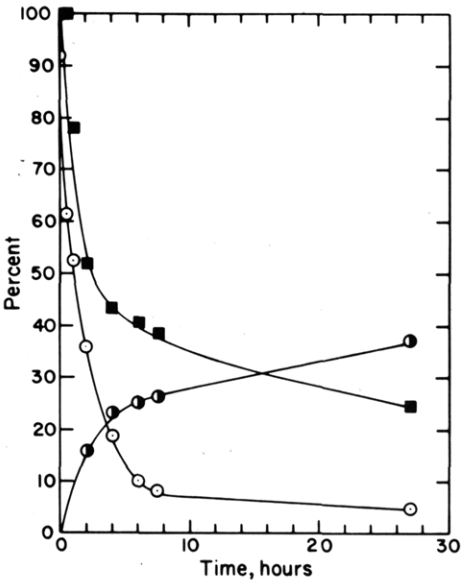

FIGURE 6: Digestion of TSP and effect on ligation. Quantification of the extents of TSP fragmentation in thrombin digests and ligation of these fragments. The digestion was done in the presence of 0.56 mM calcium chloride. At various times, the digest was analyzed by SDS–PAGE, and radioactivity in the 160-kDa subunit of intact TSP (open circles) and the 120-kDa fragment (half-filled circles) was determined as percentages of control. Duplicate samples were frozen and later added to a solution of fibrinogen, 0.27 mg/mL, containing factor XIII, 30 μg/mL. Final concentrations of proteins from the digests in these solutions were TSP, 50 μg/mL, ^{125}I-TSP, 0.6 μg/mL, and thrombin, 0.5 units/mL. After 24 h at room temperature, clots were extracted, washed, and analyzed. Squares denote the percentage of digested TSP ligated when compared to ligation of undigested TSP.

Discussion

The findings that concentrations of factor XIIIa in the range of 30–100 μg/mL mediate the covalent insertion of [^{14}C]putrescine into TSP and the ligation of TSP to itself in solution indicate that TSP is a physiological substrate for this enzyme. TSP in solution apparently forms aggregates such that lysyl residues are placed next to reactive glutaminyl residues in a way that allows formation of ϵ-(γ-glutamyl)lysine bonds. The lysyl residues of TSP also allow ligation of TSP to amidinated fibrin. In contrast, fibronectin and α-2-plasmin inhibitor contribute only glutaminyl residues to cross-links with fibrin (Mosher & Johnson, 1983a; Sakata & Aoki, 1980). Fibronectin in the extracellular matrix or in aggregates formed by disulfide rearrangement can be ligated to itself by factor XIIIa, but native fibronectin in solution, unlike native TSP in solution, is not cross-linked (Mosher, 1975; Keski-Oja et al., 1976; Mosher & Johnson, 1983b).

Because TSP contains factor XIIIa reactive glutaminyl residues and is composed of three identical subunits, intramolecular ligation is a possibility. The results suggest, however, that intermolecular ligation predominates. If intramolecular ligation is an important reaction, dimers and trimers of the 160-kDa subunit should have been present as major ligation products. Such was not the case. Further, in the presence of fibrin, subunits of TSP were ligated into complexes too large to penetrate the stacking gel when fibrin α-chain ligation occurred, and TSP molecules were ligated into complexes too large to penetrate 2% agarose gels. These two observations argue for intermolecular ligation of TSP to α-chain polymers of fibrin. The conclusion that TSP cross-links to the α chain is consistent with the finding by affinity chromatography that fibrinogen interacts with TSP through the carboxyl-terminal portion of the α chain (Tuszynski et al., 1985).

Potential ligation partners for TSP apparently occur more

frequently when fibrin oligomers are assembled into fiber bundles, because TSP was ligated preferentially to the larger complexes of soluble fibrin (Figures 3 and 4). α-Chain ligation also occurs preferentially in the larger complexes. Incomplete ligation of TSP in the clot may be due to competition between α-chain–α-chain ligation and TSP–α-chain ligation. When fibrin–fibrin interactions were limited by addition of a thrombin inhibitor prior to complete activation, TSP ligation increased.

The results indicate that each subunit of TSP has several factor XIIIa reactive glutaminyl residues located in different domains. The tryptic resistant core, which is responsible for the noncovalent incorporation of TSP into polymerizing fibrin (Bale et al., 1985), contained at least one factor XIIIa reactive glutaminyl residue that participated in fibrin–TSP ligation. At least one factor XIIIa reactive glutaminyl residue was contained in fragments removed during proteolytic digestion. The 30-kDa heparin binding domain did not contain a factor XIIIa reactive glutaminyl residue. However, the 30-kDa fragment could participate in ligation reactions by contributing reactive lysine residues. The residues predominantly involved in TSP ligation to fibrin were removed during thrombin or trypsin proteolysis.

Although ligation of TSP is not required for its incorporation into the fibrin clot (Bale et al., 1985), the action of factor XIIIa on TSP may be important in blood coagulation and associated processes. There are several lines of evidence pointing to formation of fibrinogen–TSP complexes on the surface of activated platelets (Jaffe et al., 1982; Leung, 1984). Cross-linking by factor XIIIa, which is present as a zymogen in plasma and platelet cytoplasm, could serve to stabilize this complex. Factor XIIIa may also cross-link fibrin to TSP bound on the platelet surface (Phillips et al., 1980). It is interesting to note that factor XIII is critical to isometric contraction of platelet–fibrin clots (Cohen et al., 1982). This effect could be by stabilization of platelet–TSP–fibrin interactions, although it may also be due to the increased modulus of ligated fibrin as opposed to unligated fibrin (Gerth et al., 1974). Factor XIIIa mediated cross-linking may also be important in the modulation of interactions of TSP with proteins other than fibrin, such as fibronectin, plasminogen, and histidine-rich glycoprotein. The presence of several reactive glutaminyl residues in TSP, the trimeric structure of TSP, and the propensity of TSP to interact with other macromolecules would allow ligation of several different molecules by one TSP molecule and thus the covalent stabilization of multiprotein complexes. As suggested by Lahav, Silverstein, and co-workers (Lahav et al., 1982, 1984; Silverstein et al., 1984, 1985), such complexes may occur on the platelet surface and in the extracellular matrix. Finally, factor XIIIa could ligate TSP to platelet membrane proteins, including glycoproteins IIb and IIIa, which have been identified as substrates of platelet factor XIIIa (Cohen et al., 1981).

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Phosphorylation–Dephosphorylation of Pyruvate Dehydrogenase from Bakers' Yeast†

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ABSTRACT: The pyruvate dehydrogenase complex was purified to homogeneity from bakers' yeast (*Saccharomyces cerevisiae*). No pyruvate dehydrogenase kinase activity was detected at any stage of the purification. However, the purified pyruvate dehydrogenase complex was phosphorylated and inactivated with purified pyruvate dehydrogenase kinase from bovine kidney. The protein-bound radioactivity was localized in the pyruvate dehydrogenase α subunit. The phosphorylated, inactive pyruvate dehydrogenase complex was dephosphorylated and reactivated with purified pyruvate dehydrogenase phosphatase from bovine heart. Tryptic digestion of the ^{32}P-labeled complex yielded a single phosphopeptide, which was purified to homogeneity. The sequence of the phosphopeptide was established to be Tyr-Gly-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Gly-Thr-Thr-Tyr-Arg. This sequence is very similar to the sequence of a tryptic phosphotetradecapeptide derived from the α subunit of bovine kidney and heart pyruvate dehydrogenase: Tyr-His-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Gly-Val-Ser-Tyr-Arg.

Activity of pyruvate dehydrogenase complexes from eukaryotic sources, including mammalian, avian, and plant tissues, and *Neurospora crassa* is regulated by a phosphorylation–dephosphorylation cycle [for reviews, see Reed (1974), Denton et al. (1975), and Wieland (1983)]. Phosphorylation by pyruvate dehydrogenase kinase inactivates the complex, and dephosphorylation by pyruvate dehydrogenase phosphatase reactivates the complex. The phosphorylation sites in the mammalian pyruvate dehydrogenase complex are located on three serine residues in the α subunit of the pyruvate dehydrogenase component of the complex (Yeaman et al., 1978; Sugden et al., 1979; Mullinax et al., 1985).

Attempts thus far to demonstrate pyruvate dehydrogenase kinase activity in yeast have been unsuccessful (Kresze & Ronft, 1981a). In this paper, we present evidence that highly purified pyruvate dehydrogenase complex from bakers' yeast (*Saccharomyces cerevisiae*) undergoes phosphorylation and concomitant inactivation and dephosphorylation and concom-

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