The free thiols of platelet thrombospondin (TSP) were derivatized with labeled N-ethylmaleimide (NEM) or iodoacetamide (IAM). When Ca\(^{2+}\) was chelated with EDTA, 2.9 mol of NEM or 2.6 mol of IAM reacted/mol of native TSP. No additional thiols were found after denaturation with urea. Since TSP has three apparently identical polypeptide chains, this suggests one free thiol/polypeptide chain. Ca\(^{2+}\) protected all of the thiols from reaction with IAM. In Ca\(^{2+}\) about half the thiols reacted normally with NEM and the others were unreactive, indicating that the thiols of TSP are not identical. The number of reactive thiols as a function of [Ca\(^{2+}\)] revealed a sigmoidal curve with a transition midpoint of 207 \(\mu\)M. The ability of analogs of NEM to compete for derivatization of the thiols with labeled NEM was greater with larger, more hydrophobic agents. Gel electrophoretic separation of labeled TSP that had been partially digested with thrombin and trypsin indicated that some of the label was in the C-terminal tryptic fragment but that most was in the adjacent trypsin-sensitive region. After cyanogen bromide cleavage of the labeled and reduced protein, four labeled fractions were obtained from a gel filtration column. With subsequent combinations of tryptic digestion and reversed-phase high performance liquid chromatography, labeled peptides were purified from these four fractions, and the amino acid sequences were determined. Twelve labeled cysteines were identified, each with a specific radioactivity less than that of the thiol labeling reagent, indicating that only a fraction of that cysteine in a population of TSP molecules was a free thiol at the time of derivatization. While 2 labeled cysteines are in the non-repeating C-terminal portion of the molecule, the other 10 labeled cysteines are in the adjacent trypsin-sensitive type 3 repeats proposed (Lawler, J., and Hynes, R. O. (1986) J. Cell. Biol. 103, 1635–1648) as the calcium-binding region (6); these repeats are approximately at the predicted junction of the thin connecting strand and the C-terminal globular region.

TSP is secreted from \(\alpha\)-granules by activated platelets (7), and it is released into the medium by various cultured cells, including endothelial cells (8, 9), fibroblasts (10), aortic smooth muscle cells (11), monocytes and macrophages (12), and neuroglial cells (13). TSP released from cultured cells is incorporated into their extracellular matrices (14). TSP exhibits binding affinities for a variety of molecules, including heparin (14), fibrinogen (15, 16), fibronectin (16, 17), and collagen (17–19). TSP has been implicated in several cellular activities as cell attachment and spreading (20–22), cell growth and tissue repair (13, 23–25), platelet aggregation (26–28), and tumor metastasis (29). Thus, TSP appears to be an adhesive macromolecule that is involved in cell-cell and cell-matrix interactions.

Most of these interactions are presumed to be non-covalent, but the demonstrations that TSP forms disulfide-linked multimers (30, 31) and disulfide-linked complexes with thrombin (32) suggest the possibility that intermolecular thiol-disulfide exchange between TSP and other proteins with which it interacts may be a general mechanism. Since a thiolate anion is essential for thiol-disulfide exchange, we have focused our attention on the free thiols of TSP. A thiol was first reported by Danishefsky et al. (32), who estimated that 2.6 mol of NEM reacted per mol of denatured TSP. The thiols were predicted to be Ca\(^{2+}\)-sensitive because formation of disulfide-linked complexes with other proteins and with activated thiol-Sepharose was inhibited by Ca\(^{2+}\) (31, 32), and because a spin label on thiols of TSP had low mobility in a medium

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Thrombospondin (TSP)\(^{1}\) is a large (\(M_t = 420,000\)) glycoprotein composed of three apparently identical, disulfide-linked polypeptide chains (for a review, see Refs. 1 and 2). Each chain has a globular domain at the C-terminal end connected through a long thin strand to a smaller globular region at the N-terminal end. The three chains are disulfide-linked near the N-terminal globular region. The conformation of TSP is sensitive to Ca\(^{2+}\) (3–5). TSP in the presence of Ca\(^{2+}\) is more compact (3, 4) and is less susceptible to proteolysis (3) than is TSP in the absence of Ca\(^{2+}\). On addition of EDTA to a TSP solution containing Ca\(^{2+}\), there is an increase in the length of the connecting strand in TSP and a concomitant decrease in the size of the C-terminal globular region (4). Thus, it appears that the C-terminal half of the molecule is sensitive to Ca\(^{2+}\). An amino acid sequence deduced from cDNA revealed eight repeat sequences that were proposed to be the calcium-binding region (6); these repeats are approximately at the predicted junction of the thin connecting strand and the C-terminal globular region.

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* The abbreviations used are: TSP, thrombospondin; IAM, iodoacetamide; NEM, N-maleimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate.
Thiols of Thrombospondin

**Table I**

Quantification of free thiols in TSP

TSP was reacted with 50 μM [3H]NEM or [14C]IAM at 25 °C for 45 min under nitrogen gas as described under "Materials and Method." Two methods were used. In one, TSP was isolated by heparin affinity chromatography, reacted with the reagents, and then analyzed by SDS-PAGE. In the other, the reaction occurred in the supernatant solution, and labeled TSP was resolved by SDS-PAGE. The ratios of derivative/TSP were calculated as described. The data for these two methods were apparently identical, so they were pooled. The values are ± standard deviation with the number of experiments in parentheses. Similar ratios were calculated for experiments with concentrations of reagents 40–500% of the concentrations shown here (data not shown).

| Conditions | Thiol reagent/TSP (mol/mol) |
|------------|----------------------------|
| NEM        | IAM                        |
| 6 mM EDTA  | 2.9 ± 0.4 (5)*             |
| 6 mM EDTA + 8 M urea | 3.1 ± 0.3 (6)                |
| 6 mM EDTA + 8 M urea + DTNB<sup>+</sup> | <0.1 (3)                     |
| 2 mM Ca<sup>2+</sup> | 1.4 ± 0.4 (6)                   |

*These values were calculated from amino acid analysis of the purified protein. The others were determined by SDS-PAGE analysis before purification as described under "Materials and Methods."

2 The protein was incubated with 100 μM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for 60 min prior to addition of labeled NEM or IAM.

The time courses for derivatization and the extent of derivatization with thiol reagents is shown in Fig. 1, and the time courses of derivatization are in Fig. 2. Consider reactions with NEM first. 2.9 mol of NEM reacted with 1 mol of native TSP in EDTA (Table I). Addition of 8 M urea caused the reaction to be faster (Fig. 2) but to the same extent (Table I), demonstrating the absence of completely buried thiols. Preincubation with 5,5'-dithiobis(2-nitrobenzoic acid), which is absolutely specific for free thiols (34), completely blocked derivatization with NEM in urea, indicating that the reaction of NEM was completely specific for thiols in TSP. These data suggest one thiol/polyepitope chain for the three-chain protein. In 2 mM Ca<sup>2+</sup> the rate of reaction (Fig. 2) was similar, but there was only about half as much NEM reacted with TSP (Table I). This demonstrates that the thiols were not identical; some reacted quickly and the others were unreactive.

**Materials and Methods**

RESULTS

Free thiols of secreted platelet proteins were derivatized by addition of EDTA followed by either [3H]NEM or [14C]IAM to the supernatant solution of A23187-activated platelets within 30 min of secretion. Derivatized TSP was purified by heparin-agarose chromatography as described under "Materials and Method." TSP was the major protein in the supernatant solution to react with either of the labeled thiol reagents (Fig. 1).

**Quantification of Thiols**—The extent of derivatization with thiol reagents is shown in Table I, and the time courses of derivatization are in Fig. 2. Consider reactions with NEM first. 2.9 mol of NEM reacted with 1 mol of native TSP in EDTA (Table I). Addition of 8 M urea caused the reaction to be faster (Fig. 2) but to the same extent (Table I), demonstrating the absence of completely buried thiols. Preincubation with 5,5'-dithiobis(2-nitrobenzoic acid), which is absolutely specific for free thiols (34), completely blocked derivatization with NEM in urea, indicating that the reaction of NEM was completely specific for thiols in TSP. These data suggest one thiol/polyepitope chain for the three-chain protein. In 2 mM Ca<sup>2+</sup> the rate of reaction (Fig. 2) was similar, but there was only about half as much NEM reacted with TSP (Table I). This demonstrates that the thiols were not identical; some reacted quickly and the others were unreactive.

Derivatization with IAM differed slightly. While the number of reactive groups in EDTA (2.6 mol of IAM reacted/mol of TSP) was similar, inhibition by Ca<sup>2+</sup> was nearly complete (Table I, Fig. 2). Urea caused an increase in the extent of derivatization (Table I), but this increase was not blocked by 5,5'-dithiobis(2-nitrobenzoic acid), indicating that when the protein is denatured, IAM reacts with groups other than thiols. The time courses (Fig. 2) are consistent with this conclusion; in urea the initial rate was that expected for reaction of IAM with thiols, but it was followed by a slower, nearly linear increase in reaction to a level well above 3 mol IAM/mol TSP.

The time courses for derivatization and the extent of derivatization were similar whether TSP was derivatized in the supernatant solution or after heparin purification (data not shown), suggesting that no other secreted component significantly modified the derivatization of TSP. We conclude from the data of Table I and Fig. 2 for the two reagents that (i) there are 3 equivalents of thiol/mol of TSP, presumably 1/polyepitope chain, (ii) the thiols are protected by Ca<sup>2+</sup>, (iii) partial reactivity with NEM in Ca<sup>2+</sup> indicates that the thiols are not all identical, and (iv) when the protein is denatured, IAM reacts slowly with non-thiol groups.

**Calcium Dependence of Thiol Derivatization**—The number of reactive thiols varied with [Ca<sup>2+</sup>] sigmoidally (Fig. 3), consistent with a requirement for multiple bound ligands to induce the change in conformation (29). The transition midpoint was 207 ± 21; n = 5 μM Ca<sup>2+</sup>. The shape and the midpoint are similar to those for the change in circular dichroism (midpoint = 107 μM, Ref. 5), suggesting that a similar conformation change was the basis for both. To rule
out indirect complications due to possible calcium-dependent TSP-TSP interactions, the experiment was repeated with the same TSP preparation diluted 6-fold; the results were identical.

Maleimide Derivatives and Thiol Derivatization—To evaluate steric acid hydrophobic factors in thiol reactivity, several maleimide derivatives were added simultaneously with an equal concentration of [\(^{3}H\)]NEM and allowed to compete for the thiols in TSP (Fig. 4). As a negative control, [\(^{3}H\)]NEM was added with solvent alone, and as a positive control, nonradioactive NEM was allowed to compete with labeled NEM. Inhibition increased with size and hydrophobicity until \(N\)-pyrenylmaleimide (\(N\)-pyr) was the only remaining competitor.

Localization of Thiols—Limited thrombin- and trypsin-catalyzed digestions of IAM-labeled TSP are analyzed in Fig. 5. The mass of each peptide was calculated from the mobility of the molecular weight standards as indicated. B, the 12 h sample in which 1.5 \(\mu M\) of a maleimide derivative would have bound at most 3 \(\mu M\) \(Ca^{2+}\), introducing less than 2% error in the transition midpoints observed.

Calcium Dependence of Thiol Derivatization. Aliquots of heparin-purified TSP, either 100 \(\mu g/ml\) (solid bar) or 20 \(\mu g/ml\) (open bar) in 185 mM NaCl, 13 mM bis-Tris-propane, pH 7.0, and 1 mM CaCl\(_2\), were mixed with varying amounts of EDTA. From the apparent association constant for EDTA and \(Ca^{2+}\) (1.9 \(\times\) \(10^{5}\) M\(^{-1}\) at pH 7.0; Ref. 49), we calculated the concentration of free \(Ca^{2+}\) in each aliquot. The samples were derivatized with labeled NEM and analyzed by SDS-PAGE for calculation of the ratio NEM/TSP. Assuming 12 \(\alpha^{+}\)-binding sites in TSP (5), the TSP concentration used throughout these experiments (\(\sim\)0.25 mM) would have bound at most 3 \(\mu M\) \(Ca^{2+}\), introducing less than 2% error in the transition midpoints observed.

Distribution of label among peptide fragments from partially digested labeled TSP. Proteins in the supernatant solution from activated platelets were derivatized with \([^{3}H]IAM\) in EDTA, and TSP was purified on a heparin-agarose column. TSP (400-450 \(\mu g/ml\)) was diluted by a factor of 2.5 with 6 mM EDTA, 100 mM Tris-HCl, pH 8.1, and incubated with 100 \(\mu M\) thrombin at 37°C. A, the digestion proceeded for 0 h (lane 1), 6 h (lane 2), or 12 h (lane 3) before addition of the samples to reducing SDS-PAGE sample buffer. The samples were resolved on duplicate 8–16% gels, which included a thrombin control (no TSP). One gel was stained with Coomassie Blue, and the other was prepared for fluorography. The apparent mass of each peptide was calculated from the mobility of the molecular weight standards as indicated. B, the 12 h sample in A was divided into 3 aliquots and chilled to 0°C. Trypsin (in 0.1 mM HCl) was added at a trypsin/TSP ratio (w/w) of 0 (lane 1), 1:10,000 (lane 2), and 1:1,000 (lane 3). The digestion proceeded at 0°C for 40 h, at which time 1.5 mM tosyl-lysyl chloromethyl ketone, 1 mM phenylmethylsulfonyl fluoride, and 20 mM HCl were added. SDS-PAGE analysis of the digested samples was similar to that in A. Similar results were obtained with [\(^{3}H\)]NEM-TSP.
was in the C-terminal end of the intact molecule (the 20-kDa thrombin peptide).

For a more precise location of the thiols, derivatized TSP was cleaved for isolation of labeled peptides in order to determine their sequence for location in the published sequence of TSP (6). We were unsuccessful in our attempts to purify NEM-labeled peptides, a problem also reported by others (35–37). We therefore used IAM-labeled TSP, resulting in more easily purified peptides. While this caused some concern about nonspecific labeling of the protein, radioactivity was recovered only in a cycle of the sequenator where a cysteine residue was identified (see Table II). [3H]IAM-TSP was heparin-purified, reduced, alkylated, and cleaved with cyanogen bromide. The cyanogen bromide peptides were separated into four labeled fractions on a gel filtration column. These fractions were further resolved on a reversed-phase (C8) HPLC column either before or after further digestion with trypsin. Additional Cs and Cl8 chromatographic separations were used to further purify some peptides. Fig. 6 is a flow diagram that shows the sequence of the digests and separations as well as our nomenclature of the peptides, and Figs. 7–16 show the separation on each column. The amino acid sequences of the labeled peptides were determined on a gas-phase sequenator; data are shown in Table II. It was apparent that there were multiple labeled peptides; they are summarized in Table III and indicated in the partial sequence of TSP in Fig. 17.

Of the total 69 cysteines in TSP, 12 were labeled. All of the labeled cysteines are in the C-terminal part of TSP at positions 687, 695, 700, 720, 838, 856, 876, 892, 912, 928, 974, and 1149. All but the final two are in the proposed calcium-binding region (type 3 repeats) identified by Lawler and Hynes (6). The specific radioactivities of the labeled cysteines were estimated (see Miniprint) to be only 3–25% of the specific radioactivity of the IAM used to label them, confirming that only a small fraction of that residue was a free thiol at the time of labeling. That is, TSP existed as a mixture of molecules with the free thiol at different cysteines. Three of the labeled cysteines may be special. Cys1149 and Cys1150 (the last cysteine of the final two type 3 repeats) and Cys974 (the first cysteine after the type 3 repeats) had specific radioactivities four to six times greater than the others. It is clear, however, that there was no single cysteine that was predominately a free thiol when labeled.

**Identification of Labile Disulfide Bonds**—The number of labeled cysteines, 12, is clearly greater than the 3 mol of NEM or IAM incorporated/mol of TSP (Table I). This suggested that, rather than a stable thiol(s) in TSP, there is a mechanism of thiol-disulfide exchange among the various labeled cysteines, most of which are in the putative calcium-binding region of the molecule. Is this because the disulfide bonds in this region of the molecule are less stable than those in the remainder of the molecule? To investigate this possibility, the number of free thiols was measured after incubation of TSP with low concentrations of dithioerythritol (Fig. 18). Disulfide bonds were more readily reduced when TSP was in a solution with EDTA compared with a solution with Ca++. That is, the most labile disulfide bonds were stabilized by Ca++, and therefore, are likely to be in the Ca++-sensitive part of the molecule.

**DISCUSSION**

There are 3 mol of thiol/mol of TSP (Table I), implying one thiol per polypeptide chain. We identified, however, 12 different labeled cysteine residues, indicating that each of the labeled cysteines was a free thiol in only a small fraction of TSP molecules. That is, the position of the thiol must vary through thiol-disulfide exchange. This is consistent with our observations that the thiols are not identical and that the specific radioactivity of the labeled cysteines was much less than the specific radioactivity of the labeling reagent.

Each of the labeled cysteines (i.e. cysteine with a free thiol) of TSP is in the Ca++-sensitive C-terminal part of the molecule, and all but 2 (Cys974 and Cys1149) are in the repeat sequences (Fig. 17) proposed to be the Ca++-binding part of the molecule (6). Of the eight repeat sequences, we failed to detect a labeled cysteine in only three, encompassing residues 741–822. These repeats contain 17 of the total 69 cysteines in TSP, and 10 of the 17 were labeled. The other 2 labeled cysteines are in the adjacent 120-residue C-terminal sequence, with 1 the fourth residue from the C terminus. Location of the thiol(s) in the C-terminal part of the molecule apparently is related to the ability of Ca++ to modify the reactivity of thiols in TSP and to modify the stability of disulfide bonds.

A basic principle of protein chemistry is that disulfide bond pairing is established by the most stable conformation of the

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**Fig. 17.** Amino acid sequence of TSP from residue 656 to the C-terminal end residue 1152 showing labeled cysteine residues. The residue number of the beginning amino acid of each line is on the left. The alignment of the sequence from residues 672 to 682 is from Lawler and Hynes (6), who identified repeat homologies; shown are those they identified as “type 3” homologies. They further designated the type 3 repeats as type 3A or 3B, with type 3/I defined by the sequence (Fig. 17) proposed to be the Ca++-binding part of the molecule. Included are summarized in Table III and indicated in the partial sequence of TSP in Fig. 17.

**Fig. 18.** Measurement of labile disulfide bonds in TSP. The supernatant solution of activated platelets was made 100 mM with bis-Tris-propane, pH 7.0, and 6 mM with EDTA (●) or 2 mM with Ca++ (○). Dithioerythritol was added to the indicated final concentrations, and the samples were incubated for 1 h at 25 °C under N2 gas. The samples were derivatized and analyzed by SDS-PAGE as described under “Materials and Methods,” except that the final concentration of [3H]NEM (the concentration of the stock solution was 50 mM with a specific radioactivity of 12–14 cpm/pmol) was 200 μM greater than twice the concentration of dithioerythritol for that sample.
protein. Why then did we find multiple locations of the thiol and, by inference, multiple disulfide pairings? The likely explanation is that we labeled thiol(s) during a transition from one stable conformation (with stable disulfide bonds and a single thiol) to another stable conformation (with different disulfide bonds and a different thiol). There could, for example, be a change from a conformation that exists when TSP is packed in a platelet α-granule, possibly with little or no calcium, to another conformation after it is secreted into a dilute solution containing Ca"++. Similarly, there could be a change from the Ca"+-bound to the Ca"+-free conformation when EDTA was added prior to labeling. An example is serum albumin; it contains 17 disulfide bonds and one thiol (38), and in a solution of low ionic strength and alkaline pH, there is a disulfide isomerization (39).

A second possibility for multiple disulfide pairing is that TSP exists in multiple, equally stable conformations, each with its own disulfide pairings. Thus, rather than a stable thiol(s) in TSP, there may be an equilibrium thiol-disulfide exchange among various residues labeled, with the traction of a cysteine existing as a free thiol determined by the relative stability of that conformation. Such multiple conformations could help explain the ability of TSP to bind to so many proteins. TSP could alter its disulfide bond pairings to maximize its interactions with other proteins. An example of an equilibrium thiol-disulfide exchange is β-lactoglobulin (40), which exists as an equilibrium of two forms, each with its own disulfide bond pattern and own thiol.

It is clear from the studies described here that TSP has thiol(s) that react with disulfide bonds within the molecule, and it has been reported in other studies that thiol(s) on TSP react with disulfide bonds on other protein molecules (30–32). What is the physiological significance of these reactions? The greatest reactivity with other molecules occurs in a Ca"+-free medium, but after secretion TSP presumably is in a Ca"+-containing medium. It is possible that the Ca"+-sensitive region of the TSP molecule is also sensitive to other environmental factors. Non-covalent interactions with other proteins or adhesion to cell surfaces might induce conformational changes that permit intermolecular thiol-disulfide exchange. For example, Narasimhan et al. (41) reported that one of the two buried thiols of fibronectin became accessible to 5,5'-dithiobis(2-nitrobenzoic acid) when the protein was adsorbed to a solid surface. A similar situation may exist with TSP, and Ca"+ may preserve reactive thiol(s) and inhibit intermolecular thiol-disulfide exchange until a signal is signaled by environmental factors.

Thiol-disulfide exchange between a thiol on TSP and a disulfide bond on another protein, such as extracellular matrix proteins or proteins on cell surfaces, obviously raises many intriguing possibilities. It is especially interesting that one of the labeled cysteines is in an Arg-Gly-Asp-Ala-Cys" sequence; Arg-Gly-Asp has been shown to mediate the attachment of several different adhesive proteins to cells (42), and Lawler et al. (43) demonstrated that it mediates attachment of TSP in a Ca"+-dependent manner to a receptor on a number of different cell lines. The covalent bonding of TSP to cells and to other matrix proteins through thiol-disulfide exchange would open nearly unlimited possibilities for physiologically significant reactions.

Thiol-disulfide exchange, or disulfide isomerization, is normally a very slow reaction unless catalyzed. It is possible that platelets secrete something that can catalyze disulfide isomerization. There are several proteins known to have disulfide isomerase activity. Protein disulfide isomerase is an enzyme of the endoplasmic reticulum that presumably catalyzes isomerization of disulfide bonds as a protein is synthesized (44). Thioredoxin is a protein with multiple functions involving a very reactive pair of cysteines that readily undergo oxidation to a disulfide and reduction to dithiols (45). It can reduce protein disulfide bonds, and it can catalyze disulfide bond isomerization (45). Protein disulfide isomerase and thioredoxin share an active center sequence (Table IV) (46), and it has been reported (47) that the β-chains of lutropin and follitropin contain a similar sequence and exhibit 300 and 60 times more protein disulfide isomerase activity than thioredoxin. The consensus sequence of these four proteins, -Cys-Gly-X-Cys-, is in TSP at position 554–557 (there are four additional -Cys-X-Cys-), but we did not identify it as reactive with labeled IAM. The same sequence is in von Willebrand factor, a protein that also is secreted by activated platelets and that also undergoes thiol-dependent multimerization (48), and TSP and von Willebrand factor share the property of forming multimers optimally at pH 6 (31, 48). Each of the sequences in Table IV also has a positive charge adjacent to a cysteine (it has been proposed that this would tend to stabilize the thiolate anion by forming a base pair, Ref. 45), and other residues may be close in the secondary structure and modify reactivity. It is possible that thiol-disulfide exchange in TSP is catalyzed by TSP itself or by some other factor secreted by activated platelets.

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SUGGESTED MATERIALS:

1. Tissue culture media, sera, antibiotics, etc.
2. Electrophoresis gels and equipment
3. Gel staining reagents
4. SDS-PAGE apparatus
5. Western blot transfer equipment
6. Immunoblotting reagents
7. DNA sequencing equipment
8. Protein purification equipment
9. Mass spectrometry equipment
10. Light microscopy equipment

For large-cell preparations, platelets were isolated from a variety of animal species. Platelets were prepared from blood obtained from normal rats, guinea pigs, rabbits, and dogs. Platelets were isolated from blood samples obtained from the New York Blood Center (New York, NY) within 24-36 hours of collection. Platelet concentrates were obtained from the Princeton New York Blood Center (New York, NY) within 24-36 hours of collection. Platelet concentrates were obtained from the Princeton New York Blood Center (New York, NY) within 24-36 hours of collection. Platelet concentrates were obtained from the Princeton New York Blood Center (New York, NY) within 24-36 hours of collection.
ThioLs of Thrombospondin

Quantification of labeled TDP. Two methods were used for quantification of thioLs in TDP: 1) thioLs were purified from the mixture of unmodified and labeled thioLs by gel electrophoresis, and the radioactivity of the purified thioLs was measured by liquid scintillation counting. The amount of protein was calculated from a standard curve of known protein concentrations and radioactivities. The specific activity of the purified thioLs was expressed as disintegrations per minute per nanogram of protein. 2) thioLs were purified from the mixture of unmodified and labeled thioLs by gel electrophoresis, and the radioactivity of the purified thioLs was measured by liquid scintillation counting. The amount of protein was calculated from a standard curve of known protein concentrations and radioactivities. The specific activity of the purified thioLs was expressed as disintegrations per minute per nanogram of protein.

Reduction and Alkylation. The reduction and alkalization of labeled thioLs were performed as described by Hirs (52) with some modification. Heparin-purified TDP (1.5-3.0 mg) was dialyzed stepwise for 48 h against 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.2% Triton X-100, and 1 mM dithiothreitol. The dialyzed TDP was adjusted to 8.4 with dibutylamine, and 0.4 M EDTA was added to 5.7 h before addition of 0.1% SDS at a final concentration of 0.2-3.0 mg/mL. The reaction was terminated by heating for 10 min at 100°C. The SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (53).

Protein-Peptide Sequencing. The protein-Peptide Sequencing was performed by the Protein Sequencing Center at Health Science Center at Brooklyn. The protein samples were labeled by TNBS (54) and subjected to HPLC on a C18 reverse-phase column. The elution was monitored by absorbance at 214 nm. The peptides were collected and subjected to analysis by mass spectrometry.

RESULTS

Isolation and Sequencing. The isolation and sequencing of peptides from labeled TDP were performed as described by Venkatesan and coworkers (55). The sequences of the labeled peptides are listed in Table 1. The sequences of the labeled peptides are listed in Table 2.

CONCLUSION

Thiolysis of Thrombospondin

Fig. 6. Fragmentation of labeled TDP and purification of the labeled fragments. Bovine platelet proteins were digested with trypsin, and the labeled tryptic cleavage peptides were isolated by heparin-Sepharose chromatography. The labeled peptides were subjected to partial urea-polyacrylamide gel electrophoresis to separate the labeled peptides from the unmodified tryptic cleavage peptides. The labeled peptides were then subjected to HPLC purification as described above.
Thiols of Thrombospondin

Fig. 8. RP-HPLC of CB-1 tryptic peptides. The CB-1 fraction from Fig. 7 was digested with trypsin and resolved at a flow rate of 0.3 ml/min by RP-HPLC on a C8 column as described in MATERIALS AND METHODS with the following gradient: 5% solvent B2 for 10 min; 5-95% for 200 min. The resulting peaks were collected as indicated.

Fig. 9. RP-HPLC of CB-2 tryptic peptides. The CB-2 fraction from Fig. 7 was resolved by RP-HPLC with a C8 column as in Figure 8 with the following gradient: 5-95% solvent B2 for 40 min; 25% for 1 min; 25-40% for 10 min.

Fig. 10. RP-HPLC of CB-3 tryptic peptides. The CB-3 fraction from Fig. 7 was digested with trypsin as described in Figure 8 with the following gradient: 55% solvent B2 for 5 min; 5-45% for 200 min.

Fig. 11. RP-HPLC of CB-4 tryptic peptides. The CB-4 fraction from Fig. 7 was purified by RP-HPLC with a C8 column as in Fig. 8 with the following gradient: 5-35% solvent B2 for 20 min; 15% for 5 min; 15-45% for 270 min.

Fig. 12. RP-HPLC of CB-2A tryptic peptides. The CB-2A fraction from Fig. 9 was digested with trypsin and the mixture was resolved on a C8 column with the following gradient: 5% solvent B2 for 10 min; 5-95% for 200 min.

Fig. 13. RP-HPLC of CB-2B tryptic peptides. The CB-2B fraction from Fig. 9 was digested with trypsin and the mixture was resolved on a C8 column with the following gradient: 5% solvent B2 for 10 min; 5-95% for 200 min.

Fig. 14. RP-HPLC of CB-2F tryptic peptides. The CB-2F fraction from Fig. 8 was purified by RP-HPLC with a C8 column. The flow rate was 0.3 ml/min with the following gradient: 55% solvent B2 for 1 min; 1-95% for 110 min.

Fig. 15. RP-HPLC of CB-2C tryptic peptides. The CB-2C fraction from Fig. 8 was purified by RP-HPLC with a C8 column with the following gradient: 5% solvent B2 for 10 min; 5-95% for 200 min.

Fig. 16. RP-HPLC of CB-2D tryptic peptides. The CB-2D fraction of Fig. 13 was resolved by RP-HPLC with a C8 column as in Fig. 14.
Thiois of Thrombospondin

Table II

| Peptide | CD-1/1-4 | CD-1/1-3 | CD-1/1-2 | CD-1/1-1 |
|---------|----------|----------|----------|----------|
| Asp     | 72       | 122      | 83       | 200      |
| Lys     | 74       | 113      | 85       | 198      |
| Pro     | 54       | 86       | 22       | 40       |
| Lys     | 59       | 98       | 11        | 20       |
| Thr     | 66       | 105      | 56       | 132      |
| Tyr     | 76       | 113      | 76       | 154      |
| Asp     | 77       | 117      | 77       | 154      |
| Lys     | 59       | 98       | 11        | 20       |
| Asp     | 77       | 117      | 77       | 154      |
| Lys     | 59       | 98       | 11        | 20       |
| Thr     | 66       | 105      | 56       | 132      |
| Tyr     | 76       | 113      | 76       | 154      |
| Asp     | 77       | 117      | 77       | 154      |

Table III

| Peptide | CD-2/1-4 | CD-2/1-3 | CD-2/1-2 | CD-1/1-1 |
|---------|----------|----------|----------|----------|
| Asp     | 72       | 122      | 83       | 200      |
| Lys     | 74       | 113      | 85       | 198      |
| Pro     | 54       | 86       | 22       | 40       |
| Lys     | 59       | 98       | 11        | 20       |
| Thr     | 66       | 105      | 56       | 132      |
| Tyr     | 76       | 113      | 76       | 154      |
| Asp     | 77       | 117      | 77       | 154      |
| Lys     | 59       | 98       | 11        | 20       |
| Asp     | 77       | 117      | 77       | 154      |
| Lys     | 59       | 98       | 11        | 20       |
| Thr     | 66       | 105      | 56       | 132      |
| Tyr     | 76       | 113      | 76       | 154      |
| Asp     | 77       | 117      | 77       | 154      |

Sequence data from Table II are summarized below with residue numbers assigned from the sequence of TSP. Where no clear identification of an amino acid was made, the one in parentheses is from the published sequence. Uniform residues were identified as reported in KG11. Amino termini are underlined and bolded; the number of trisulfides refers to the number of different preparations of TSP from which this particular peptide was sequenced.

Table IV

| Peptide | CD-2/1-4 | CD-2/1-3 | CD-2/1-2 | CD-1/1-1 |
|---------|----------|----------|----------|----------|
| Asp     | 72       | 122      | 83       | 200      |
| Lys     | 74       | 113      | 85       | 198      |
| Pro     | 54       | 86       | 22       | 40       |
| Lys     | 59       | 98       | 11        | 20       |
| Thr     | 66       | 105      | 56       | 132      |
| Tyr     | 76       | 113      | 76       | 154      |
| Asp     | 77       | 117      | 77       | 154      |
| Lys     | 59       | 98       | 11        | 20       |
| Asp     | 77       | 117      | 77       | 154      |
| Lys     | 59       | 98       | 11        | 20       |
| Thr     | 66       | 105      | 56       | 132      |
| Tyr     | 76       | 113      | 76       | 154      |
| Asp     | 77       | 117      | 77       | 154      |
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