Electron Spin Resonance and Fluorescence Studies of the Conformational Environment of the Thiol Groups of Thrombospondin: Interactions with Thrombin[†]

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ABSTRACT: The free thiols of platelet thrombospondin (TSP) were modified with thiol-specific spin labels and fluorescence probes. The conformational effects of thrombin complexation with TSP were monitored by thiol-specific spin labels covalently attached to TSP and active site specific spin labels on thrombin. The results provide evidence supporting speculations that the thiols of the three polypeptide chains in TSP are not conformationally identical. Studies on the effects of Ca^{2+} and temperature confirm that TSP exists in multiple conformations which are under dynamic equilibrium. The ESR spectra of spin-labeled TSP are sensitive to the proteolytic effects of thrombin in the presence and absence of calcium. Phenylsulfonyl fluoride spin labels specific for the active site of thrombin are excellent indicators of thrombin:TSP complex formation in the absence of calcium. The anticoagulant thrombin inhibitor hirudin competes with TSP for the same binding locus on thrombin (which includes the requirement of an intact anion exosite). The results suggest that the species observed here is the noncovalent complex formed during the first step of the TSP−thrombin interaction, showing also that thrombin activity is not essential for complex formation. ESR and fluorescence studies of thiol-labeled TSP indicate that the sulfhydryls are not affected in the noncovalent thrombin:TSP complex, although they must be playing a major role in the second step, i.e., formation of the covalent complex, through intermolecular thiol exchange.

Thrombospondin (TSP)[1] is one of several protein constituents secreted in the postcoagulation period when the platelets are exposed to thrombin (Holmsen et al., 1977). Released thrombospondin becomes associated with the developing fibrin clot, where it modulates the structure of fibrin fibrils (Phillips et al., 1980; Bale, 1987). TSP, also known as thrombin sensitive protein and glycoprotein G (Lawler et al., 1982), is a 420-kDa protein composed of three polypeptide chains (of equal molecular weight) that are cross-linked by disulfide bonds. Each subunit is composed of 1150 amino acids and can be divided into three different structural regions: globular region N, extended connecting region, and globular region C (Lawler, 1986). TSP is a multifunctional protein with binding domains for heparin (Dixit et al., 1984), fibronectin (Lahav et al., 1984; Zafar et al., 1992), fibrinogen (Walz et al., 1981; Bacon-Baguley et al., 1987), collagen (Galvin et al., 1987), and plasminogen (Silverstein et al., 1984). TSP binds at least 12 calcium ions [$K_{app} \approx 120\ \mu M$] which markedly affect the protein conformation (Lawler & Simons, 1983). Upon removal of Ca^{2+} the globular region becomes more compact by a factor of about one-third with a concomitant increase in the length of the filamentous region (Lawler et al., 1982; Lawler, 1985). The globular region contains a free thiol and an intrachain disulfide bond, both of which become more exposed in the absence of calcium (Danishefsky et al., 1984).

The manifestation of a calcium-induced conformational change raises the possibility that the binding of calcium to TSP may affect its interactions with macromolecules and hence its adhesive role in cell−cell and cell−matrix interactions. Detwiler and co-workers have extensively studied the thiols of thrombospondin and their functional role in complex formation with other molecules (Turk & Detwiler, 1986; Detwiler et al., 1987; Speziale & Detwiler, 1990, 1991; Chen et al., 1992). They reported that (i) there are 3 equiv of thiol/mol of TSP, indicative of 1 thiol per polypeptide chain; (ii) the thiols are protected upon calcium binding; (iii) the three thiols are not identical; (iv) the intrachain disulfide bonds are stabilized by Ca^{2+} against dithiothreitol (DTT) reduction; and (v) there is evidence for intramolecular disulfide isomerization where the 1 equiv of free thiol/polypeptide chain may be distributed among 12 different cysteine residues (Detwiler et al., 1987). According to Detwiler and co-workers (Danishefsky et al., 1984; Miller et al., 1988; Detwiler et al., 1987, 1992; Chang & Detwiler, 1992) thrombin forms a strong non-covalent complex with TSP in the absence of calcium ($K_d \approx 35$ nM) with a consequential intermolecular thiol exchange between throm-

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[1] Abbreviations: TSP, thrombospondin; ESR, electron spin resonance; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMMTS, (1-oxy-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methyl methanethiosulfonate; MANS, (2-[(4-maleimidophenyl)amino]naphthalene-6-sulfonic acid, sodium salt); 4-maleimido-TEMPO, 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); S-2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide hydrochloride; PPACK, D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone; m-IV, (*m*-CO-6NH), 4-(2,2,6,6-tetramethylpiperidine-1-oxyl)-*m*-(fluorosulfonyl)-benzamide; p-V, (*p*-CO-5NH), 3-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*p*-(fluorosulfonyl)benzamide.

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bin and TSP in a second step, and thiol blocking reagents prevent this exchange.

This paper describes a detailed investigation of TSP and its interactions with thrombin using electron spin resonance (ESR) and fluorescence techniques. A preliminary report with a less sensitive spin-labeled TSP derivative was only able to suggest a calcium-induced conformational change, but did not expand on the nature of the thiol groups or the interaction(s) of TSP with thrombin (Slane et al., 1988).

MATERIALS AND METHODS

Proteins and Reagents. Thrombospondin was isolated from A23187-released bovine platelets as previously described (Walz et al., 1981; Ciaglowski & Walz, 1986). The purity was checked by SDS−PAGE (5% or 7.5% gels) by the Laemmli (1970) method. Human α- and γ-thrombins either were prepared and characterized after the literature procedures of Fenton et al. (1977) or were gifts from Dr. John W. Fenton, II. Pure bovine α-thrombin was a gift from Dr. Sudish Chandra, Armour Pharmaceuticals, Kankakee, IL. Phenylsulfonyl fluoride nitroxide spin labels were synthesized in our laboratory as described previously (Wong et al., 1974). Recombinant hirudin (HV2-Lys47) was generously donated by Dr. Carolyn Roitsch, Transgene S.A., Strasbourg, France. Dr. John L. Krstenansky, Marion-Merrell Dow Pharmaceuticals, Cincinnati, generously provided the two synthetic C-terminal peptide fragment analogs utilized in these studies (Mao et al., 1988). These peptide fragments, $hirudin_{45-65}$ (21-mer) and $hirudin_{54-65}$ (12-mer) (Mao et al., 1988), as well as the HV2-Lys47 were not sulfated at tyrosine 63. Their concentrations were also determined by absorbance at 280 nm using $\epsilon = 3082\ M^{-1}\ cm^{-1}$ for HV2-Lys 47 (M_r 6848), $\epsilon = 1297\ M^{-1}\ cm^{-1}$ for 21-mer (M_r 2514), and $\epsilon = 1254\ M^{-1}\ cm^{-1}$ for 12-mer (M_r 1468). The reversible thiol-specific spin label (1-oxy-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methyl methanethiosulfonate (PMMTS) was a generous gift from Dr. K. Hideg, University of Pecs, Hungary. The fluorescent probe MANS (2-[(4-maleimidophenyl)amino]-naphthalene-6-sulfonic acid, sodium salt) was from Molecular Probes, Inc. The spin label 4-maleimido-TEMPO (4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl) and DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] were from Sigma Chemical Co. The chromogenic substrate S-2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide hydrochloride) was from Kabi Diagnostics, Sweden. PPACK (D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone) was purchased from Chemica Alta Ltd. (Canada). Chelex-100 was from Bio-Rad. All other chemicals were of analytical grade.

Methods. TSP and thrombin concentrations were measured on a Kontron Instruments UVIKON 860 spectrophotometer using extinction coefficients at 280 nm of 1.0 mg^{-1} mL cm^{-1} (Margossian et al., 1981) and 1.83 mg^{-1} mL cm^{-1}, respectively (Fenton et al., 1977). Thrombin catalytic activity was measured by the S-2238 amidolytic assay (Lottenberg et al., 1982). Free thiol content was estimated spectrophotometrically using DTNB (Ellman, 1959).

ESR spectra were measured in quartz flat cells on a Varian E-109 or E-112 X-band spectrometer with an E-257 variable temperature accessory. Typical instrument settings: microwave power, 20−50 mW; modulation frequency, 100 MHz; modulation amplitude, 0.63 G. Measurements of the maximal separation in hyperfine extrema, $2T_{\|}$, were taken from "high gain" spectra, which were typically run at 5−10-fold higher receiver gain and 1−4-fold greater modulation amplitude to enhance these peaks. Spin-label stoichiometry was determined by computer double integration or by the "spin count" technique (Berliner, 1981). Fluorescence spectra were measured on a Perkin-Elmer LS-50 spectrofluorimeter at 25 °C.

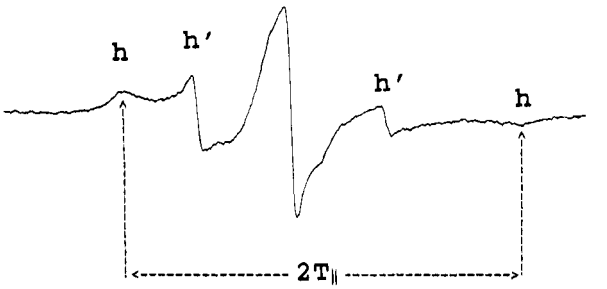

FIGURE 1: ESR spectrum of 8 μM PMMTS−TSP. The buffer was 15 mM Hepes, 0.55 M NaCl, and 1 mg/mL PEG 6000, pH 7.6. ESR instrumental parameters: microwave power, 40 mW; microwave frequency, 9.5 GHz; modulation amplitude, 0.63G; time constant, 0.25 s. Twenty-five scans (4 min each) were time-averaged to improve the signal/noise ratio. The broad peak at the extrema arising from the strongly immobilized nitroxides (marked as h) has an anisotropic hyperfine splitting ($2T_{\|}$) of 68 ± 0.5 G. The ESR spectrum of TSP labeled with 4-maleimido-TEMPO was identical.

A typical TSP thiol modification with either spin label or fluorescent probe was carried out as follows: TSP (6−8 μM) was incubated with a 10−20-fold molar excess of label/-SH group in 15 mM Hepes buffer, 0.55 M NaCl, and 1 mg/mL PEG 6000 (pH 7.6). The reaction was initiated by adding EDTA to a final concentration of 10 mM in order to deplete the TSP of calcium. After 3−4 h at 25 °C, excess reagent was removed by exhaustive dialysis in the cold room against the same buffer with or without 2 mM $CaCl_2$.

α- or γ-thrombin was inhibited with a 20-fold excess of phenylsulfonyl fluoride spin label in 50 mM Tris, 0.75 M NaCl, and 10% (v/v) acetonitrile, pH 7.2, for 1−2 h at room temperature. The extent of spin labeling was estimated from the S-2238 assay, where typically ≥95% inhibition was observed. Unreacted label was removed by dialysis in the cold against the same buffer, but at pH 6.5.

RESULTS AND DISCUSSION

Spin Label Modification of the Free Thiols of Thrombospondin. Labeling of TSP with the two thiol-specific spin labels, PMMTS (65 ± 4%) and 4-maleimido-TEMPO (35 ± 4%), by both ESR spin count and DTNB titrations indicated that only two of the three thiols per TSP molecule had reacted with PMMTS and that 4-maleimido-TEMPO had modified only one thiol under identical conditions and time courses of labeling. This actually supports the earlier finding by Detwiler et al. (1987) that the three thiols are *not all identical* in accessibility or reactivity.

Interestingly, both thiol-labeled TSP derivatives (PMMTS and 4-maleimido-TEMPO) coincidentally gave rise to identical ESR spectra containing two spectral components, i.e., a strongly immobilized species with hyperfine extrema, $2T_{\|}$ = 68 ± 0.5 G (denoted as h in Figure 1), and a second weakly immobilized species (denoted as h′ in Figure 1). This indicates that there are spin labels distributed over two different environments, which may be due either to two

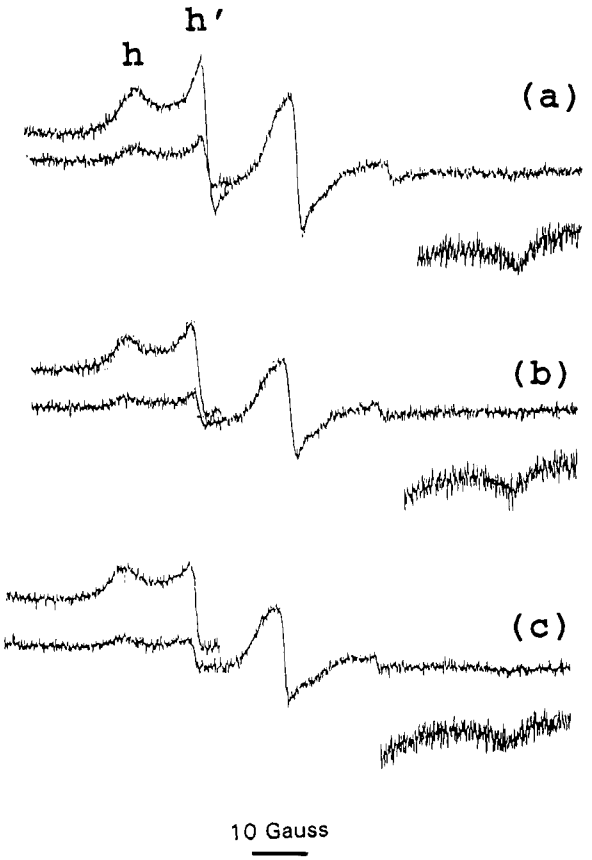

FIGURE 2: ESR spectra of 8 μM PMMTS−TSP in the absence and presence of Ca^{2+}. The buffer was 15 mM Hepes, 0.55 M NaCl, and 1 mg/mL PEG 6000, pH 7.6 in the absence of Ca^{2+} (a) and in the presence of 0.25 mM $CaCl_2$ (b) and supersaturated $CaCl_2$ (c). ESR instrumental parameters: microwave frequency, 9.5 GHz; microwave power, 40 mW; modulation amplitude, 0.63 G; time constant, 0.25 s; scan time, 16 min. The high gain spectra were recorded with a modulation amplitude of 2 G. The peak height ratios, h/h′, were (a) 0.23, (b) 0.25, and (c) 0.29. The ESR spectra of 4-maleimido-TEMPO−TSP exhibited similar behavior with Ca^{2+}.

classes of thiols or to two different conformations of the polypeptide chains. Another possibility is that the two components may arise from the structural dissimilarity of the thiols in the three different polypeptide chains, but this has been ruled out since both PMMTS and 4-maleimido-TEMPO gave identical ESR spectra, although the number of thiols modified by these labels was different. This is in sharp contrast with the previously reported ESR spectrum of 4-maleimido-TEMPO spin-labeled TSP, which showed none of these large hyperfine features (Slane et al., 1988).

Calcium and Temperature Dependence. Figure 2a depicts the ESR spectrum of PMMTS−TSP in the absence of calcium. Upon addition of Ca^{2+}, the weakly immobilized h′ component decreased with a proportional, hyperbolic increase in the broad, strongly immobilized h peak (Figure 2b). At saturating concentrations (K_d = 120 μM), the peak height ratio (h/h′) reached a maximum (Figure 2c), indicating that calcium affects the partitioning between the two components. This strongly supports the hypothesis that the spectral components are due to two different conformations which are in equilibrium with one another, not to two different classes of thiols. Both PMMTS and 4-maleimido-TEMPO derivatives of TSP showed analogous calcium dependence.

In order to ascertain whether the two spin label environments arise from two distinct equilibrium conformations, we followed the temperature dependence of the ESR spectra as shown in Figure 3a. Note how the h/h′ ratio diminishes with temperature, consistent with the strongly immobilized conformer decreasing at higher temperatures (Figure 3b). Furthermore, these changes were completely reversible, again confirming the existence of a dynamic equilibrium between the two components.

TSP Interactions with Thrombin. Since according to Detwiler et al. (1987) thiol-blocking reagents prevent the intermolecular thiol exchange between thrombin and TSP, we would not expect spin-labeled TSP to form a covalent complex with thrombin. We did not observe any changes in the ESR spectra of either PMMTS−TSP or 4-maleimido-TEMPO−TSP in the presence of thrombin except for proteolysis of the TSP in the absence of calcium. This was evidenced by a weakly immobilized spectral component that appeared which reflected a nitroxide group near the free tumbling motional limit (data not shown). Spectral changes of this nature are often observed when a spin-labeled protein has been proteolyzed (Berliner, 1981, 1984). Takahashi et al. (1984) have shown that thrombin progressively and completely cleaves TSP chains into three fragments of molecular masses 130, 95, and 65 kDa in the absence of calcium, whereas only the heparin-binding fragment (30 kDa) is released in the presence of 2 mM Ca^{2+}. α-Chymotrypsin, which digests TSP more rapidly into smaller fragments (Lawler & Hynes, 1986), produced similar changes in which the entire ESR spectrum rapidly converted to freely tumbling spin labels. Direct evidence for the cleavage of spin-labeled TSP samples was also confirmed by SDS−PAGE (data not shown). In an attempt to distinguish binding interactions of thrombin with TSP versus proteolysis, a sample of PMMTS−TSP or 4-maleimido-TEMPO−TSP was incubated with PPACK−thrombin (which was devoid of any enzymatic activity). We observed no changes in the ESR spectra, suggesting that the thiol labels do not sense the thrombin interactions, despite the evidence for a TSP−thrombin interaction from the proteolysis data as well as the active site labeled thrombin studies discussed in the next section.

The fluorescence emission of MANS-labeled TSP was monitored during titrations with either calcium or thrombin at room temperature. There were only slight changes in the fluorescence intensity upon addition of calcium, whereas exposure to thrombin, with or without calcium, caused no changes (data not shown).

TSP Interactions with Active Site Spin-Labeled Thrombin. Since Detwiler et al. (1992) suggested the requirement for active thrombin in TSP−thrombin complex formation, we examined the effects at the thrombin active site upon TSP binding using spin-labeled sulfonyl fluoride inhibitor analogs. These spin labels inhibit thrombin by covalently blocking Ser 195 and thereby preventing proteolysis of TSP. Consequently any ESR spectral changes observed with these spin-labeled thrombins would correspond to conformational communication at the active site upon TSP binding. Figure 4a depicts the ESR spectrum of m-IV-inhibited human α-thrombin, a derivative where the nitroxide ring has been shown to occupy an exosite region that corresponds to differences between the α and γ forms of human thrombin (Berliner, 1984). When this sample was treated with TSP in 2 mM Ca^{2+} and a 2-fold molar excess of thrombin, no changes were observed in the ESR spectrum (Figure 4a). The very small increase in the narrow line peak (denoted by ↓) was due to hydrolysis (i.e., desulfonylation) which releases

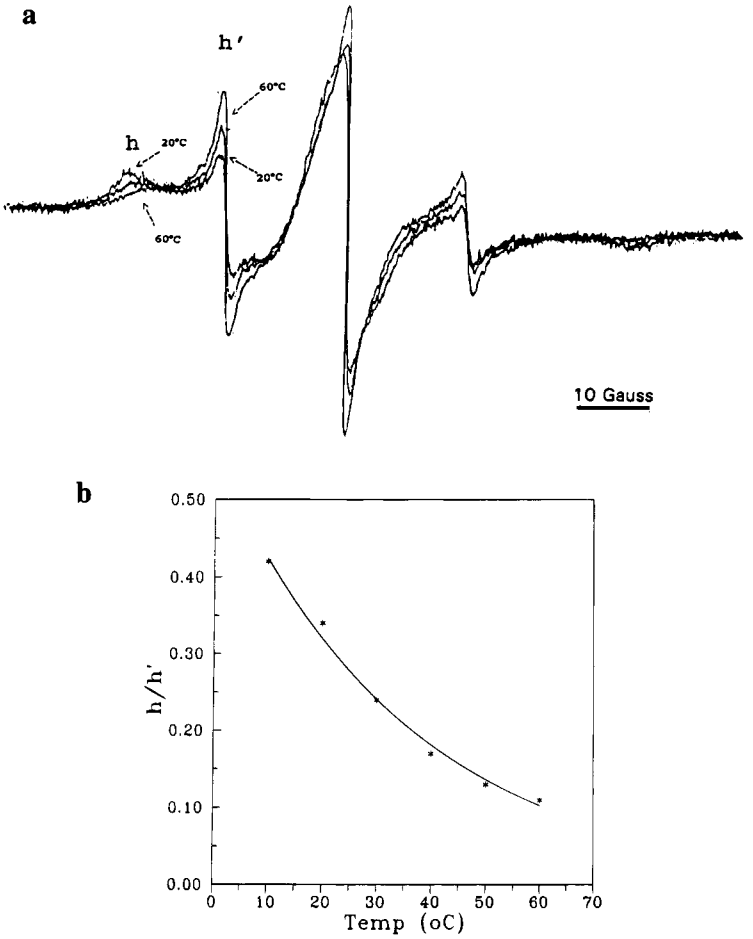

FIGURE 3: (a) Temperature dependence of the ESR spectra of 8 μM PMMTS−TSP. The temperature was varied from 10 to 60 °C. ESR instrumental parameters: microwave frequency, 9.14 G; microwave power, 50 mW; modulation amplitude, 2.0 G; time constant, 0.5 s; scan time, 16 min. (b) Plot of relative peak height versus temperature. All other conditions were identical to those in Figure 2.

a small amount of free nitroxide into solution (Berliner & Wong, 1974). When this mixture was treated with 5 mM EDTA to remove Ca^{2+}, a new spectral component appeared that was distinguished by its hyperfine splitting ($2T_{\|}$) of 54 ± 0.5 G (Figure 4c). After dialysis against 15 mM Hepes (0.55 M NaCl and 1 mg/mL PEG 6000, pH 7.6) in the presence of Chelex-100 to remove free Ca^{2+}, spectrum 4d was obtained where the hyperfine extrema were again well pronounced. Thus the nitroxide at the α-thrombin active site becomes more immobilized upon TSP binding (in the absence of calcium), suggesting that the thrombin active site is effected upon forming the TSP−thrombin complex. In order to identify whether this complex was non-covalent or covalent (i.e., formed by intermolecular thiol exchange), we treated this complex with dithiothreitol but observed no changes in the ESR, suggesting the possibility of a non-covalent complex. Since slowing down the motion of a spin-labeled protein can cause an increase in the hyperfine extrema (Shimshick & McConnell, 1972), an isokylindric control experiment was performed in which the motion of m-IV−human α-thrombin was reduced to approximate that of the thrombin−TSP complex by exposure to 48% (w/v) sucrose. The hyperfine extrema did not match the above value obtained for incubation with TSP, indicating that the ESR spectral change observed was not due to the decrease of molecular motion consequent to the docking of the huge TSP molecule.

Hirudin, the potent protein inhibitor from the leech, forms an extremely tight complex with thrombin that encompasses the catalytic site as well as the anion-binding exosite (Markwardt, 1970). When the TSP−α-thrombin complex in Figure 4d was mixed with a 1.3-fold excess of hirudin, the hyperfine coupling $2T_{\|}$ increased to 65 ± 0.5 G (Figure 4e), which was identical to that of the human α-thrombin−hirudin control alone. Thus the data are totally consistent with hirudin competing with TSP for the same binding locus. Since the hirudin-binding locus also includes the anion exosite, we tested this region selectively by employing saturating concentrations of the hirudin C-terminal peptides, $hirudin_{45-65}$ (21-mer) and $hirudin_{54-65}$ (12-mer), which bind solely at the anion exosite; however, no effects in the TSP−human α-thrombin ESR spectra were observed (data not shown). Thus our interpretation is that TSP binding only involves the immediate active site region and not the anion exosite. In addition, m-IV-labeled human γ-thrombin (which is a proteolyzed, nonclotting, amido/esterolytically active thrombin which no longer has an intact anion exosite) showed no changes in the ESR spectrum upon incubation with TSP (data not shown). This result is not surprising in light of the data from Takahashi et al. (1984) which showed that TSP is not a substrate for γ-thrombin; however, this does not preclude nonproductive binding of γ-thrombin with TSP.

We extended these experiments to bovine thrombin since we had previously shown with these spin labels that subtle differences can be detected in the extended active site regions of theses thrombins. Panels a−c of Figure 5 show ESR spectra for m-IV-inhibited bovine α-thrombin alone and

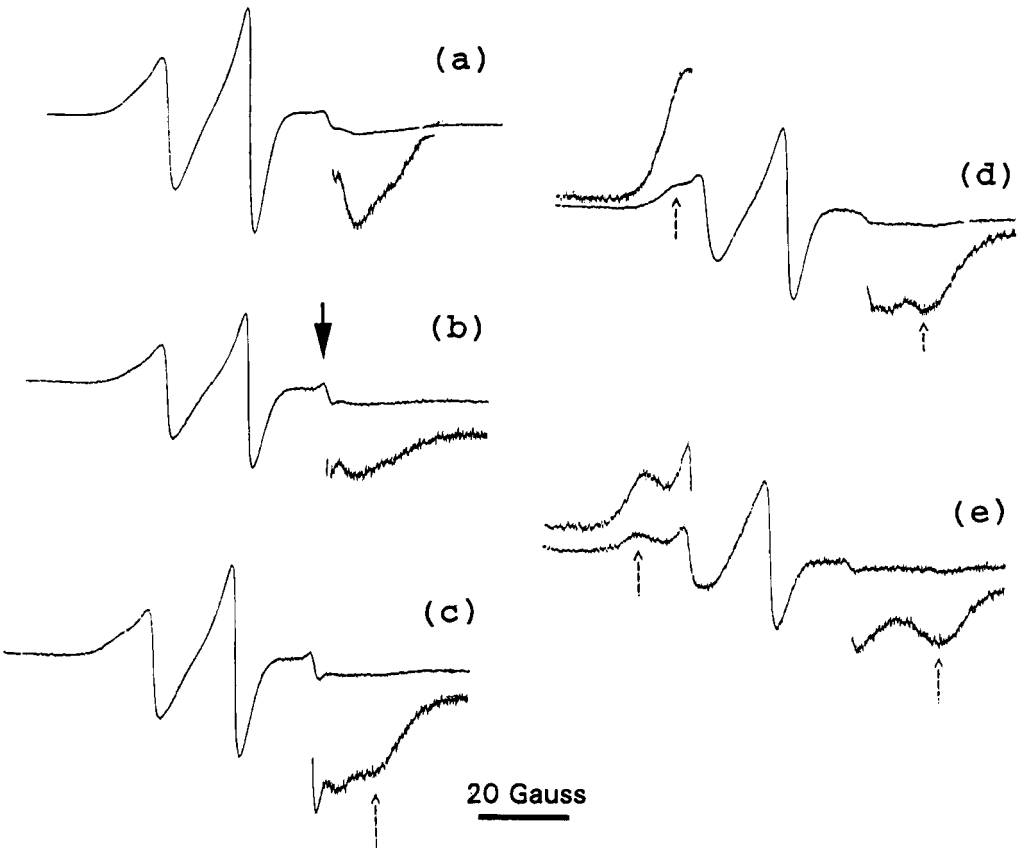

FIGURE 4: ESR spectra of m-IV spin-labeled human α-thrombin. (a) m-IV α-thrombin (25 μM), 50 mM Tris, and 0.75 M NaCl, pH 6.5. There are minor amounts of autodigested spin-labeled thrombin which give rise to the sharp peak overlapping the main broad immobilized label peak. (b) 15 μM m-IV human α-thrombin + 6 μM TSP in Hepes buffer, pH 7.6 with 2 mM $CaCl_2$. A very small increase in the sharp peak (solid arrow) was due to label hydrolysis (see text). (c) Sample (b) treated with 5 mM EDTA in the same buffer. The new immobilized label peak has a $2T_{\|}$ value of 54 ± 0.5 G. (d) Sample (c) after dialysis against Hepes buffer (pH 7.6) and treatment with Chelex-100 to remove Ca^{2+}. The anisotropic hyperfine extrema ($2T_{\|}$) are marked with dashed arrows. (e) The sample from (d) treated with 25 μM hirudin in the same buffer.

complexed with TSP and hirudin, respectively, where the results were qualitatively similar to those with the human species (Figure 4a,d,e).

We also examined TSP complexes with p-V-labeled human and bovine thrombins. This derivative did not show any changes in its ESR spectrum upon complexation with TSP. The p-V label has been shown to be sensitive to indole binding site in thrombin (Berliner, 1984), which is structurally distinct from the α−γ difference region where the m-IV label detected significant changes upon binding TSP in the absence of calcium (Figure 4c). Taken together, the results with these two spin labels support a model where the α−γ difference region of thrombin (but not the indole site) is involved in TSP binding.

Intermolecular Distance Constraints. 4-Maleimido-TEMPO−TSP and m-IV−thrombin were complexed (in the absence of calcium) in order to assess whether the labels are close enough in the complex to be detected by paramagnetic dipolar interactions. However, the resultant ESR spectra showed no new spectral features apart from the motional effects observed earlier for m-IV−thrombin complexed with unlabeled TSP. Thus, this suggests that the two nitroxide moieties must be greater than *ca.* 15 Å apart in the complex.

Summary of These Results. These present studies provide several new and interesting results about the nature of the thiols of TSP as well as details about the interactions between TSP and thrombin. The results also complement the studies of Detwiler and co-workers (Turk & Detwiler, 1986; Detwiler et al., 1987, 1992; Speziale & Detwiler, 1990, 1990; Chen et al., 1992; Miller et al., 1988; Chang & Detwiler, 1992) by providing direct physical evidence for many of the speculations about conformational states of TSP. For example, Speziale and Detwiler (1990) suggested that the thiols from the three polypeptide chains were nonidentical even though they had almost the same primary and secondary structure. Our spin-labeling experiments with thiol-specific labels confirm these suggestions by virtue of the differences in the reactivities of the thiols. Moreover, within a particular chain, Detwiler et al. (1987) identified multiple thiol and disulfide pairing locations which they speculated were due to two possible reasons: (i) during the TSP secretion, there is a transition from one conformer (with one set of disulfide bonds and a single thiol) to another stable conformer (with a different set of disulfide bonds and different free thiol); or (ii) TSP exists in multiple, equally probable conformations, each with its own disulfide pairings. The calcium titrations and temperature-dependence studies described here corroborate the latter multiple conformational model. The ESR spectra of thiol-spin-labeled TSP also confirmed that calcium stabilizes the molecule against digestion by thrombin (and other serine proteases).

Lastly, our studies shed more light on the TSP−thrombin interaction. The ESR results provided direct physical evidence for TSP−thrombin complex formation in the absence of calcium as noted by Detwiler et al. (1987). On the contrary, however, the results showed that thrombin activity was not a prerequisite for non-covalent complex

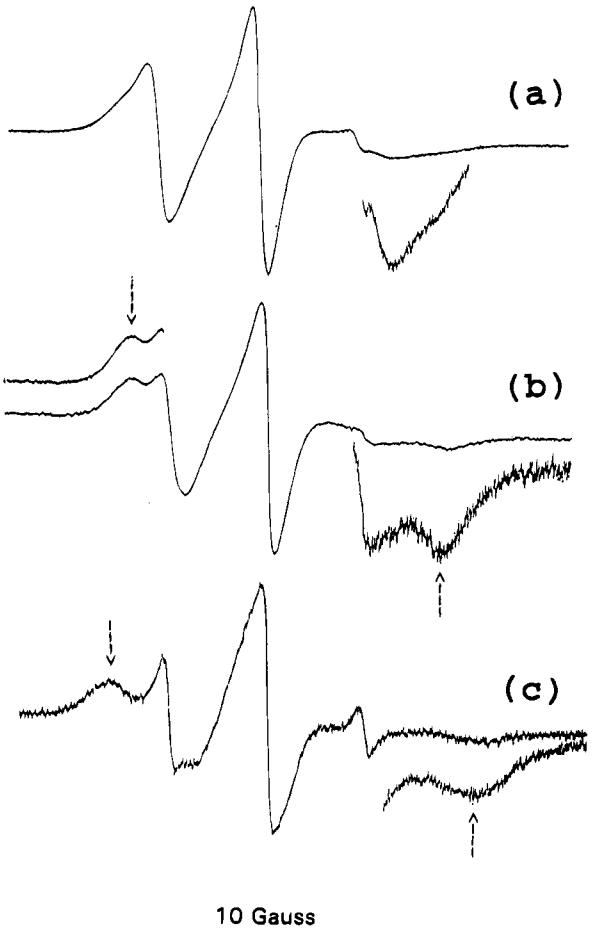

FIGURE 5: ESR spectra of m-IV spin-labeled bovine α-thrombin. (a) 30 μM bovine α-thrombin in Hepes buffer, pH 7.6. (b) 20 μM bovine α-thrombin + 7 μM TSP in the same buffer (calcium removed by Chelex-100). The anisotropic hyperfine extrema ($2T_{\parallel}$) are marked with dashed arrows. (c) The sample from (b) treated with 25 μM hirudin in the same buffer.

formation, although it might be essential for covalent intermolecular thiol interchange (Detwiler et al., 1992). Our results also suggest that complex formation does not necessarily require an initial association between thrombin and a serpin. Regardless of the nature of the interaction between TSP and thrombin, our ESR and fluorescence data both showed that the conformational consequences were not sensed by the reactive thiol region of TSP.

The studies described herein suggest that the thiols of TSP are not conformationally identical among the three chains of a TSP molecule. The molecule appears to exist in multiple conformations, each with its own thiol environment. These conformers are under a dynamic equilibrium, which is modulated by calcium, and probably play an important role in the interaction of TSP with other macromolecules. Thrombin plays two different roles in its interactions with TSP, i.e., digestion and complex formation, both of which are influenced by the effect of calcium on TSP. The thrombin active site senses complex formation, although a free catalytic site is not essential.

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