Defining the Native Disulfide Topology in the Somatomedin B Domain of Human Vitronectin*

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The N-terminal 44 amino acid residues of the human plasma glycoprotein vitronectin, known as the somatomedin B (SMB) domain, mediates the interaction between vitronectin and plasminogen activator inhibitor 1 (PAI-1) in a variety of important biological processes. Despite the functional importance of the Cys-rich SMB domain, how its four disulfide bridges are arranged in the molecule remains highly controversial, as evidenced by three different disulfide connectivities reported by several laboratories. Using native chemical ligation and orthogonal protection of selected Cys residues, we chemically synthesized all three topological analogs of SMB with predefined disulfide connectivities corresponding to those previously published. In addition, we oxidatively folded a fully reduced SMB in aqueous solution, and prepared, by CNBr cleavage, the N-terminal segment of 51 amino acid residues of intact vitronectin purified from human blood. Proteolysis coupled with mass spectrometric analysis and functional characterization using a surface plasmon resonance based vitronectin-PAI-1-SMB competition assay allowed us to conclude that 1) only the Cys5–Cys21, Cys9–Cys39, Cys19–Cys32, and Cys25–Cys31 connectivity is present in native vitronectin; 2) only the native disulfide connectivity is functional; and 3) the native disulfide pairings can be readily formed during spontaneous (oxidative) folding of the SMB domain in vitro. Our results unequivocally define the native disulfide topology in the SMB domain of human vitronectin, providing biochemical as well as functional support to the structural findings on a recombinant SMB domain by Read and colleagues (Zhou, A., Huntington, J. A., Pannu, N. S., Carrell, R. W., and Read, R. J. (2003) Nat. Struct. Biol. 10, 541–544).

Vitronectin is a multidomain plasma glycoprotein involved in a variety of biological processes such as cell adhesion, cell migration, modulation of the immune system, and regulation of blood coagulation and fibrinolysis (1, 2). Many regulatory functions of vitronectin result from its ability to interact with plasminogen activator inhibitor 1 (PAI-1), a member of the serine protease inhibitor superfamily that inhibits both tissue- and urinary-type plasminogen activators (3–6). PAI-1 plays important roles in thrombosis and fibrinolysis and has been implicated in hemostasis, angiogenesis, and tumor metastasis (7–11). Low abundant PAI-1 circulates in blood complexed with vitronectin (12); unliganded PAI-1 undergoes rapid "self-inactivation" into an inactive "latent" form in which the inhibitory reactive-site loop inserts as a new strand into the main β-sheet of the molecule (13–15). Ample evidence suggests that vitronectin regulates the activity of PAI-1 and PAI-1-mediated cellular events by stabilizing the active form of the inhibitor and delaying its conformational transition to the latent state (16–18). Conversely, PAI-1 mediates, via binding, vitronectin-dependent cell adhesion and migration (19–23). Thus, molecular recognition between vitronectin and PAI-1 is of great importance in biology.

Loskutoff and colleagues (24–27) first pinpointed a high affinity PAI-1-binding site in vitronectin to the N-terminal somatomedin B (SMB) domain of the adhesive glycoprotein. The SMB domain consists of 44 amino acid residues (28–31), including eight conserved cysteines that form four functionally indispensable intramolecular disulfide bridges (24). Kamikubo et al. (32) reported that an N-terminal fragment of VN of 97 amino acid residues, expressed in the cytoplasm of Escherichia coli and purified by immuno-affinity chromatography, showed activity in PAI-1 binding and antibody recognition similar to urea-activated vitronectin (uVN)3 purified from human blood. CNBr cleavage of the recombinant VN fragment released the SMB domain elongated C-terminally by an extra seven-residue RGD motif (we term rVN1–51-1). Partial reduction and S-alkylation of rVN1–51-1 coupled with proteomics techniques identified a consecutively arranged, uncrossed pattern of disulfide bridges in the SMB domain, i.e. Cys5–Cys9, Cys19–Cys21, Cys25–Cys31, and Cys32–Cys39 (32). As rVN1–51-1 was functionally indistinguishable from intact uVN, the linear disulfide topology was thought to exist in native vitronectin (32).

Read and colleagues (18) subsequently reported the crystal structure of PAI-1 complexed with a similarly obtained recombinant VN1–51 (we term rVN1–51-2), which was shown to contain a crossed pattern of disulfides, i.e. Cys5–Cys9, Cys19–Cys21, Cys39–Cys4, and Cys32–Cys39 (32). As Zhou et al. (18) did not address the difference in disulfide topology between rVN1–51-1
and rVN1–51-2, the question of which disulfide linkages represent the topology in native vitronectin was left unanswered.

Added to the brewing controversy was a recent finding by Peterson and colleagues (33) that the first 51 amino acid residues cleaved by CNBr from natively purified vitronectin (32) had a third type of disulfide topology (Cys5–Cys9, Cys19–Cys31, and Cys25–Cys39), which matched neither the pattern reported for rVN1–51-1 nor for rVN1–51-2. Horn et al. (33) further suggested that rVN1–51-1 and rVN1–51-2 were stable folding intermediates with non-native disulfide linkages, a view hotly contested in a latest report by Kamikubo et al. (34).

We strategically selected Cys residues to be protected by Acm in the SMB sequence so that discerning the pattern of the first two disulfides after Me2SO oxidation could be achieved by one-step proteolysis coupled with liquid chromatography-MS analysis. Bovine chymotrypsin and trypsin and Staphylococcus aureus Glu-specific V8 protease were purchased from Worthington Biochemical Co. Enzymatic digestion was carried out for 1 h at 37 °C in 50 mm Tris, 20 mm CaCl2, 0.005% Triton X-100, and rVN1–51-2.

**MATERIALS AND METHODS**

**Peptide Synthesis, Native Chemical Ligation, and Oxidative Folding—**Stepwise chemical synthesis of the 44-residue SMB domain on solid phase by t-butoxycarbonyl chemistry turned out to be extremely difficult if not impossible. We took advantage of the native chemical ligation technique pioneered by Kent and colleagues (35–37), assembled the following four full-length peptides in high purity (hereinafter referred to as peptides 1–4; C denotes Acm-protected Cys; ↓ depicts the site for native chemical ligation).

Specifically, the C-terminal peptides were assembled on t-butoxycarbonyl-Thr(benzyl)-OCH2-phenylacetamidomethyl resin using the n,N-disopropylethyamine in situ neutralization/2-(H-benzotriazol-1-yl)-1,1,3,3-tetramethyloxonium hexafluorophosphate activation protocol developed by Kent and colleagues (38). The N-terminal peptides were synthesized on custom-made thioester resin (trityl)SCH2CH2COO-Leu-OCH2-phenylacetamidomethyl using an otherwise identical chemistry. After HF cleavage and deprotection, crude peptides were purified to homogeneity by preparative C18 HPLC, and their molecular masses were verified by electrospray ionization mass spectrometry (ESI-MS). Two-segment native chemical ligation was performed as described previously (39, 40), resulting in the four full-length SMB peptides with correct molecular masses.

To obtain sSMB-1, sSMB-2, and sSMB-3, Acm-protected peptides 1–3 were first oxidized at 0.25 mg/ml in phosphate-buffered saline buffer, pH 7.4, by 20% (v/v) Me2SO. An overnight oxidation of each peptide at room temperature yielded three chromatographically distinct species. After disulfide mapping, three desired folding intermediates with Cys19–Cys21/Cys25–Cys31 (for sSMB-1), Cys5–Cys21/Cys25–Cys31 (for sSMB-2), and Cys5–Cys9/Cys21–Cys32 (for sSMB-3) were selected for Acm deprotection/disulfide formation. Specifically, the two-disulfide-bridged peptides at 0.5 mg/ml in an acidic solution containing 0.1 M citric acid, 0.2 M HCl, and 20% methanol were treated by 1 mM iodine for 15 min, each resulting in three fully oxidized and topologically different SMB domains, from which sSMB-1, sSMB-2, or sSMB-3 was eventually decoded. Spontaneous folding of the fully unprotected peptide 4 in aqueous solution under redox control of reduced and oxidized glutathione was carried out essentially as described for rVN1–51-1 (34), giving rise to a predominant folding species termed sSMB-4. All four synthetic SMB domains were purified to homogeneity on RP-HPLC and verified by ESI-MS. Quantification of SMB domains was carried out by UV absorbance measurements at 280 nm using molar extinction coefficients calculated according to a published algorithm (41).

**Dissection of Two-disulfide-bridged Oxidation Intermediates—**We strategically selected Cys residues to be protected by Acm in the SMB sequence so that discerning the pattern of the first two disulfides after Me2SO oxidation could be achieved by one-step proteolysis coupled with liquid chromatography-MS analysis. Bovine chymotrypsin and trypsin and Staphylococcus aureus Glu-specific V8 protease were purchased from Worthington Biochemical Co. Enzymatic digestion was carried out for 1 h at 37 °C in 50 mm Tris, 20 mm CaCl2, 0.005% Triton X-100,
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pH 8.3. The desired oxidation intermediate derived from peptide 1 contained Cys\(^{19}\)–Cys\(^{21}\)/Cys\(^{32}\)–Cys\(^{39}\), and was readily identified by digestion with chymotrypsin that cleaved (Tyr\(^{27}\)/Tyr\(^{28}\) or Tyr\(^{26}\)/Gln\(^{29}\)) between Cys\(^{21}\) and Cys\(^{32}\). The desired oxidation intermediates derived from peptides 2 and 3 both contained two linear and uncrossed disulfides, Cys\(^{5}\)–Cys\(^{21}\)/Cys\(^{25}\)–Cys\(^{31}\), and Cys\(^{5}\)–Cys\(^{7}\)/Cys\(^{21}\)–Cys\(^{25}\), respectively. For the peptide 2 derivative, Glu-specific V8 protease made a clean split (Glu\(^{23}\)/Leu\(^{24}\)) between Cys\(^{21}\) and Cys\(^{25}\), whereas trypsin cleaved (Lys\(^{17}\)/Lys\(^{18}\) or Lys\(^{18}\)/Cys\(^{19}\)) between Cys\(^{9}\) and Cys\(^{21}\) of the peptide 3 derivative. Using this highly simplified approach, all undesired Me\(_2\)SO-oxidized intermediates with crossed patterns of disulfide bonding were readily excluded.

Dissection of Fully Oxidized sSMB-1, sSMB-2, and sSMB-3—To confirm the linear and uncrossed disulfide pattern in sSMB-1, the peptide was treated with chymotrypsin/Glu-specific V8 protease for 1 h at 37 °C, generating, as judged by ESI-MS, the following four major fragments: [DQESC\(^{5}\)K][C\(^{19}\)QC\(^{21}\)DEL][QSC\(^{31}\)C\(^{32}\)TDY][C\(^{39}\)KPQVT], and [LC\(^{25}\)SYYSC\(^{31}\)C\(^{32}\)TDYTAE][QSC\(^{31}\)C\(^{32}\)TDY]. For sSMB-2, cleavage by chymotrypsin/trypsin yielded three major fragments: [DQESC\(^{5}\)K][C\(^{19}\)QC\(^{21}\)DEL][QSC\(^{31}\)C\(^{32}\)SY][QSC\(^{31}\)C\(^{32}\)TDY], [C\(^{25}\)TE][AQSC\(^{31}\)C\(^{32}\)TDY], and [DQESC\(^{5}\)K][C\(^{19}\)QC\(^{21}\)DEL][QSC\(^{31}\)C\(^{32}\)SY][QSC\(^{31}\)C\(^{32}\)TDY], thus confirming the presence of Cys\(^{19}\)/Cys\(^{32}\) and Cys\(^{3}–Cys\(^{39}\) in sSMB-2. A combination cleavage of sSMB-3 by chymotrypsin and Glu-specific V8 protease resulted in [SC\(^{5}\)KGRG\(^{9}\)TE], [NVKKC\(^{19}\)QC\(^{21}\)DE][QSC\(^{31}\)C\(^{32}\)TDY][C\(^{39}\)KPQVT], and [LC\(^{25}\)SYYSC\(^{31}\)C\(^{32}\)TDYTAE][QSC\(^{31}\)C\(^{32}\)TDY], thus confirming the presence of Cys\(^{19}\)–Cys\(^{31}\) and Cys\(^{25}\)–Cys\(^{39}\) in the molecule.

Isolation of VN\(^{1–51}\) from Intact Vitronectin—Natively purified and urea-activated vitronectins of human blood source were purchased from Molecular Innovations, Inc. The SMB-containing VN\(^{1–51}\) was released from vitronectin (0.5 mg/ml in 2.5% trifluoroacetic acid) by CNBr (20 mg/ml). After an overnight cleavage at room temperature, VN\(^{1–51}\) was purified by analytical C18 RP-HPLC. Two products resulted, the major component containing a C-terminal homoserine lactone and the minor species ending with homoserine at basic pH, spontaneous hydrolysis quickly converts homoserine lactone to homoserine.

Proteolytic Fingerprinting of sSMB-2, sSMB-4, nVN\(^{1–51}\), and uVN\(^{1–51}\)—50 µg of sSMB-2 or sSMB-4 was dissolved at 1 mg/ml in 50 mM Tris/HCl buffer containing 20 mM CaCl\(_2\) and 0.005% Triton X-100, pH 8.3, to which 1 µg of each enzyme was added in one of the following three binary combinations: chymotrypsin/trypsin, chymotrypsin/V8 protease, and trypsin/V8 protease. After an 18-h incubation at 37 °C, the cleavage reaction was terminated by addition of 50 µl of 10% acetic acid, followed by liquid chromatography-MS analyses. Due to limitations in quantities, much less nVN\(^{1–51}\) or uVN\(^{1–51}\) was used in otherwise identical experiments.

Surface Plasmon Resonance Spectroscopy—The ability of synthetic SMB domains and VN\(^{1–51}\) to interact with PAI-1 (purchased from Oxford Biomedical Research) in solution was quantified on a Biacore 3000 surface plasmon resonance instrument according to a competition assay protocol developed by Loskutoff and colleagues (32, 34, 42). Briefly, 1100 resonance units of urea-activated vitronectin were immobilized (in 10 mM acetic buffer, pH 4.0) to a CM5 sensor chip using the 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride/N-hydroxysulfosuccinimide coupling chemistry and procedures recommended by the manufacturer. Kinetic analysis of the binding to vitronectin of PAI-1, either alone or in the presence of sSMB, was carried out at 25 °C in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4). For competition, 25 nM PAI-1 was incubated at room temperature for 15 min with varying concentrations of synthetic SMB and injected at a flow rate of 20 µl/min for 5 min, followed by 5-min dissociation. The concentration of free PAI-1 in solution (not complexed with sSMB) was deduced, based on the initial rate (slope) of VN association, from a calibration curve established by resonance unit measurements of different concentrations of PAI-1 injected alone. Non-linear regression analysis was performed using GraphPad Prism 4 to give rise to IC\(_{50}\) values, concentrations of SMB at which 50% of PAI-1 was sequestered in SMB-PAI-1 complexes, thus unavailable for VN binding.

RESULTS

The SMB domain with eight Cys residues possesses 105 unique disulfide connectivities. We selectively protected four Cys residues in SMB with the orthogonal protecting agent Acm and oxidatively folded the resultant peptide containing four free cysteines, yielding three two-disulfide-bridged intermediates separable on reversed phase HPLC. After the disulfide linkages were discerned for all three species by mass mapping of peptide fragments generated by proteolysis, the folding intermediate with desired disulfide bridges was selected for Acm deprotection and simultaneous formation of two remaining disulfide bonds, also in three unique combinations. Mass mapping aided by enzymatic digestion was performed again to definitively establish disulfide connectivities in three fully oxidized products. The strategy for the synthesis of sSMB-1, sSMB-2, and sSMB-3 with predefined disulfide connectivities corresponding to the ones previously reported for rVN\(^{1–51}\)-1, rVN\(^{1–51}\)-2, and nVN\(^{1–51}\)-3, respectively, is illustrated in Fig. 1.

For comparison, a fully reduced and unprotected synthetic SMB was oxidatively folded in aqueous solution according to the published protocol (34), yielding conformationally homogeneous and thermodynamically stable sSMB-4. In addition, nVN\(^{1–51}\) and uVN\(^{1–51}\), which contain the SMB domain C-terminally connected to a seven-residue RGD motif (RGDVTFTM), were prepared by CNBr cleavage, at the Met\(^{51}\)-Pro\(^{52}\) peptide bond, of natively purified and urea-treated vitronectin from human blood, respectively. All synthetic SMB domains as well as nVN\(^{1–51}\) and uVN\(^{1–51}\) were purified by RP-HPLC to homogeneity, and their molecular masses ascertained by ESI-MS. Shown in Fig. 2 are sSMB-1, sSMB-2, sSMB-3, and sSMB-4 analyzed by C18 RP-HPLC and ESI-MS. The molecular masses of synthetic sSMB domains were found to be 5003.2 ± 0.3 Da, in agreement with the expected value of 5003.5 Da calculated on the basis of average isotopic compositions of fully oxidized SMB.

High resolution analytical RP-HPLC is a powerful tool for chromatographically differentiating peptides with different disulfide connectivities (43). As shown in Fig. 2, the three topological analogs sSMB-1, sSMB-2 and sSMB-3, at least 1.5 min
apart from each other, were fully separated on analytical C18 RP-HPLC. One immediate anomaly was that sSMB-4 had different retention from sSMB-1 (1.6 min apart), suggesting that sSMB-4 and sSMB-1 were topologically different. This finding was surprising because, based on the recent report by Kamikubo et al. (34), we had expected sSMB-4 to be identical to sSMB-1. Proteolytic digestion of sSMB-1 and sSMB-4 by Glu-specific V8 protease confirmed their difference in disulfide bonding pattern (data not shown). Notably, sSMB-4 and sSMB-2 showed identical retention on RP-HPLC (Fig. 2), and, in fact, co-eluted when injected as a mixture. We therefore hypothe-

FIGURE 1. Strategy for the synthesis of sSMB-1, sSMB-2, and sSMB-3 with predefined disulfide connectivities. Filled circles represent Acm-protected cysteines and unfilled circles free Cys residues. DMSO, dimethyl sulfoxide.
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FIGURE 3. Identical proteolytic fingerprints for sSMB-2 and sSMB-4 generated by chymotrypsin/trypsin (left column), chymotrypsin/V8 protease (center column), and trypsin/V8 protease (right column). The chromatographic conditions were the same as described in the legend of Fig. 2. All the peaks numbered were identified by simulated proteolytic digestion and mass spectrometric analysis, and the peptide fragments generated were consistent with the disulfide connectivity Cys5–Cys21, Cys9–Cys39, Cys19–Cys32, and Cys25–Cys31 (Table 1).

TABLE 1
Peptides fragments from sSMB-2/sSMB-4 (Fig. 3) identified by simulated proteolysis and mass mapping

| Peak # | Chymotrypsin/Trypsin | Chymotrypsin/V8 protease | Trypsin/V8 protease |
|--------|-----------------------|--------------------------|---------------------|
| 1      | [9–13][36–42]         | [12–13]                  | [9–11][39–44]       |
| 2      | 1329.4 ± 0.4 (1329.5) | 222.4 (222.2)            | 1024.0 ± 0.0 (1024.2) |
| 3      | 2469.5 ± 0.2 (2469.7) | 4043.6 ± 0.0 (4043.5)    | 807.4 ± 0.3 (806.9)  |
| 4      | 1529.5 ± 0.3 (1529.7) | 4197.8 ± 0.0 (4197.7)    | 127–17              |
| 5      | 2584.7 ± 0.2 (2584.8) | 4016.7 ± 0.3 (4016.5)    | 678.7 ± 0.3 (678.7)  |
| 6      | 1–44 with one internal cut | 4179.5 ± 0.2 (4179.7) | 2476.6 ± 0.2 (2476.7) |
| 7      | 3–51–2                | 14–23                    | 1–11–19–44          |
| 8      | 4436.5 ± 0.2 (4436.9) | 4835.2 ± 0.2 (4835.3)    | 4232.6 ± 0.2 (4232.6) |
| 9      | NA                    | 1–11–14–44              | NA                  |

esized that folding of fully reduced SMB under redox control in aqueous solution had produced, in contrast to the published assumption (34), a non-linear disulfide-bonding pattern identical to the one reported by Zhou et al. (18) for the crystal structure of rVN1–51-2 complexed with PAI-1.

Two powerful lines of evidence support this hypothesis. First, sSMB-2 and sSMB-4 were structurally identical. We incubated sSMB-2 and sSMB-4 with three different binary combinations of chymotrypsin, trypsin, and Glu-specific V8 protease and generated identical proteolytic “fingerprints” for the two SMB domains (Fig. 3). Results from mass spectroscopic analyses of all peptide fragments generated were consistent with enzyme specificities and the disulfide connectivity, Cys5–Cys21, Cys9–Cys39, Cys19–Cys32, and Cys25–Cys31 (Table 1). Second, sSMB-2 and sSMB-4 were functionally indistinguishable with respect to PAI-1 binding. We quantified the ability of sSMB-2 and sSMB-4 to interact with PAI-1 by using a previously established competition assay in which urea-activated vitronectin was immobilized on an optical sensor chip for kinetic analysis (34, 42). As shown in Fig. 4, both sSMB-2 and sSMB-4 strongly bound to PAI-1 in solution and inhibited the binding of PAI-1 to immobilized uVN in an identical fashion. Non-linear regression analysis yielded identical IC$_{50}$ values of 6.6 and 6.5 nM for sSMB-2 and sSMB-4, respectively.

In sharp contrast, the disulfide connectivity deduced by Peterson and co-workers (33) was hugely deleterious functionally. As shown in Fig. 4, sSMB-3 showed a drastically reduced binding affinity for PAI-1 compared with sSMB-2/sSMB-4, with an IC$_{50}$ value of 39.5 μM, at least 6000-fold higher than those of sSMB-2/sSMS-4. The linear and uncrossed pattern of
disulfides reported by Loskutoff and colleagues (32) fared a little better with an IC50 value of 10.8 \( M \), still 1600-fold higher than those of sSMB-2/sSMB-4. Thus, functionally, it is unlikely that the disulfide-bonding pattern in sSMB-1 and sSMB-3 represents the native topology in vitronectin.

The central question remains: what is the native disulfide topology in vitronectin? To tackle this controversial issue, we biochemically and functionally characterized nVN1–51 and uVN1–51 prepared by CNBr cleavage of nVN and uVN. As shown in Fig. 5, two different forms of VN1–51, one ending with homoserine (minor peak) and the other with homoserine lactone (major product), were generated, whose experimentally determined molecular masses of 5780.2 ± 0.4 and 5762.4 ± 0.1 Da were in agreement with the calculated values of 5780.4 and 5762.4 Da, respectively. We focused on the properties of the major cleavage product ending with homoserine lactone because of its quantity (homoserine lacton was rapidly converted to homoserine at basic pH where most characterizations were performed).

Both nVN1–51 and uVN1–51 had identical retention on analytical RP-HPLC (Fig. 5), and, when injected as a mixture, co-eluted as a single peak, suggesting that nVN1–51 and uVN1–51 shared the same disulfide topology. This was further verified by identical proteolytic fingerprints generated from combination...
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cleavages of the two SMB domain-containing peptides by chymotrypsin/trypsin and by trypsin/Glu-specific V8 protease (data not shown). Importantly, the proteolytic fingerprints of nVN1–51/uVN1–51 matched those of sSMB-2/sSMB-4, and the peptide fragments generated were consistent with the disulfide connectivity Cys5–Cys21, Cys9–Cys39, Cys19–Cys32, and Cys25–Cys31, as judged by mass spectroscopic analyses (Table 2). Furthermore, results obtained from surface plasmon resonance-based competition assays of nVN1–51 and uVN1–51 using PAI-1 and immobilized vitronectin fully corroborated the structural data. As shown in Fig. 6, nVN1–51 and uVN1–51 bound PAI-1 equally well, with almost identical IC50 values of 4.0 and 3.6 nM, respectively, slightly lower than the values previously determined for sSMB-2 and sSMB-4. The small difference in binding affinity for PAI-1 between sSMB-2/sSMB-4 and nVN1–51/uVN1–51 may result from the extra seven-residue RGD motif at the C terminus of VN1–51 making favorable contacts with PAI-1. Although disordered and invisible in the crystal structure of rVN1–51–2 complexed with PAI-1, the RGD motif is indeed positioned close to PAI-1 in the complex (18).

Taken together, our data unequivocally demonstrate that the native disulphide topology in the SMB domain of vitronectin is the same as that in sSMB2, sSMB4, and rVN1–51–2, i.e., Cys5–Cys21, Cys9–Cys39, Cys19–Cys32, and Cys25–Cys31. Furthermore, this crossed pattern of disulfide bonding, first identified by Read and colleagues structurally (18), represents the only functional topology in the adhesive glycoprotein. Finally, as was demonstrated by Loskutoff and co-workers (34), the correctly folded and fully active SMB domain can be readily obtained via oxidative folding of a fully reduced peptide in aqueous solution.

DISCUSSION

The studies in this report clarified a hotly debated issue with respect to the native disulfide topology in the SMB domain of human vitronectin, paving the way for a better understanding of the molecular recognition between vitronectin and PAI-1 in a variety of important biological processes. Our work, while providing direct biochemical and functional support to the structural findings by Read and colleagues, sharply contrasts the reports of Kamikubo et al. (32) and Horn et al. (33). Naturally, the biggest remaining question is how did the Loskutoff and the Peterson laboratories arrive at two different, non-native and inactive disulfide topologies for SMB when both groups clearly had correctly folded and fully functional molecules, rVN1–51–1 and nVN1–51–3, in the first place?

We strongly suspect that the partial reduction/S-alkylation procedures used by both teams to decode disulfide bonding in SMB caused unobserved disulfide-thiol exchanges under the experimental conditions used. The problem appears to be 2-fold. First, thiol-disulfide exchanges, known to occur much more rapidly at basic pH, cannot be eliminated entirely at pH 4.6 and pH 6.5, where limited disulfide reduction of rVN1–51–1 and nVN1–51–3 by tris(2-carboxyethyl)phosphine was conducted by Kamikubo et al. (32) and Horn et al., respectively (33). Second, we found that S-alkylation of free Cys residues by N-ethylmaleimide at pH 4.6 was an incomplete reaction itself and that singly and doubly derivatized SMB peptides co-eluted chromatographically. Consequently, contamination by partially alkylated peptides with free thiols can give rise to ambiguous disulfide assignments using protein sequencing. To push the alkylation reaction to completion, Horn et al. (33) maintained pH at 6.5 for hours after tris(2-carboxyethyl)phosphine reduction, which likely intensified disulfide shuffling. Using sSMB-2 with the native disulfide topology, we replicated the partial reduction/S-alkylation procedures used by Kamikubo et al. (32) and were indeed able to identify the presence of the disulfide bond between the two N-terminal cysteine residues (Cys5–Cys9) (data not shown). Notably, Cys5–Cys9 is the only common disulfide bond shared by the two differently

### TABLE 2

Peptides fragments from VN1–51 (Fig. 5) identified by simulated proteolysis and mass mapping

| Peak # | Chymotrypsin/Trypsin | Trypsin/V8 protease |
|--------|----------------------|---------------------|
| 1      | [9–13][36–42]        | [9–11][39–45]       |
| 2      | [9–13][36–45]        | [12–17]             |
| 3      | 1684.7 ± 2.7 (1685.9) | 679.8 ± 1.6 (678.7) |
| 4      | [3–6][18–27][29–35]  | [46–51] (homoserine) |
| 5      | 2469.3 ± 0.3 (2469.7) | 638.4 (638.6)       |
| 6      | [1–6][19–29][39–45]  | [4–6][19–23][24–36] |
| 7      | 2584.7 ± 0.2 (2584.8) | 2476.7 ± 0.4 (2476.7) |
| 8      | [46–49] and [6–19][28–39] | 0.4 (2476.7) |
| 9      | 463.5 (463.5) and 2747 ± 4 (2748.0) | 436.5 (436.5) |
| 10     | [1–45]               | [1–11][19–45]       |
| 11     | 5159.3 ± 0.1 (5159.7) | 4388.4 ± 0.6 (4388.8) |
| 12     | [1–13][19–45]        | [4–6][19–36]        |
| 13     | 4592.8 ± 0.4 (4593.1) | 2458.2 ± 0.2 (2458.7) |
| 14     | NA                   | [1–45]              |

FIGURE 6. Competition binding kinetics of 25 nM PAI-1, in the presence of varying concentrations of uVN1–51 or nVN1–51, on immobilized vitronectin analyzed by surface plasmon resonance spectroscopy. Non-linear regression analysis of the data yielded IC50 values of 3.6 and 4.0 nM for uVN1–51 and nVN1–51, respectively.
topologies reported by Kamikubo et al. (32) and Horn et al. (33), suggesting an inherent propensity of disulfide shuffling to form the Cys5–Cys9 bond in the molecule.

Interestingly, rVN1–51-1, which presumably contained the native disulfide topology, was characterized by NMR spectroscopy, showing a compact solution structure stabilized by packing in the core of four linearly arranged disulfides (42). A charge-hydrophobic patch encompassing residues from the third disulfide loop (DELC25SYQSC31), previously mapped to be the PAI-1-binding site by mutagenesis (24, 44), clustered on the surface of the molecule to form the putative binding interface (42). The overall solution structure of rVN1–51-1 was highly similar to the crystal structure of rVN1–51-2 except for the disulfide bonding pattern. Importantly, it was noted in the same study that several different disulfide arrangements, including the native topology, had comparable stabilization energies in conformational energy calculations and could all satisfy the same NMR constraints, thus raising serious doubts about the structural certainty of the linear and uncrossed pattern of disulfide bonding reported for rVN1–51-1 based on obviously ambiguous NMR data.

The solution structure of nVN1–51-3 was also studied by NMR spectroscopy (45). As was the case with rVN1–51-1, direct observation of the disulfide bridges was not possible based on nuclear Overhauser effect cross-peaks involving Cys residues. Consequently, the two disulfide bonds biochemically identified by Horn et al. (33) for nVN1–51-3, i.e. Cys5–Cys9 and Cys25–Cys39, were used as constraints in simulated annealing calculations, yielded a low-resolution model significantly different from the structures of rVN1–51-1 and rVN1–51-2 (18, 42). The structural studies of nVN1–51-3 by NMR spectroscopy, in retrospect, appeared seriously flawed due to the use of the incorrect disulfide constraints.

Two important lessons can be learned from the studies of the SMB domain of human vitronectin. First, limited tris(2-carboxyethyl)phosphine reduction/S-alkylation, while a widely used biochemical procedure for decoding disulfide bonding pattern in peptides and proteins, can cause, under certain conditions, thiol-disulfide shuffling not easily detected by conventional analytical techniques, perhaps a far more serious problem than commonly led to believe by the existing literature. To completely eliminate the possibility of disulfide scrambling, we avoided using any reduction step in dissecting the disulfide connectivities for synthetic SMB domains. Second, since disulfide bonds are often difficult to observe directly from NMR data, to minimize ambiguity and subjectivity, extra care ought to be exercised when “ruling in” or “ruling out” structural constraints for energy calculations. X-ray crystallography clearly enjoys the upper hand in this particular case.

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