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Characterization of the Denaturation and Renaturation of Human Plasma Vitronectin

II. INVESTIGATION INTO THE MECHANISM OF FORMATION OF MULTIMERS*

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Unfolding and refolding of plasma vitronectin appear irreversible under near physiological conditions, with rearrangements of disulfides and self-association to a multimeric form observed as prominent structural alterations which accompany denaturation. A mechanism for the folding reactions of vitronectin has been proposed (Zhuang, P., Blackburn, M. N., and Peterson, C. B. (1996) *J. Biol. Chem.* 270, 14323–14332) in which vitronectin acquires a partially folded intermediate structure which is highly prone to oligomerize into a multimeric form. Strongly oxidizing conditions adopted for refolding from urea were effective at preventing disulfide rearrangement which disrupts distal disulfides near the C terminus of the protein. Prohibiting disulfide rearrangement under these conditions, however, was not sufficient to achieve reversibility in folding. In contrast, variations in the ionic strength of the refolding medium affect the partitioning of species so that refolded monomers are obtained at high ionic strength, and self-association is precluded. The effects of ionic strength on the partially folded intermediate in the vitronectin folding pathway appear to favor intramolecular hydrophobic collapse to form a stable hydrophobic core for the monomer *versus* intermolecular hydrophobic interactions which stabilize multimeric vitronectin. Although both ionic and hydrophobic interactions presumably contribute to subunit interfaces within the multimer, the basic heparin-binding region near the C terminus of the protein does not provide binding interactions which are important for self-association of vitronectin.

Vitronectin is a multi-functional protein which is found in a plasma form, which circulates in the bloodstream at high concentrations, and in tissue forms, which are present within platelets, deposited into the extracellular matrix of endothelial cells, or localized to other extravascular sites. The multiple functions of vitronectin are a manifestation of specific interac-

tions with many other macromolecules, including the integrin class of receptors on cells, the inactive antithrombin-thrombin complex, the terminal membrane attack complex of complement, collagen and other extracellular matrix components, plasminogen activator inhibitor type 1, and the mucopolysaccharide, heparin. Binding of these target ligands appears to be modulated through alterations in the conformation of vitronectin, an observation that was originally noted for interactions with heparin (1, 2) and subsequently extended to other physiologically relevant macromolecules (reviewed in Refs. 3–6).

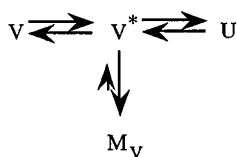
Monoclonal antibodies have been useful to demonstrate the conformational lability of vitronectin (7–10), which has been characterized using chemical or thermal denaturation methods to mimic the more physiologically relevant stimuli for structural rearrangement. Justification for this approach came early in the evaluation of structural changes in vitronectin, with the demonstration by Tomasini and Mosher (7) using monoclonal antibody 8E6 that conformational alterations induced upon treatment with urea were similar to those observed upon association of vitronectin with the antithrombin-thrombin complex. Comparison between plasma vitronectin and tissue-associated forms is by no means complete, but several recent studies have indicated that non-circulating forms of the protein exist in the structurally-altered form, detected by several conformation-specific monoclonal antibodies, including the 8E6 prototype (11).

The structural basis for the conformational lability of vitronectin is incompletely understood. Alterations in the conformation of vitronectin induced by chemical denaturation are notably accompanied by self-association to form vitronectin multimers *in vitro* (9, 11–13). In a companion paper (12), spectroscopic methods were used to characterize structural changes which accompany unfolding and subsequent refolding of purified plasma vitronectin. Unfolding and refolding curves generated by varying concentrations of chemical denaturants were non-coincident, although precautions were taken to ensure complete unfolding prior to renaturation. Refolding from high denaturant concentrations was accompanied by association to a multimeric form of vitronectin with an average molecular weight of 420,000. The hysteresis observed is indicative of different pathways for unfolding *versus* refolding and/or the conversion of vitronectin from one stable form to a second, differently folded structure.

Scheme 1 can be used to describe the folding and self-association of vitronectin. V represents the native, monomeric form of vitronectin, V[†] an intermediate, partially folded form of the protein, U the fully unfolded form, and M_v, the multimeric form of vitronectin. Folding of vitronectin appears to be regulated by partitioning of folding intermediates toward either of two conformations, one that exists as a stable monomer and another

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SCHEME 1

that associates into a multimeric form. Partial unfolding of vitronectin results in a pronounced tendency for the protein to self-associate, and refolding of the protein following chemical or thermal denaturation is invariably accompanied by oligomerization to the multimeric form at physiological ionic strengths. This study expands on the work which germinated Scheme I to consider the following questions. Does intramolecular disulfide rearrangement stabilize an alternative fold with a tendency to multimerize? Are there conditions which favor refolding of vitronectin to a monomeric form without self-association as a prominent side reaction? What effect do charged biomolecules or salts have on the distribution of refolded vitronectin between monomeric and multimeric forms? What contributions do ionic and hydrophobic interactions make to the process of self-association of vitronectin? Does the heparin-binding region of vitronectin contribute intermolecular interactions which participate in oligomerization to the multimeric form? Analytical and immunochemical approaches have been used to address these questions and gain insight into the mechanism by which multimeric vitronectin is produced.

EXPERIMENTAL PROCEDURES

Materials—Vitronectin was purified by a modification of the original procedure of Dahlback and Podack (14), essentially as described by Bittorf *et al.* (9). The modifications of Bittorf *et al.* (9) include addition of DTNB¹ during the preparation to block available sulfhydryls on vitronectin which were originally reported in the Dahlback and Podack (14) purification. Note that Bittorf *et al.* (9) indicate that there is not clear evidence that DTNB is maintained on vitronectin throughout the protein preparation, since reduction of the purified vitronectin sample with dithiothreitol was not accompanied by release of the thionitrobenzoate anion (9). Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of β -mercaptoethanol (15). A molecular weight for the protein of 72,000 and an extinction coefficient of 1.02 ml·mg⁻¹·cm⁻¹ were used for purified vitronectin (12). The number of free sulfhydryls in purified vitronectin was determined under native, denaturing, or reducing conditions using DTNB (16). Multimeric vitronectin was prepared by treatment of protein in 8 M urea at room temperature for 2 h, with subsequent removal of denaturant by dialysis into standard phosphate buffer (0.1 M sodium phosphate, pH 7.5, containing 0.15 M NaCl and 1 mM EDTA). Heat-denatured vitronectin was prepared by incubating the protein in phosphate-buffered saline (40 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl) or standard phosphate buffer at 55 °C for 1–2 h and subsequent cooling on ice (10, 11).

The 18-residue peptide which corresponds to amino acids 346 to 363 in vitronectin, LAKKQRFRRHRNRKGYRSQ, was purchased from Chiron Mimotopes. The peptide from the commercial vendor was greater than 85% pure and was used without further purification. The amino acid sequence of the peptide was confirmed by amino acid analysis and NMR characterization, and heparin binding activity was established by fluorescence methods during the initial structural work on the peptide.² Ultrapure guanidine hydrochloride was purchased from Life Technologies, Inc. Urea was a product of ICN Biomedical. ¹²⁵I-labeled sheep anti-mouse IgG was from Amersham; biotin-conjugated goat anti-

mouse IgG, streptavidin-alkaline phosphatase conjugate, and *p*-nitrophenyl phosphate were from Zymed. All other chemicals were of reagent grade quality.

Immunochemical Analyses—Monoclonal antibodies (mAbs) 153 and 1244 were obtained by standard hybridoma techniques using denatured vitronectin as the immunogen (17), and the hybridoma clone producing mAb 8E6 was kindly provided by Dr. Deane Mosher (University of Wisconsin). MABs were produced as ascites, and the resulting IgG was purified by using protein A-agarose (mAbs 153 and 1244) or by the caprylic acid method (mAb 8E6) (18).

Native PAGE was performed using a 3% polyacrylamide stacking gel and 7.5% polyacrylamide resolving gel run in the Laemmli buffer system (15) without SDS. For Western blotting, proteins in the gel were transferred onto nitrocellulose membranes and analyzed using mAb 1244 as described (17). Binding of the anti-vitronectin antibodies was detected using ¹²⁵I-labeled sheep anti-mouse IgG.

Conformational changes in vitronectin were quantified by a competitive ELISA method principally as described (7, 9, 10), in which mAb binding is partitioned between denatured vitronectin coated on microtiter wells and competing vitronectin in solution. Bound IgG was detected with biotin-conjugated goat anti-mouse IgG, followed by streptavidin-alkaline phosphatase conjugate, and the substrate *p*-nitrophenyl phosphate in a final step. In the concentration range used for the experiments, the addition of amino acids or NaCl did not interfere with the binding of mAbs to immobilized vitronectin; neither did the added reagents remove coated vitronectin from the microtiter wells.

Variation of Redox Conditions for Unfolding—Vitronectin at a concentration of 5 μ M was treated overnight with 5 M urea, a concentration of denaturant very near the midpoint in the unfolding curve (12) which has been shown to lead to rearrangement of disulfides (9). Various combinations of oxidized and reduced glutathione were added along with 5 M urea to individual vitronectin samples to vary the redox potential during unfolding from strongly oxidizing to strongly reducing potentials. Urea solutions were made fresh for each experiment. Redox potential was calculated from the Nernst equation as follows,

$$E_n = E^\circ + (2.303RT/nF) \times \log([GSSG]/[GS^-]^2) \quad (\text{Eq. 1})$$

in which E° , the standard redox potential for glutathione is -0.24 V (19), n equals 2, R is the gas constant, 8.314 J·K⁻¹·mol⁻¹, and F is Faraday's constant, 97,485 C·mol⁻¹. Samples were analyzed for disulfide rearrangement on 10% SDS-PAGE gels in which samples were prepared in loading buffer without added reducing agents (*i.e.* non-reducing SDS-PAGE). Electrophoresis was performed according to Laemmli (15).

Analytical Gel Filtration Analyses—Native and multimeric proteins were analyzed by HPLC using a Beckman ultraspherogel SEC2000 column (7.5 mm x 30 cm) at a flow rate of 1 ml·min⁻¹ in standard phosphate buffer. Samples were generally 1 mg·ml⁻¹ in concentration, and 20- μ l samples were analyzed per run. Elution of protein from the column was monitored by absorbance at 280 nm. Retention times characteristic of monomeric and multimeric vitronectin were 6.8 \pm 0.1 and 5.6 \pm 0.1 min, respectively.

In a similar fashion, analytical gel filtration was performed in some experiments using a Superose-12 column (1 x 30 cm; 23.7-ml bed volume) with a Pharmacia FPLC system. Vitronectin (1 mg·ml⁻¹) was denatured for 16 h in 5 M urea in standard phosphate buffer, and the same denaturant solution was used for chromatography at flow rates of 0.5 ml·min⁻¹. Duplicate experiments were performed in 5 M urea in standard phosphate buffer plus 10 mM GSSG. Initial sample volume applied to the column was 0.5 ml or less. Protein elution patterns were detected by monitoring absorbance at 280 nm.

Fluorescence Analysis of Chemical Denaturation of Vitronectin—Unfolding and refolding of vitronectin were induced by GdnHCl and monitored by changes in intrinsic protein fluorescence, as described (12). Buffer used for the unfolding and refolding experiments was standard phosphate buffer containing 0.04% Tween 20. For experiments to test the effects of limiting disulfide rearrangement, the same buffer was used with the addition of 10 mM GSSG. Aliquots from a concentrated protein stock were diluted to a final concentration of 0.5 μ M protein in varying concentrations of denaturant. Samples were incubated at room temperature overnight to ensure complete unfolding before the measurements were made. For refolding studies, vitronectin was first unfolded in 7 M GdnHCl in standard phosphate buffer plus 10 mM GSSG for 16 h. Aliquots of vitronectin in 7 M GdnHCl were then diluted 10-fold into mixtures of buffer and GdnHCl solution, both containing 10 mM GSSG, and again incubated overnight. GdnHCl concentrations were

¹ The abbreviations used are: DTNB, dithionitrobenzoate; GdnHCl, guanidine hydrochloride; PAGE, polyacrylamide gel electrophoresis; GSH and GSSG, the reduced and oxidized forms of glutathione, respectively; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; Cpn60 and Cpn70, chaperonins 60 and 70 (sometimes referred to as Hsp60 and Hsp70); HPLC, high performance liquid chromatography.

² J. A. Lamerdin, C. B. Peterson, and E. H. Serpersu, submitted for publication.

TABLE I
Determination of free sulphydryls in native and denatured vitronectin

Experimental conditions	(mol TNB)/(mol protein) ^a
Buffer only	0.08
	0.00
Denaturing ^b	2.00
Denaturing and reducing ^c	12.87
	11.28

^a Mol of thionitrobenzoate anion released per mol of protein. Values listed represent separate determinations on replicate samples.

^b Vitronectin was unfolded in 6 M urea for 4 h as described under "Experimental Procedures."

^c Vitronectin was unfolded in 6 M urea with 10 mM dithiothreitol as described under "Experimental Procedures."

calculated using equations relating refractive indexes to concentrations (20), and refractive indexes were measured using a Zeiss refractometer.

RESULTS

Adjustments in Redox Conditions Limit Disulfide Rearrangements Which Are Observed upon Urea Treatment of Vitronectin—Although vitronectin has been proposed to contain cysteines in a combination of oxidized disulfides and reduced -SH forms, the number of free sulphydryls in vitronectin has been disputed (9, 14, 21). In order to quantitate free sulphydryls in the purified protein used for these experiments, vitronectin was treated with DTNB under native and denaturing conditions. Native vitronectin was found to have essentially no available free sulphydryls, consistent with previous observations (9), whereas vitronectin denatured in 6 M urea contained 2 sulphydryls (Table I). These results indicate that vitronectin contains a mixture of oxidized and reduced sulfur groups, and that the free sulphydryls are buried in the native protein. It is not until vitronectin is denatured that these sulphydryls are exposed and available to promote disulfide rearrangement in the protein. DTNB reactions on fully denatured and reduced vitronectin gave an average of 12.1 -SH groups per protein molecule, in fair agreement with the 14 sulphydryls expected from the known sequence of the protein (22).

Under near physiological buffer conditions, refolding of vitronectin from either a chemically-denatured or heat-denatured state proceeds via an intermediate which is partially folded and highly prone to self-associate into a multimeric form. It was noted early in the work on urea denaturation of vitronectin that disulfide rearrangement occurs upon chemical treatment (7, 8). This observation was made from SDS-PAGE run under non-reducing conditions in which disulfide rearrangement disrupts interchain cross-links between the 62,000 and 10,000 polypeptide chains of vitronectin and two polypeptides are observed on gels rather than a single 72,000 species. Although disulfide rearrangement could result in intermolecular cross-linking of constituent proteins within multimeric vitronectin, only a minority of chains within the multimer are linked by disulfide bonds (12) so that the multimer is not uniformly cross-linked.

Although intermolecular disulfides are not uniform within the multimer and thus do not fully account for assembly of the multimer, the possibility exists that intramolecular disulfide rearrangement stabilizes an alternative conformation of vitronectin which is prone to self-associate upon refolding. To test this hypothesis, variations in redox potential during unfolding and refolding were used to define conditions which prohibit disulfide rearrangement. Fig. 1 shows the effects of varying redox potential with GSH and GSSG during unfolding. A clear trend is observed in which appearance of the 62,000 chain upon release of the 10,000 fragment is minimized as unfolding reactions are conducted under strongly oxidizing conditions. Under the most highly oxidizing conditions tested,

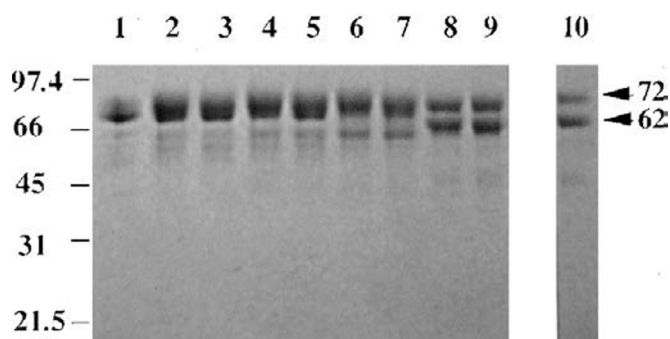


FIG. 1. Variations in redox potential affect rearrangement of disulfides in vitronectin induced by urea treatment. Vitronectin was unfolded in 5 M urea for 16 h in the absence or presence of added glutathione. Gel-loading buffer (50 mM Tris, pH 6.8, containing 2% SDS, 0.1% bromophenol blue, and 20% glycerol) without reducing agents was added in an equal volume to the samples. The samples were boiled for 5 min before loading onto the 10% polyacrylamide gel. Following electrophoretic separation, proteins were visualized by staining with Coomassie Brilliant Blue R-250. Lane 1 contains untreated vitronectin with no denaturant or glutathione added, and lane 10 represents vitronectin which has been denatured in urea without addition of glutathione. Concentrations of GSSG and GSH were adjusted to vary the redox potential as follows: lane 2, 10 mM GSSG; lane 3, 10 mM GSSG, 0.01 mM GSH; lane 4, 10 mM GSSG, 0.1 mM GSH; lane 5, 5 mM GSSG, 0.01 mM GSH; lane 6, 5 mM GSSG, 0.1 mM GSH; lane 7, 1 mM GSSG and 0.1 mM GSH; lane 8, 1 mM GSSG and 1 mM GSH. Redox potentials for unfolding solutions in lanes 2-8 are 0, 0, -60, -10, -70, -90, and -150 mV, respectively. The sample in lane 10 is native vitronectin which was prepared in gel-loading buffer containing 100 mM dithiothreitol as a control for migration of the 72,000 and 62,000 forms of vitronectin upon disruption of the disulfide-bonded (62,000 + 10,000) form.

disulfide rearrangement appears to be prohibited. Exposed sulphydryls are prevented from catalyzing intramolecular disulfide rearrangement within vitronectin due to the great excess of glutathione, which provides a small molecular weight substrate for disulfide exchange and formation of mixed disulfides.

The results of the SDS-PAGE were confirmed by using analytical gel filtration to test for dissociation of the disulfide cross-linked two-chain form of vitronectin to separated 62,000 and 10,000 chains upon denaturation in 5 M urea plus or minus 10 mM GSSG (Fig. 2). Under normal denaturation conditions without added glutathione, urea treatment results in disruption of the covalent disulfide cross-link between the 62,000 and 10,000 chains of vitronectin, so a late eluting peak corresponding to the 10,000 polypeptide is clearly seen on analytical gel filtration. In contrast, addition of oxidized glutathione to the denaturation mixture prevented the disulfide rearrangement which leads to release of the 10,000 C-terminal fragment, and a single peak corresponding to a 72,000 species was observed in analytical gel filtration under these conditions. Within the limitations of these assays, it appears that highly oxidizing conditions conferred during unfolding of vitronectin prohibit intra- or intermolecular disulfide rearrangement.

Irreversibility of Unfolding/Refolding of Vitronectin Is Not Solely due to Disulfide Rearrangement—Hysteresis between unfolding and refolding curves was noted in the absence of GSSG and can be attributed either to the possibility that the two processes proceed via different pathways or to the prospect of a different fold for native compared to denatured/renatured vitronectin. Since oxidizing conditions prevented disulfide rearrangement, GSSG was included in the denaturation mixtures, and unfolding and refolding behavior were tested as a function of GdnHCl by monitoring changes in intrinsic fluorescence of the protein (12). The results shown in Fig. 3 exhibit hysteresis between unfolding and refolding curves, demonstrating that inclusion of GSSG in the reactions to stem disul-

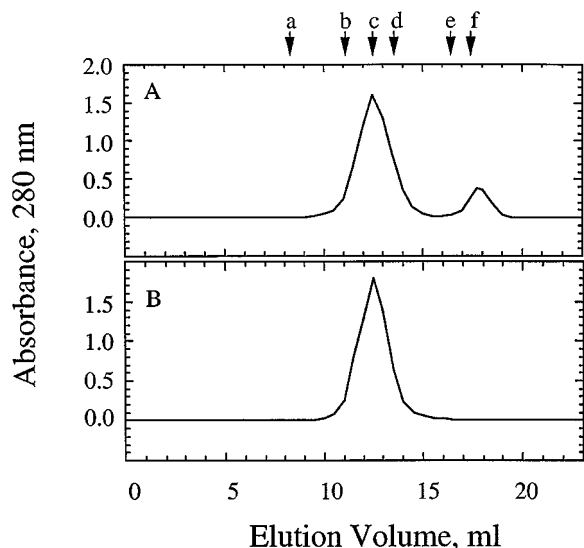


FIG. 2. Analytical gel filtration indicates that strongly oxidizing conditions prevent release of the 10,000 C-terminal fragment via disulfide rearrangement. Vitronectin (1 mg·ml⁻¹; 0.2 ml) was denatured overnight in 5 M urea (panel A) or 5 M urea containing 10 mM GSSG (panel B). The sample was subsequently chromatographed by HPLC at room temperature on a Superose 12 column equilibrated in the urea solution with or without GSSG as appropriate for the two samples. The elution profile was monitored by absorbance at 280 nm, shown on the y axis. Arrows mark the elution volume for molecular weight standards: a, ferritin ($M_r = 440,000$); b, catalase ($M_r = 232,000$); c, bovine serum albumin ($M_r = 67,000$); d, ovalbumin ($M_r = 43,000$); e, chymotrypsinogen A ($M_r = 25,000$); f, ribonuclease A ($M_r = 13,700$).

fide rearrangement does not lead to reversible unfolding/refolding behavior. Midpoints for the denaturation curves and intensity-averaged wavelengths corresponding to folded and unfolded protein are summarized in Table II. Values are also listed in Table II for GdnHCl unfolding/refolding curves generated without GSSG (12); parameters describing unfolding and refolding of vitronectin in GdnHCl are virtually indistinguishable regardless of addition of GSSG.

Charged Amino Acids Influence the Partitioning between Monomers and Multimers upon Refolding of Heat-denatured Vitronectin—Comparison of unfolding curves for vitronectin in GdnHCl and urea indicated differences in the cooperativity of denaturation (*i.e.* different slopes in the transition region in unfolding curves), in which unfolding in urea occurred over a much broader concentration range than in GdnHCl (12). Although both denaturants are chaotropes, GdnHCl is also a salt. Differences in the nature of the denaturants might account for the differences in the unfolding curves, with the possibility that ionic effects of GdnHCl may affect the folding of vitronectin. Postulated influences of ionic strength on folding of the protein prompted evaluation of effects of ionic compounds, including charged amino acids and NaCl, on denaturation and renaturation.

Native vitronectin was denatured by heat treatment in the presence or absence of selected amino acids, and the extent of the resulting multimerization was evaluated by native PAGE (Fig. 4). As has been previously observed (11), heat treatment induced the formation of high molecular weight multimers that only partially entered the separating gel and thus were easily distinguished from native vitronectin which migrates as two or three predominant bands in native gels.³ Upon heat treatment

³ Native vitronectin appears polydisperse on these native gels due to reasons that are incompletely understood. It has been our observation that monomeric vitronectin which is determined to be homogeneous with respect to molecular weight upon equilibrium analytical centrifuga-

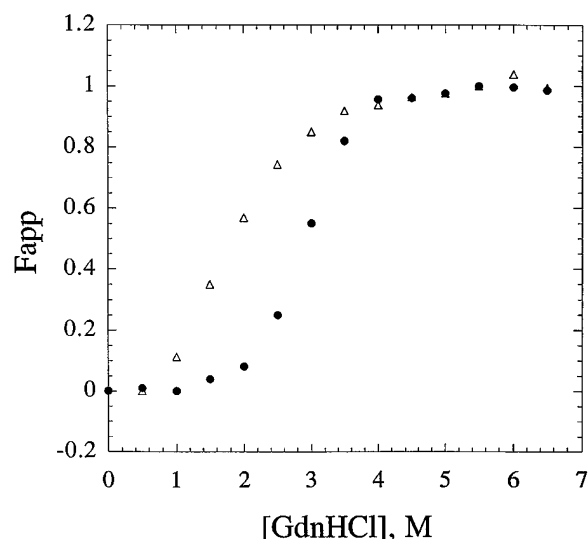


FIG. 3. GdnHCl unfolding/refolding curves for vitronectin in the presence of GSSG. Unfolding of purified native vitronectin (closed circles) in varying concentrations of GdnHCl with a constant concentration of 10 mM GSSG was monitored by average tryptophan emission wavelength, as described under "Experimental Procedures." For refolding data (open triangles), proteins were initially denatured in 7 M GdnHCl plus 10 mM GSSG, and refolding was subsequently initiated by diluting a 5 μ M protein stock 10-fold in mixtures of buffer and denaturant to give final GdnHCl concentrations from 0.7–7 M GdnHCl with GSSG. Following overnight incubation of vitronectin at each concentration of denaturant in buffer, emission spectra were recorded from 300 to 450 nm using an excitation wavelength of 290 nm. Data are expressed as F_{app} , the apparent fraction unfolded, by normalization of individual readings to λ_F and λ_U as given in Table II, to facilitate comparison between unfolding and refolding data.

of vitronectin in the presence of charged amino acids, self-association of vitronectin was not observed as a prominent side reaction accompanying refolding, and the electrophoretic mobilities of the resultant vitronectin preparations were relatively unchanged in comparison to native, untreated vitronectin. In contrast, the non-polar amino acid glycine had no effect on the redistribution toward a monomeric form upon refolding, and multimers were readily formed. The prevention of self-association of vitronectin by charged amino acids was not restricted to a purified protein system, but was also apparent in plasma or serum (data not shown). Control experiments were used to demonstrate that the amino acids had no effect on the migration of multimeric vitronectin if added subsequent to heat treatment and cooling (data not shown).

Significantly, the monomeric vitronectin obtained after refolding at high concentrations of arginine appeared stable as a monomer, even after dialysis to remove the charged amino acid (Fig. 5, panel A). Thus, it is the process of refolding, rather than the stability of the refolded monomer, that is affected by L-arginine. The refolding pathway toward a monomer is preferred *versus* self-association to a multimer when arginine is present, and the final folded protein is stable in the absence of added charged amino acids.

gation (12) migrates as at least two bands on native polyacrylamide gels. The fact that purified plasma vitronectin exists as a mixture of single-chain and two-chain, disulfide cross-linked forms may contribute to the observed homogeneity. Also, it is not known how much potential variability in glycosylation contributes to anomalies in electrophoretic migration of vitronectin in this gel system. Furthermore, it remains unclear to what extent the formation of high local vitronectin concentrations at the interface between stacking and separating phases of the gel may facilitate partial multimerization during electrophoresis. Fractionation of the vitronectin used for these gels by SDS-PAGE under non-reducing conditions revealed that 5% or less of the vitronectin preparation existed as disulfide-linked oligomers.

TABLE II
Behavior of native and multimeric vitronectin in chemical denaturation/renaturation experiments

	Vitronectin sample ^a	Denaturant	Midpoint (M)	$\bar{\lambda}_F^b$	$\bar{\lambda}_U^b$
Unfolding conditions	0.5 μ M native ^c	GdnHCl	3.2	349.7	363.8
	0.5 μ M native	GdnHCl/GSSG ^d	3.0	350.0	363.5
	0.5 μ M multimer ^c	GdnHCl	2.2	352.2	360.0
	0.5 μ M heat-treated ^c	GdnHCl	2.6	351.9	361.9
	0.5 μ M heat-treated/refolded in 1 M NaCl	GdnHCl	3.0	351.1	363.0
	0.5 μ M urea treated/refolded in 1 M NaCl	GdnHCl	3.0	349.5	362.2
Refolding conditions ^e	0.5 μ M native ^c	GdnHCl	2.1	352.1	360.7
	0.5 μ M native	GdnHCl/GSSG ^d	2.0	352.0	360.5

^a Samples are labeled according to the state of the starting material for the experiment as: native, corresponding to the untreated monomeric protein; multimer, corresponding to urea treated protein which is subsequently renatured at physiological ionic strength; and heat treated, corresponding to the protein heated to 55 °C and cooled at physiological ionic strength as described under "Experimental Procedures," heat-treated/refolded protein has been heated at 55 °C and cooled to room temperature in buffer containing 1 M NaCl; urea-treated/refolded protein has been denatured overnight in 8 M urea followed by dialysis into 0.1 M sodium phosphate, pH 7.5, containing 1 mM EDTA and 1 M NaCl.

^b The intensity averaged wavelength corresponding to folded, F, or unfolded, U, protein. Error limits on these measurements are ± 0.2 nm.

^c Unfolding parameters taken from Zhuang *et al.* (12).

^d Unfolding or refolding experiments were conducted by varying the GdnHCl concentration in standard phosphate buffer containing 10 mM GSSG.

^e For refolding experiments, protein was initially denatured in 7 M GdnHCl for 16 h. The vitronectin sample types listed thus correspond to the starting material which was initially denatured prior to the refolding experiment.

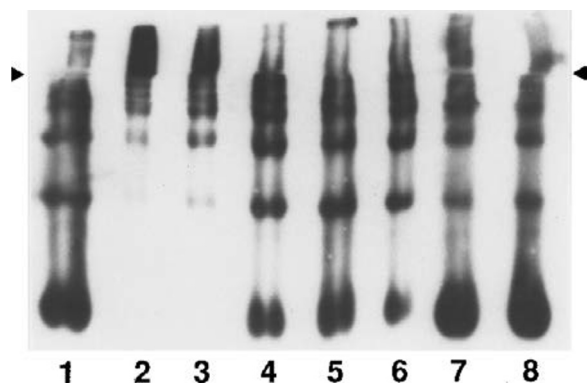


FIG. 4. Prevention of vitronectin self-association by charged amino acids. Native vitronectin was heat treated at 55 °C for 1 h at pH 7.4 and fractionated by native PAGE. Immunoblotting was performed using mAb 1244 followed by ¹²⁵I-labeled sheep anti-mouse IgG. The interface between stacking and resolving gel is indicated by the arrowheads. The untreated native protein is shown in lane 1. Heat treatment of vitronectin was performed in phosphate-buffered saline (lane 2), 500 mM glycine (lane 3), 500 mM L-lysine (lane 4), 500 mM L-arginine (lane 5), 500 mM L-histidine (lane 6), 500 mM L-aspartic acid (lane 7), or 500 mM L-glutamic acid (lane 8).

Refolding of Vitronectin to a Monomeric versus Multimeric Form Is a Function of Ionic Strength—Preferential refolding to a monomeric form was dose-dependent with respect to the charged amino acid concentration (Fig. 5, panel B, and Fig. 7, inset). A significant alteration in the bias away from self-association toward a preferred monomeric form was observed at concentrations of arginine greater than 100 mM. The effect of charged amino acids was compared to that of NaCl, and dose-response experiments with NaCl demonstrate that NaCl was as effective as the charged amino acids in preventing self-association of vitronectin (Fig. 5, panel B). The effects of charged amino acids thus appear most likely to be manifestations of an ionic strength effect similar to that observed with NaCl. Indeed the ionic strength of a solution of 0.5 M L-arginine, pH 7.4, was calculated to be equivalent to that for a 1.0 M NaCl solution.⁴

⁴ Ionic strength was calculated as: $\mu = 1/2\sum(m_i z_i^2)$, in which m_i represents the molar concentration, and z_i represents the charge on the species, i . pK values of 2.3, 9.6, and 12.0 were used to calculate the effective ion concentrations at pH 7.4 for the carboxyl, α -amino, and guanidino side chain. The pH of the arginine solution was adjusted with measured aliquots from standardized HCl solutions so that the contri-

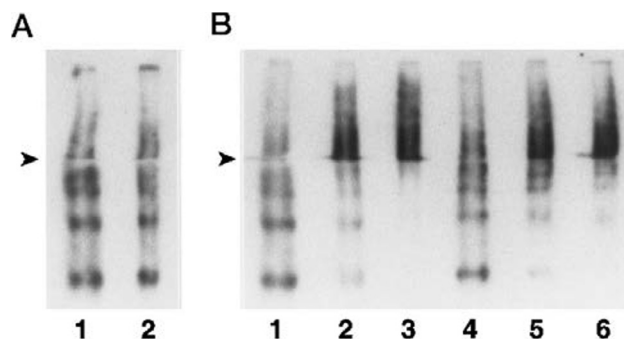


FIG. 5. Variations in ionic strength have similar effects to varying concentrations of L-arginine in preventing multimerization of vitronectin. Panel A, native vitronectin was heat-treated in the presence of 500 mM L-arginine and analyzed by native PAGE (lane 1). Following heat treatment with arginine, a duplicate sample was dialyzed into phosphate-buffered saline and analyzed on the gel (lane 2). Panel B, native vitronectin was heat-treated in the presence of 500 mM L-arginine (lane 1), 250 mM L-arginine (lane 2), 125 mM L-arginine (lane 3), or 500 mM NaCl (lane 4), 250 mM NaCl (lane 5), or 125 mM NaCl (lane 6). For both panels, the vitronectin samples were fractionated by native PAGE followed by immunoblotting using mAb 1244 as in Fig. 4. The interface between stacking and resolving phases of the gel is indicated by the arrowheads.

Because of the difficulties in a straightforward interpretation of the electrophoretic migration patterns of native vitronectin on native gels,³ a more analytical approach was sought to characterize the size and quantitate the molecular species achieved after refolding vitronectin under various conditions. Analytical gel filtration by HPLC has proven to be an ideal method to characterize the conditions which lead to multimerization, as the multimer and monomer are adequately separated and quantitated by the system. The effect of variations in NaCl concentration on the distribution of refolded vitronectin species between multimers and monomers is shown in Fig. 6. As had been previously established, refolding at physiological ionic strengths yields predominantly a multimeric form of vitronectin. However, increasing NaCl concentration from 0.15 to 1.5 M yielded essentially all monomeric vitronectin upon refolding.

tribution of the counterion to solution ionic strength could be directly quantitated. The ionic strength of heparin ($M_r \sim 6000$) was calculated using an average charge density of 3 negative charges per disaccharide repeat at near neutral pH values and an approximate molecular weight of 432 for a disaccharide unit.

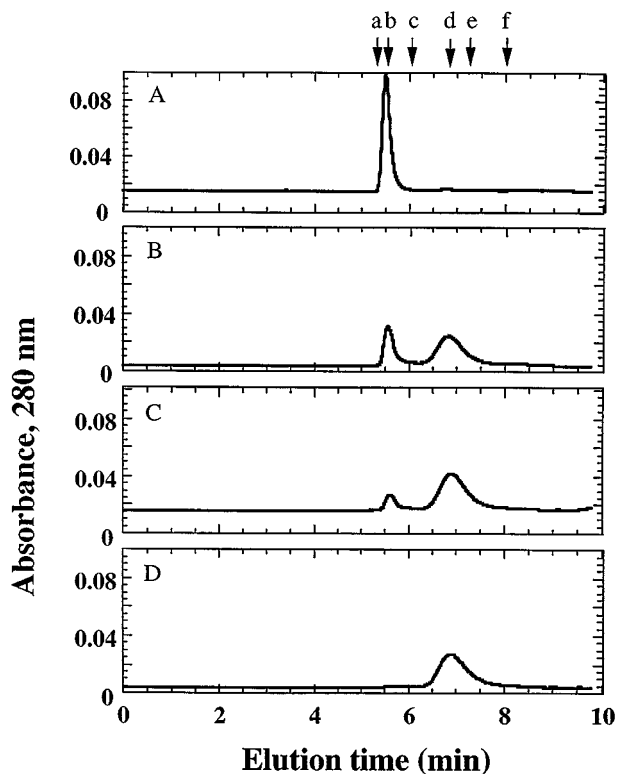


FIG. 6. Effects of ionic strength on the partitioning between monomer and multimer upon renaturation of vitronectin. Vitronectin ($1 \text{ mg}\cdot\text{ml}^{-1}$) was heat treated for 1 h in 0.1 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and 0.15 M NaCl (panel A), 0.5 M NaCl (panel B), 1 M NaCl (panel C), or 1.5 M NaCl (panel D). Samples were cooled to room temperature and $20\text{-}\mu\text{l}$ volumes were injected onto the SEC2000 column as described under "Experimental Procedures." Elution was monitored at 280 nm and is plotted as a function of time following sample injection. Arrows mark the elution times for molecular weight standards: a, blue dextran 2000 ($M_r = 2 \times 10^6$); b, ferritin ($M_r = 440,000$); c, catalase ($M_r = 232,000$); d, bovine serum albumin ($M_r = 67,000$); e, ovalbumin ($M_r = 43,000$); f, chymotrypsinogen A ($M_r = 25,000$).

Results from the HPLC analyses are summarized in Table III. As shown in the table, the effects of NaCl or L-arginine are not restricted to refolding of vitronectin following heat-induced denaturation, as unfolding/refolding experiments performed using urea with high concentrations of NaCl or L-arginine result in preferential folding to a monomeric species. Furthermore, the analytical HPLC method clearly substantiates the result initially observed on gels, that removal of salt following refolding to a monomer is not conducive to self-association. That is, once a monomeric form is achieved upon refolding at high ionic strength, the salt is no longer necessary to stabilize the monomer and can be removed by dialysis with no effects on the molecular size or aggregation state of vitronectin. As had been initially observed on native gels, addition of 1 M NaCl does not dissociate multimeric vitronectin into constituent monomers, even at long time periods. Also, heat treatment of the multimer at 55°C for 1 h in the presence of added NaCl in high concentrations does not reverse the effects of self-association to yield a monomeric form of vitronectin.

Conformationally Sensitive MAbs Indicate a Native-like Structure for Vitronectin Refolded in Vitro at High Ionic Strength—Multimerization of vitronectin is closely linked to structural alterations which can be detected using conformationally sensitive mAbs (9–11). The effect of L-arginine on the heat-induced expression of conformationally sensitive epitopes was determined in competitive ELISA experiments (Fig. 7). Vitronectin was heat-treated in the presence of varying concen-

trations of L-arginine and the resulting vitronectin samples were assessed for their ability to compete for binding of mAbs with immobilized, denatured vitronectin. Arginine prevented the expression of the conformationally-sensitive mAb 8E6 epitope, localized to the connecting region/first hemopexin repeat, in a dose-dependent manner similar to the dose-dependent behavior observed on native gels (see Fig. 7, inset). Similarly, another charged amino acid, L-glutamic acid, was observed to interfere with heat-induced expression of the 8E6 epitope; effects of selected amino acids on epitope expression following heat-denaturation are summarized in Table IV. Thermally-induced expression of another conformationally sensitive epitope, located in the N-terminal somatomedin B domain (mAb 153), was also prevented by charged amino acids, whereas expression of either epitope was not influenced by the presence of L-glycine during denaturation/renaturation. The inclusion of high concentrations of NaCl during thermal denaturation was shown to prevent expression of these epitopes in a similar fashion to the charged amino acids (Table IV), again supporting the notion that the charged amino acids influence the distribution of refolded species to monomeric and multimeric forms via an ionic strength effect. It should be noted that in control experiments, polar amino acids added to multimeric vitronectin after heat denaturation and cooling did not affect the ability of the multimer to bind mAbs in the competitive ELISA (data not shown).

Monomers Recovered from Thermal or Chemical Denaturation/Renaturation at High Ionic Strength Exhibit Similar Stabilities—Thermal and chemical denaturation of vitronectin has been shown to lead to multimers which differ in size and stability after self-association at physiological ionic strength (12). An obvious point of curiosity, therefore, is whether the monomers, which can be recovered upon either heat or chemical treatment followed by renaturation in high salt, differ in folding and stability. To compare stability of the renatured monomers, intrinsic fluorescence of the proteins was monitored as a function of GdnHCl concentration (Fig. 8). In striking contrast to the unfolding curves comparing multimers generated by heat or chemical denaturation, which exhibit different midpoints and shapes, the unfolding curves for the two types of renatured monomers are identical. Midpoints for denaturation and average emission wavelengths associated with the folded and unfolded monomers are summarized in Table II for comparison with other folding curves (12). GdnHCl-induced unfolding of the refolded monomers closely resembles the denaturation of native vitronectin and differs markedly from the behavior of multimers upon GdnHCl treatment.

Neither Heparin, nor a Peptide Representing the Heparin-binding Region of Vitronectin, Is Effective at Biasing the Refolding Pathway toward Monomeric Vitronectin—To test the involvement of the heparin-binding region of vitronectin in self-association, vitronectin was denatured and renatured in the presence of saturating concentrations of heparin. A rationale for this experiment was that heparin will bind to the arginine-rich heparin-binding sequence near the C terminus which is exposed upon denaturation of vitronectin. It is well established that denatured vitronectin binds to heparin with reasonable affinity (1, 2). If heparin is bound to a site involved in intermolecular interactions which stabilize the multimeric form of vitronectin, then its binding should interfere with the self-association process. In contrast, as shown by the results of analytical gel filtration in Table III, heparin did not shift the distribution of vitronectin toward the monomeric form upon refolding. This experiment was performed at approximately 50

TABLE III
Analytical gel filtration analysis by HPLC on vitronectin samples

Denaturation conditions ^a	Vitronectin sample ^b	Folding/multimerization conditions ^c	Percentage multimeric vitronectin ^d		
Heat treatment	Native	0.15 M NaCl	100		
		0.5 M NaCl	36		
		1.0 M NaCl	12		
		1.5 M NaCl	8		
		2.0 M NaCl	7		
		500 mM L-arginine ^e	15		
		100 μ M heparin	100		
		500 μ M peptide ^f	100		
		1.0 M NaCl	100		
		1.5 M NaCl, changed to ^g 0.15 M NaCl	9		
Urea treatment	Native	0.15 M NaCl	100		
		1.0 M NaCl	40		
		1.5 M NaCl	5		
		500 mM L-arginine	26		
		100 μ M heparin	100		
		490 μ M peptide ^f	100		
		1.5 M NaCl, changed to ^g 0.15 M NaCl	5		
		No denaturation	Native	5 μ M heparin ^h	0
				100 μ M heparin ^h	0
				5 μ M peptide ^{f,h}	0
500 μ M peptide ^{f,h}	0				
Multimer	Multimer			1.0 M NaCl ⁱ	100

^a For heat treatment, 1 mg · ml⁻¹ vitronectin was heated to 55 °C for 1 h and then cooled to room temperature. Urea treatment was performed by unfolding vitronectin (1 mg · ml⁻¹) in 8 M urea overnight.

^b Samples are labeled according to the state of the starting material for the experiment as: native, corresponding to the untreated monomeric protein; multimeric, corresponding to the chemically denatured then renatured form of the protein; and refolded, corresponding to vitronectin that was heat-treated at 55 °C for 1 h and cooled to room temperature in 0.1 M sodium phosphate, pH 7.5, containing 1 mM EDTA and 1.0 M NaCl.

^c Unless otherwise noted, refolding reactions were conducted in 0.1 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and the reagents indicated. Renaturation following heat treatment of vitronectin occurred as the protein was cooled to room temperature, and samples were analyzed after refolding for at least 5 h. Renaturation following urea treatment of vitronectin was achieved after removing urea by overnight dialysis of the protein into 0.1 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and the other additives listed.

^d Errors in percent multimer are $\pm 5\%$.

^e The pH of 0.5 M arginine solutions in buffer or dH₂O was adjusted to 7.5 with HCl.

^f The 18-residue synthetic peptide representing amino acids 346 to 363 within the heparin-binding sequence of vitronectin (see under "Experimental Procedures").

^g For heat treatment, protein was incubated at 55 °C for 1 h in 0.1 M sodium phosphate buffer, pH 7.5, containing 1.5 M NaCl and 1 mM EDTA. Upon cooling in the high salt solution, the protein was monomeric. The salt concentration in the solution was adjusted to 0.15 M NaCl by ultrafiltration into standard phosphate buffer before analysis by HPLC. For urea treatment, protein was incubated in 5 M urea overnight and then subsequently refolded by ultrafiltration into 0.1 M sodium phosphate buffer, pH 7.5, containing 1.5 M NaCl and 1 mM EDTA. After refolding in 1.5 M NaCl, the protein was dialyzed into standard phosphate buffer to verify the stability of the refolded monomer.

^h Native vitronectin in standard phosphate buffer was incubated at room temperature for up to 48 h with the indicated concentrations of either heparin or the 18-residue peptide. HPLC analysis at all time points indicated that a monomeric species was present, with no evidence for multimerization under these conditions.

ⁱ Multimeric vitronectin was treated with 1.0 M NaCl at room temperature for up to 48 h to test for salt-induced dissociation of the multimer. HPLC analysis at all time points indicated no dissociation of the multimer under these conditions.

× the K_d for the heparin-vitronectin interaction,⁵ a concentration which does not significantly affect the ionic strength of the solution. Furthermore, if the heparin-binding region is involved in self-association of vitronectin, it was reasoned that binding of subsaturating amounts of heparin should interrupt the hypothesized ionic interactions between the C-terminal basic region and N-terminal acidic residues (23). According to the proposed model, which has been widely adopted in spite of a paucity of experimental evidence in support, binding of heparin at the C-terminal basic region would expose the N-terminal acidic multimerization site and promote self-association. This was not observed, and native protein remained in a monomeric form following incubation of 1 mg·ml⁻¹ vitronectin with 5 μ M heparin for up to 48 h (Table III). Consistently, Stockmann and co-workers (11) also observed that incubation of native vitronectin with heparin does not induce multimer formation.

In a similar experimental design, an 18-residue peptide

which represents the heparin-binding sequence of vitronectin was tested to determine whether it would affect partitioning of denatured vitronectin between a monomeric and multimeric form upon renaturation. If this region of the protein is involved in self-association, the peptide should compete with the same site on vitronectin for binding sites of opposite charge on other chains which assemble to form the multimer. The peptide is present at an approximate 40-fold excess over vitronectin, so it should compete effectively for potential intermolecular binding sites which stabilize the multimer, if, indeed, these interactions are important for self-association. However, the peptide had no effect on the exclusive formation of a multimeric species upon refolding at physiological ionic strength. Also, incubation of the peptide with native vitronectin in standard phosphate buffer for long time periods did not promote oligomerization of the protein. These results are summarized in Table III.

The effect of heparin on the kinetics of formation of the multimer in renaturation experiments is shown in Fig. 9. In a buffer of physiological ionic strength, a refolded form of the protein that has a molecular size characteristic of the monomer is observed transiently. Most of the vitronectin monomers

⁵ P. Zhuang, A. I. Chen, and C. B. Peterson, manuscript in preparation.

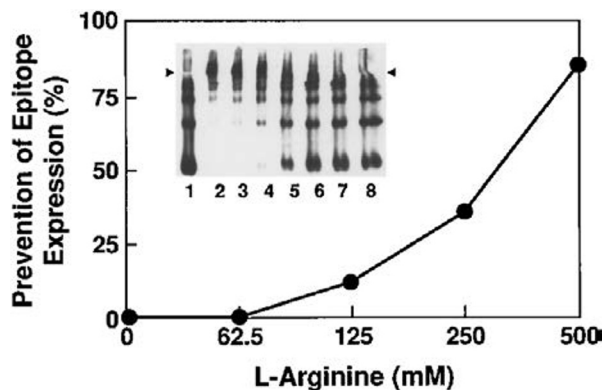


FIG. 7. Conformational changes associated with the altered form of vitronectin are prevented upon heat treatment with L-arginine. Native vitronectin was heat treated at 55 °C for 1 h in the presence of varying concentrations of L-arginine. Expression of the mAb 8E6 epitope was determined by competitive ELISA and results are expressed as percentage of prevention of epitope expression (see “Experimental Procedures”). *Inset*, native vitronectin was heat treated in the presence of phosphate-buffered saline (*lane 2*), or concentrations of L-arginine equal to 50 mM (*lane 3*), 100 mM (*lane 4*), 200 mM (*lane 5*), 300 mM (*lane 6*), 400 mM (*lane 7*), or 500 mM (*lane 8*). Untreated vitronectin is shown in *lane 1*. Samples were fractionated by native PAGE and analyzed as in Fig. 4. The interface between stacking and separating gels is shown by the arrowheads.

TABLE IV
Prevention of exposure of conformationally sensitive epitopes by amino acids and NaCl

Antibody	Epitope	Conditions ^a	% Prevention of epitope expression ^b
MAb 153	Amino acids 1–40	Gly	0
		L-Arg	61
		L-Glu	71
		0.5 M NaCl	69
mAb 8E6	Amino acids 52–239	Gly	0
		L-Arg	67
		L-Glu	83
		0.5 M NaCl	75

^a Native vitronectin was heat-treated in the presence of the indicated amino acids (500 mM final concentration) or 0.5 M NaCl for 1 h at 55 °C.

^b The competition of the heat-treated protein for binding of conformationally sensitive mAbs to wells precoated with denatured vitronectin was determined by a competitive ELISA method as described in the legend to Fig. 7. Results are expressed as percentage of prevention of epitope expression as defined under “Experimental Procedures.” Duplicate measurements varied less than 10%.

which are initially present progressively oligomerize into a multimeric form. The half-life for assembly under these experimental conditions is approximately 8 h. Note that the monomer does not completely self-associate into a multimeric form over the duration of this experiment, in which refolding is conducted in 0.5 M urea. Recall that low concentrations of chaotropic agents are disruptive to the oligomeric structure of multimeric vitronectin, inducing dissociation into monomers (12). Thus, it would be expected that the presence of denaturant upon refolding would bias the monomer/multimer distribution so that some monomers persist under these conditions.

At very high concentrations of heparin, equal to 1 mM, the kinetics of self-association of vitronectin and partitioning between monomeric and multimeric forms are affected. As in buffer without heparin, a refolded species which elutes as a monomer is observed initially; the rate of self-association of this intermediate form of vitronectin into a multimer is decreased upon addition of extremely high concentrations of heparin which equal 500 × the K_d for the heparin-vitronectin interaction.⁵ The half-life for self-association is estimated to be

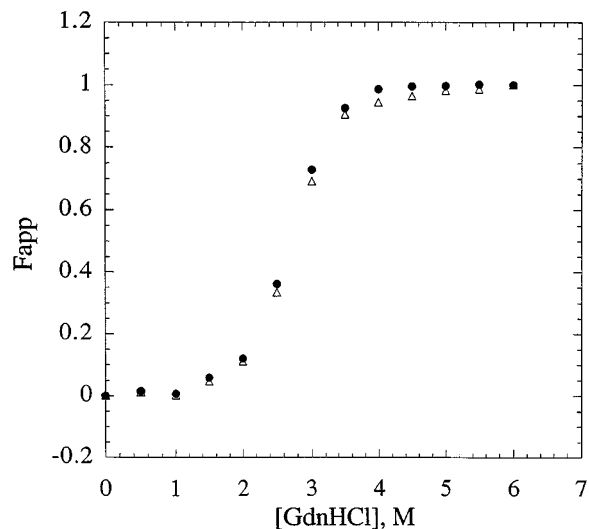


FIG. 8. GdnHCl unfolding curves for refolded monomers isolated following thermal or chemical denaturation and refolding of vitronectin in 1.5 M NaCl. Unfolding curves are shown for refolded monomers generated upon refolding after heat (*open triangles*) or chemical (*solid circles*) denaturation. Heat-treated vitronectin (5 μM) was prepared by heating protein in 0.1 M sodium phosphate, pH 7.5, containing 1.5 M NaCl and 1 mM EDTA, at 55 °C for 2 h, followed by cooling on ice. Chemical denaturation of vitronectin was performed by treatment of protein at a 5 μM concentration with 8 M urea in standard phosphate buffer; refolding of the urea-treated protein was achieved by dialysis into 0.1 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and 1.5 M NaCl. Refolded monomers were diluted to a 0.5 μM concentration in mixtures containing various concentrations of GdnHCl in standard phosphate buffer plus 0.04% Tween 20. Unfolding was estimated from fluorescence emission spectra by calculating the intensity-averaged emission wavelength, as described under “Experimental Procedures.” Following overnight incubation of vitronectin at each concentration of denaturant in buffer, emission spectra were recorded from 300 to 450 nm using an excitation wavelength of 290 nm. Data are expressed as F_{app} , the apparent fraction unfolded, by normalization of individual readings to λ_F and λ_U as given in Table II.

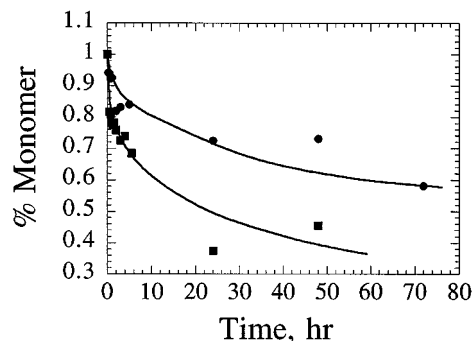


FIG. 9. Kinetics of self-association of refolded vitronectin in the absence and presence of added heparin. Vitronectin (1 mg·ml⁻¹) was unfolded in 5 M urea in standard phosphate buffer, and refolding was initiated by diluting the protein 10-fold in standard phosphate buffer plus or minus 1 mM heparin. At various times after refolding was initiated, 20-μl samples were injected onto the SEC2000 column and chromatographed by HPLC in standard phosphate buffer. Multimerization in the absence of heparin is shown *squares*, and in the presence of added heparin with *circles*. Peaks on a printed copy of the elution profile with elution times of 5.6 and 6.8 min, corresponding to the multimer and monomer, respectively, were integrated manually by weight. Fraction monomer is plotted on the y axis as a function of time. Lines drawn through the data are for illustrative purposes and do not represent fits to exponentials.

increased approximately 2-fold to near 20 h. Notably, the oligomerization process to form the multimer is not prohibited, with an equal partitioning between multimer and monomer over the time course of this refolding experiment. The distribution between monomer and multimer upon refolding is per-

turbed so that a higher fraction of monomer is observed compared to refolding in buffer of physiological ionic strength and low concentrations of urea. This partitioning is reminiscent of the distribution observed at NaCl concentrations intermediate between physiological concentrations and 1.5 M NaCl. Since heparin itself is a polyelectrolyte, the refolding to a monomeric fraction in addition to the multimeric form is likely a function of ionic strength. Indeed, the ionic strength of a 1 mM heparin solution was calculated to approximately equal 0.88 (data not shown). Clearly, the heparin-binding site on vitronectin, which is saturated with ligand under these conditions, cannot participate in self-association into the multimeric form.

DISCUSSION

The biophysical analyses presented in an accompanying article (12), which were used to characterize the *in vitro* unfolding, refolding, and multimerization behavior for vitronectin, were expanded in this work to investigate the mechanism by which vitronectin self-associates. Particular attention was paid to the effects of varying redox conditions and ionic strength on the self-association of vitronectin upon refolding. As summarized in Scheme 1, the monomeric form of vitronectin isolated from human plasma partially unfolds at intermediate concentrations of denaturant to an altered conformation with a high propensity to associate into multimers. Likewise, refolding of vitronectin proceeds via the partially folded intermediate which highly prefers the branch pathway in Scheme 1 leading to multimer formation over the pathway leading to a folded monomeric form. Since monomeric and multimeric forms of vitronectin are associated with different tissues, folding of vitronectin *in vivo* appears to be regulated by partitioning of folding intermediates via these pathways to either of two conformations, one that exists as a stable monomer and another that associates into a multimeric form. Discussion of the results and conclusions drawn from this work is organized according to the questions posed in the Introduction.

Does Intramolecular Disulfide Rearrangement Stabilize an Alternative Fold with a Tendency to Multimerize?—Intermolecular disulfides do not appear to make an important contribution to stabilizing the multimeric form of vitronectin, since disulfides do not uniformly cross-link the vitronectin protomers within the multimeric form. Unfolding of native vitronectin by chaotropes exposes two buried sulfhydryls which presumably promote the inter- and intramolecular rearrangement which has been observed among some of the 14 cysteines in vitronectin. In order to establish that disulfide cross-links were not essential for stabilization of multimeric vitronectin, Bittorf *et al.* (9) conducted experiments intended to limit disulfide exchange upon denaturation. These investigators blocked available free sulfhydryls on native vitronectin with *N*-ethylmaleimide prior to unfolding in urea and refolding to generate multimeric vitronectin. Since Bittorf and co-workers (9) blocked available sulfhydryls on the native protein, which is devoid of free sulfhydryls, potential disulfide rearrangement upon denaturation would not have been avoided in their experiments. This presumably accounts for the observation by these investigators that the average molecular weight of the constituent subunits isolated following dissociation of the multimer was equal to 130,000, considerably higher than the molecular weight expected for monomeric vitronectin. This molecular weight is in fair agreement with that from ultracentrifugation experiments presented in the accompanying article (12), in which the higher molecular weight is attributed to a mixture of vitronectin monomers and a small fraction of disulfide cross-linked species which assemble to form the multimer.

In a series of experiments designed to test the possibility that perturbed disulfide-bonding patterns in vitronectin con-

tribute to an altered fold which is prone to multimerize, redox conditions were varied in order to find conditions which prevent the rearrangement of the C-terminal disulfide linking the 62,000 and 10,000 polypeptides comprising the two-chain form of vitronectin. Strongly oxidizing conditions employed during chemical denaturation of vitronectin were found to be effective at preventing the disruption of the distal disulfide bond(s) normally associated with denaturation. However, precluding disruption of the covalent link between the 62,000 and 10,000 polypeptide chains was not sufficient to achieve reversibility in unfolding and refolding of vitronectin *in vitro*. Hysteresis comparing unfolding and refolding curves in GdnHCl is still apparent, so unfolding and refolding apparently proceed via different pathways, even when disulfide rearrangement is avoided. The hypothesis that an altered disulfide-bonded fold may promote intermolecular interactions in formation of multimeric vitronectin is not supported by these results. Alternatively, perturbations in folding at sites within the protein which are not particularly sensitive to the presence or absence of native disulfides appear to characterize the partially folded intermediate which has a propensity to oligomerize.

Conclusions about the role of disulfide rearrangement and its effect in stabilizing an altered conformation which is prone to self-association must acknowledge the limitations of the experimental measurements which measure disulfide shuffling in the protein. Assays which have been used in this work and by others (7–9) are solely sensitive to disruption of the distal disulfide(s) which covalently link the 62,000 and 10,000 polypeptides of two-chain vitronectin. The identity of the disulfide(s) which cross-link the heavy and light chains of vitronectin is not known. Furthermore, it has not been rigorously established whether other disulfide rearrangements accompany disruption of the distal C-terminal disulfides. It is unclear whether denaturation of vitronectin under highly oxidizing conditions promotes limited disulfide rearrangement that is not propagated to the C-terminal disulfides.

Are There Conditions Which Favor Refolding of Vitronectin to a Monomeric Form without Self-association as a Prominent Side Reaction?—For the first time, conditions have been found which disfavor self-association upon refolding of vitronectin *in vitro* so that a monomeric species can be recovered after denaturation. The irreversibility of changes which are induced by denaturation had been observed without exception, from the early work of Barnes *et al.* (1), on changes in heparin-binding properties of vitronectin, and Tomasini and Mosher (7, 8), on changes detected immunochemically, to the more recent studies in which self-association irrevocably accompanies denaturation/renaturation of the protein using physiological renaturation conditions (9, 11–13). However, it has now been established that high concentrations of charged amino acids or NaCl included during refolding reactions are effective at prohibiting assembly into a multimeric form. Since both charged amino acids and NaCl have similar influences which favor refolding to a monomeric vitronectin, the effects are presumed to result from increased ionic strength of the refolding medium.

What Effect do Charged Biomolecules or Salts Have on the Distribution of Refolded Vitronectin between Monomeric and Multimeric Forms?—Remarkably, the refolded monomer acquired upon renaturation at high ionic strength is stable, so that ions can be removed by dialysis, and the protein retains its monomeric character. The observation that removal of high salt from the medium (after refolding has been achieved) does not lead to multimer formation indicates that it is the pathway for refolding, rather than the final folded product, which is being influenced by the ionic strength effects. Another way to construe this argument is to consider that ionic strength effects

are exerted on the partially folded vitronectin species at the branch point which determines whether the final refolded product will be monomeric or multimeric in nature. It is helpful to consider these possibilities with regard to Scheme 1 as drawn; high ionic strengths disfavor partitioning toward the multimeric species and clearly favor the refolding pathway that restores vitronectin to its initial monomeric form. It is only under these higher ionic strength conditions that there is a true equilibrium distribution between multimer and monomer, justifying the reversible reactions between V , V^* , and M_v depicted in the scheme.

Reversibility of the folding/unfolding equilibria in Scheme 1 are supported by the immunochemical and analytical characterization of vitronectin species undertaken in this work. As already discussed, the analytical gel filtration results clearly demonstrate that the refolded vitronectin species is identical in molecular size to the native monomer. In the presence of high arginine or NaCl concentrations, epitopes exposed in the altered conformation of vitronectin which is prone to multimerization are not expressed. By these criteria, the native and refolded proteins are indistinguishable. Furthermore, GdnHCl unfolding curves which are monitored by changes in the intrinsic fluorescence of vitronectin demonstrate that proteins that are refolded at high ionic strengths subsequent to thermal or chemical denaturation exhibit identical unfolding behavior. In other words, the refolded structure acquired at high ionic strength is the same regardless of the denaturation method used to unfold the protein initially. This result is in stark contrast to the observation that renaturation of thermally or chemically denatured vitronectins in physiological ionic strength yields multimers that are clearly different with respect to molecular size and sensitivity to denaturation (12).

What Contributions Do Ionic and Hydrophobic Interactions Make to the Process of Self-association of Vitronectin?—The types of interactions which contribute prominently to self-association for vitronectin are not fully evident from the ionic strength data for several reasons. First, partitioning between the two branch pathways from V^* in Scheme 1 involves intermolecular interactions to form M_v and solely intramolecular interactions to refold to V . Therefore, the influence of solvent conditions to favor intra- versus intermolecular interactions must be considered. Second, the effects of ions in the medium are opposite for ionic interactions, which are disrupted by solvent ions, and hydrophobic interactions, which are strengthened at high ionic strength. A naive interpretation of the ionic strength effects on partitioning of refolded vitronectin between multimers and monomers would be that hydrophobic interactions do not appear to be the driving force for self-association, as these interactions should be relatively strong in high salt. Ionic interactions would perhaps be more important. Were this interpretation of the data adequate, it would be anticipated that treatment of multimeric vitronectin with high salt should dissociate the protein into monomeric constituent chains. This was not observed in practice, indicating that intermolecular interactions within the multimer are not exclusively ionic in nature.

It is more meaningful to consider the ionic strength effects as they influence the folding intermediate, V^* . Note that V^* is monomeric, a precursor to self-assembled forms of vitronectin. In high ionic strengths, this intermediate experiences the same effects described above, namely weakened ionic interactions and strengthened hydrophobic forces. Hydrophobic collapse is considered to be a driving force in acquisition of protein structure, and high ionic strength should favor intramolecular hydrophobic interactions which lead to formation of a stable hydrophobic core. Ionic interactions are probably less important

in the initial stages of folding and do not contribute significantly to the hydrophobic core of a protein. For the partially folded intermediate, V^* , a refolding medium containing high salt should favor collapse of the hydrophobic core which presumably directs the folding of the monomer. Potential hydrophobic interactions with other partially folded vitronectin chains, which may indeed stabilize M_v , are not as highly favored as intramolecular interactions due to entropic considerations. In other words, intramolecular events are favored over diffusion-limited processes under these solvent conditions. Strengthening of the hydrophobic effect for the monomeric intermediate, V^* , is thus proposed to be the principal determinant of partitioning V^* toward a multimeric or monomeric form. Further experimentation will be necessary to determine whether inter- versus intramolecular hydrophobic forces indeed predominate in determining the refolding pathway for vitronectin.

Does the Heparin-binding Region of Vitronectin Contribute Intermolecular Interactions Which Participate in Oligomerization to the Multimeric Form?—Other workers have also suggested that both hydrophobic and ionic bonds are disrupted upon unfolding of vitronectin (11), and furthermore, contribute to assembly of the multimer (13). Partial proteolysis of vitronectin within the heparin-binding region was observed to preclude self-association, supporting the idea that the heparin-binding region is involved in intermolecular interactions between vitronectin chains. The arguments were taken further to suggest that basic residues near the C-terminal heparin-binding site, as well as acidic residues, near the N terminus of the protein, comprise parts of the multimerization sites (11).

These conclusions were re-evaluated by taking a somewhat different approach in this study. Experiments were conducted in which either saturating concentrations of heparin or 500 μM concentrations of a peptide from the heparin-binding site of vitronectin were included upon refolding of vitronectin following thermal denaturation. It was reasoned that saturation of the protein with heparin would block the heparin-binding site on the protein and interfere with its proposed participation in the multimerization process. Likewise, binding of the heparin-binding peptide should occur at sites on vitronectin which are complementary and which comprise part of the multimerization sites, as proposed by Stockmann *et al.* (11). Although it has been suggested in the literature that acidic residues near the N terminus interact with basic residues within the heparin-binding site, there is no experimental evidence to support this contention, and potential binding sites for the basic peptide have not been identified. The heparin-binding peptide used in this study did not promote self-association by binding to vitronectin; neither did it preclude self-association by competing for binding sites on other vitronectin chains during the oligomerization process. The refolding experiments demonstrate that neither heparin, nor the peptide, prevented formation of multimeric vitronectin, disproving the hypothesis that parts of the heparin-binding site provide multimerization interfaces for self-assembly.

Conclusions—In summary, the mechanism for unfolding/refolding and self-association of vitronectin has been evaluated in this study. Effects of inter- and intramolecular disulfide rearrangement, which normally accompany denaturation of the protein, are not the sole deterrent to reversibility of unfolding/refolding of monomeric vitronectin. However, high ionic strengths favor reversible refolding of denatured vitronectin to a monomeric form and prevent self-association into a multimeric form. Ions apparently influence the pathway for refolding via effects on a partially folded intermediate. High ion concentrations strengthen hydrophobic interactions within the hydro-

phobic core that drives folding of the monomer; the effect is much greater on intramolecular hydrophobic forces than for potential hydrophobic interactions between chains that assemble to form the multimer. Although there may be both ionic and hydrophobic interactions involved in oligomerization of vitronectin, the heparin-binding site near the C terminus of the protein does not appear to contribute binding interactions in the multimer.

Proper redox conditions have been observed to be important for correct *in vitro* folding of proteins in many cases. Optimized redox conditions using a mixture of oxidized and reduced glutathione have been effective at reactivation of antibodies from a completely unfolded and reduced state, providing conditions which allow for correct disulfide formation (24–26). It is interesting to note that urea or L-arginine were also used as additives in these experiments to suppress aggregation as a side reaction. Folding of vitronectin *in vivo* is controlled by accessory factors and intracellular targeting, and the expression of monomeric *versus* multimeric forms of the protein with different functional properties is tissue-specific. The set of helper proteins that may assist vitronectin folding *in vivo* include folding catalysts, such as peptidyl-prolyl isomerase and protein disulfide isomerase, and chaperones, such as those in the Cpn60 and Cpn70 classes.⁶ The work cited above concerning refolding of antibodies was expanded to demonstrate that addition of protein disulfide isomerase during *in vitro* refolding reactions facilitates formation of correct disulfides (27). Furthermore, the effects of protein disulfide isomerase *in vitro* have been observed to parallel acquisition of native disulfide bonds *in vivo* in cases where *in vitro* and *in vivo* pathways have been characterized (19). It should be interesting to test *in vitro* effects of protein disulfide isomerase on the refolding of human vitronectin.

As this discussion has progressed, points for future work have become apparent and many questions remain to be answered. For example, is the monomer acquired upon refolding at high ionic strength folded identically to native vitronectin isolated from plasma? Are all of the native disulfide bonds intact? Does the refolded monomer exhibit the same functional properties, especially in terms of macromolecular interactions, that native vitronectin exhibits? Do vitronectin monomers and multimers contain unique arrangements of oxidized and reduced sulfhydryls within the two populations of vitronectin species? What specific interactions contribute to stabilization at interfaces between vitronectin subunits in the multimer? How does the fold of the native monomer differ from that of the individual vitronectin chains which comprise the multimer? Partial answers to these questions can be gained by determin-

ing the disulfide-bonded structure of native and conformationally-altered vitronectin. The potential for using site-directed mutagenesis on vitronectin to analyze folding properties and intermolecular interactions is great now that vitronectin can be expressed in a recombinant form (28, 29). Since the self-association exhibited by vitronectin to acquire a multivalent nature is a property shared by many of the adhesive glycoproteins involved in hemostasis, a thorough understanding of the folding pathway for the protein and intra- and intermolecular interactions which contribute to stabilization of the two forms of vitronectin should be fruitful and relevant to regulation of function *in vivo*.

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⁶ *In vitro* experiments in which the molecular chaperone GroEL (a molecular chaperone in the Cpn60 class) was included upon initiation of refolding did not affect the observed self-association of refolded vitronectin exclusively into a multimeric form.