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Studies of Sulphydryl Chemistry in Human Vitronectin

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I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Cynthia Peterson, Faculty Mentor
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Studies of Sulfhydryl Chemistry in Human Vitronectin

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Senior Thesis
**Introduction:**

Vitronectin, or S-protein, is a regulatory glycoprotein found in the circulation and the extra-cellular matrix. It is known to be involved in a number of diverse physiological processes including the immune response, cell adhesion, blood coagulation, and fibrinolysis. There are few detailed studies involving the structure of vitronectin or how its structure mediates the regulatory properties associated with the protein. The remainder of this introduction is intended to relate what is known about the structural elements of vitronectin and the role of the protein *in vivo*.

**Physiological Importance:**

*The Immune Response*— Vitronectin was first isolated and characterized as a component in the complement cascade of the immune response (1). It was named S-protein, soluble protein, on account of its ability to associate with the terminal complexes of the membrane attack complex and maintain these molecules in solution (1). Furthermore, vitronectin was found to associate with the C9 molecule, possibly through amino acid residues in the carboxyl terminal region of the protein, and prevent C9 polymerization (2). It is the C9 polymer that inserts into the cell membrane and forms the lytic pore. Therefore, as it is understood, vitronectin prevents formation of the membrane attack complex (2). This anti-cytolytic property is believed to function as a safeguard mechanism, thus preventing injury to bystander cells (2).

*Cell Adhesion*— Vitronectin has also been associated with the property of cell adhesion and was subsequently characterized as “serum spreading factor (3,4).” Vitronectin was found to be the major serum component in tissue culture media that was
responsible for cell adhesion (3,4). Vitronectin itself adheres to the surface of the culture dish through non-covalent interactions (5). Upon adhesion of vitronectin to the surface conformational changes occur (5). It is believed that the tripeptide RGD, which is recognized by the integrin class of cell receptors, becomes surface exposed during this conformational change allowing for cell attachment (5).

Coagulation-- Vitronectin acts as a procoagulant through its neutralization of the activity of the anticoagulation drug heparin (6-10). Heparin accelerates the rate of deactivation of a coagulation protease, thrombin, by a serine protease inhibitor, antithrombin, by supplying a surface on which these molecules can interact. The inactivation of thrombin by antithrombin is a diffusion limited process. Heparin, a glycosaminoglycan chain, binds both thrombin and antithrombin thus limiting their diffusion to one dimension, that of the heparin chain, instead of the three dimensions allowed in solution. Vitronectin destroys the catalytic property of heparin by binding to the heparin chain and preventing the movement of thrombin and antithrombin along the chain. The heparin binding site of vitronectin was localized to residues 340 through 379 by cyanogen bromide digestion (7).

Fibrinolysis-- Vitronectin is also known to be involved in the fibrinolytic pathway (11, 12-16). Fibrinolysis is the degradation of blood clots. Blood clots are formed when thrombin, the terminal protease of the coagulation cascade, cleaves soluble fibrinogen into insoluble molecules of fibrin. The blood clot is composed mainly of this fibrin. During fibrinolysis, plasmin digests the clot into small soluble fragments. Plasmin, however, must first be activated from its inactive form, plasminogen.
Plasminogen is activated by a group of serine proteases called plasminogen activators. Furthermore, these activator molecules can be inhibited by a molecule called plasminogen activator inhibitor type one (PAI-1). PAI-1 is in a class of molecules called serine protease inhibitors (SERPINS). These molecules have a common structure and mode of inactivation of their target proteases. SERPINS have a conserved "reactive loop" that contains the bond that is a pseudosubstrate for the serine proteases. Instead of cleaving this bond and releasing cleaved products, the protease forms a stable complex with the inhibitor. For the SERPIN to effectively block the proteolytic activity of the activators, however, the "reactive loop" must be surface exposed. In newly synthesized PAI-1 the "reactive loop" is surfaced exposed, but quickly self inserts into central beta sheet. Upon insertion of the "reactive loop" into the beta sheet, PAI-1 looses its inhibitory activities and is said to become latent. Vitronectin plays an important anti-fibrinolytic role in this process by binding to active PAI-1 and preventing the insertion of the "reactive loop" into the core of the protein (17,18).

**Structural Elements:**

**General Structure**—Mature vitronectin consists of 459 amino acids after the cleavage of a 19 amino acid signal sequence that instructs the liver cells, where vitronectin is synthesized, to secrete the protein into the circulation (19-23). The protein is glycosylated and highly charged containing a large number of arginines and lysines. Vitronectin also has nine tryptophan residues that can be used to monitor intrinsic protein fluorescence. In addition, vitronectin contains 14 cysteines of which the oxidation states are disputed. Vitronectin has a molecular weight of approximately 72 kilodaltons (24).
The protein is believed to exist largely in a “closed” or folded form in the circulation and as an “open” or unfolded form in the extra-cellular matrix. Moreover, vitronectin exists as a single chain form (72 kD) and as a cleaved two chain form (62 kD + 10 kD) that is disulfide cross-linked (19-23).

*Primary Sequence*— Although no crystal structure has been resolved for vitronectin much can be learned by analyzing the primary amino acid sequence of the protein. The 44 residues at the amino terminus of the protein comprise the “somatomedin B region.” This region of vitronectin has sequence homology with another plasma protein, somatomedin B. Eight of the 14 cysteine residues in vitronectin are in this region. Through the homology with somatomedin B, each of these cysteines is believed to interact with one of the other seven cysteines contained within the region. This forms what is essentially a “disulfide knot” (25). Immediately adjacent to the “somatomedin B region” is the RGD tripeptide associated with the integrin class of cell receptors and the property of cell adhesion (5). Following the RGD sequence is an “acidic domain” that has been postulated to interact with a basic region in the carboxyl terminal region of the protein thus stabilizing the folded form of the vitronectin. This hypothesis, however, was proposed on the basis of sequence analysis without any experimental evidence (7). After the “acidic region” are two “hemopexin repeats.” These regions have homology with the heme binding plasma protein, hemopexin (26). The second of these homologous repeats is imperfect, interrupted by the “heparin binding domain.” The “heparin binding domain” is, of course, the region of the protein that binds to heparin. This highly basic region is proposed to interact with the “acidic region” that
is more amino terminally located (7). At the carboxyl terminal edge of the "heparin binding domain," after residue 379, is the endogenous cleavage site. Cleavage at this site, by a trypsin-like protease, produces the 62 kD and 10 kD two chain form of the molecule (27-28).

Effects of Denaturation Upon Structure-- It has been suggested that denaturation of vitronectin, by heat or chemicals, results in the protein adopting a structure similar to that of the molecule when it is in the extra-cellular matrix (1-5). Some groups argue that vitronectin binds some ligands more tightly in this "open" conformation (29-31), but this observation is debatable. Experimental evidence does suggest, however, that other processes do occur upon denaturation (32). One phenomenon is the failure of folding and unfolding curves to overlap as shown in the figure below. This historesis suggests that the pathways of unfolding and refolding are quite different. This observation agrees with the fact that upon removal of denaturant (or cooling if thermal denaturation) vitronectin has a tendency to irreversibly self-associate into multimers with a molecular weight of approximately 420 kilodaltons (24).
It has also been observed that disulfide rearrangement occurs upon denaturation (32). The SDS polyacrylamide gel below (cartoon) shows that non-reduced native vitronectin migrates as a single band corresponding to both the single chain form and two chain disulfide linked form. Upon treatment of native vitronectin with a reducing agent, dithiothreitol (DTT), the disulfide bonds are disrupted. On account of the disruption of the disulfide bonds, including the one that links the 62 kD fragment and the 10 kD fragment of the two chain form, vitronectin migrates as a doublet corresponding to the single chain form and the 62 kD fragment of the two chain form. When vitronectin is denatured with urea, it migrates as a doublet even though no reducing agent has been added to the protein. This illustrates the disruption of the disulfide bond linking the large and small fragments of the two chain form, evidence that upon denaturation, disulfide bond rearrangement occurs.

Research Objective-- The primary objective of this study is to determine the oxidation states of the cysteine residues in native and chemically altered vitronectin. An
understanding of the oxidation states may be helpful in folding and binding studies, and elucidating the disulfide bonded structure of the protein, which has not yet been determined. A second goal is to attach a sulfhydryl specific fluorescent probe to the protein that may be useful for monitoring protein/protein and protein/ligand interactions.

**Materials and Methods:**

Vitronectin was purified by a modification of the original procedure of Dahlback and Podack, as described by Bittorf *et al.* (33). The modifications of Bittorf *et al.* (33) include addition of dithionotrobenzoic acid (DTNB) during the preparation to block free sulfhydryls in the native protein that were originally reported in the Dahlback and Podack purification (34). Bittorf *et al.* indicate there is no clear evidence that DTNB is maintained on vitronectin throughout the preparation, since reduction of the purified protein sample with DTT was not accompanied by the release of the thionitrobenzoate anion (33). Purity of the protein was assessed by SDS-polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol. A molecular weight of 72 kD and an extinction coefficient of 1.02 ml mg⁻¹ cm⁻¹ were used for the quantification of the final purified vitronectin (24). Multimeric vitronectin was prepared by treating the protein with 8 M urea at room temperature for two hours, followed by extensive dialysis into phosphate buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, 1 mM EDTA, pH 7.5) to remove the denaturant.

Urea was purchased from ICN Biomedical. Acrylodan was a product of Molecular Probes Inc. Oxidized glutathione was from Calbiochem. Sephadex G-25 resin
was purchased from Pharmacia Biotech. Both DTNB and DTT were from Sigma. All other chemicals were of reagent grade quality.

**Cysteine Oxidation State Determination** -- The number of reduced cysteines in vitronectin was determined using DTNB. The protein was denatured by adding 90 mg of solid urea to 0.200 ml of vitronectin (1 mg ml\(^{-1}\)) in 20 mM Tris, 20 mM NaCl, 1 mM EDTA, pH 7.4, to give a final urea concentration of approximately 6 M. After dissolution of the denaturant, the reaction mixture was allowed to incubate at 25°C for 4 hours. If reducing conditions were desired as well, 10 mM DTT was added to the reaction mixture prior to incubation. If only reducing conditions were desired the denaturant was omitted. Following the incubation period, urea and DTT were removed by desalting the reaction mixture over a 5 ml Sephadex G-25 column equilibrated with 0.1 M acetate, pH 6.0. Eluted vitronectin (0.725 ml) was treated with 0.25% SDS for 5 minutes. The pH of the reaction mixture was then adjusted to 8.0 by the addition of 0.235 ml 2 M ammonium phosphate, pH 9.25. The resulting free sulfhydryls were then reacted with 20 mM DTNB and the released thionitrobenzoate anion was quantitated at 412 nm using an extinction coefficient of 1.14 x 10\(^4\) M\(^{-1}\) cm\(^{-1}\) (35). Protein was quantitated by absorption at 280 nm prior to the addition of DTNB using an extinction coefficient of 1.02 ml mg\(^{-1}\) cm\(^{-1}\) (24). Quantitation of reduced cysteines in native protein was determined in the same manner, omitting the addition of urea and DTT to the incubation mixture.

**Fluorescent Labeling** -- Labeling was performed by first partially reducing vitronectin with mildly reducing conditions and then reacting the reduced protein with
Acrylodan. Vitronectin was reduced by adding 0.2 mM DTT to 0.200 ml of protein (1 mg ml\(^{-1}\)) in 20 mM Tris, 20 mM NaCl, 1 mM EDTA, pH 7.4. This reaction mixture was allowed to incubate for 30 minutes at 4°C. Following the incubation the reducing agent was removed by desalting the mixture over a 5 ml Sephadex G-25 column equilibrated with 0.1 M acetate, pH 6.0. The eluted protein (1 ml) was then reacted with 20 mM Acrylodan in 0.1% dimethyl formamide for 24 hours at 4°C in the dark with gentle stirring. Unreacted probe was removed by centrifugation at 14,000 rpm for 10 minutes followed by desalting over a 5 ml sephadex G-25 column equilibrated with 20 mM Tris, 20 mM NaCl, 1 mM EDTA, pH 7.4. The success of the labeling was determined by analysis of an emission spectrum from 400 nm to 600 nm when excited at 390 nm on a Perkin-Elmer LS-50B spectrofluorimeter. The stoichiometry of the labeling was resolved from the ratio of the protein, determined by Bradford assay (Bio-Rad kit), to that of the bound probe, determined spectrophotometrically at 390 nm with an extinction coefficient of 2.00 x 10\(^4\) M\(^{-1}\) cm\(^{-1}\) (36).

Results:

_Cysteine Oxidation States--_ Although vitronectin has been proposed to contain cysteines in both the reduced sulphydryl state and the oxidized disulfide state, the number of free sulphydryls has been disputed (33,34). The number of reduced cysteines in purified vitronectin were determined by treating the protein with DTNB under native and chemically altered conditions. Consistent with previous observations (33), native vitronectin was found to have essentially no free sulphydryls (see table). The reduced
form, however, showed the presence of two free sulfhydryls. These results indicate that native vitronectin does indeed contain a mixture of reduced and oxidized cysteine residues. When vitronectin was treated with both denaturant and a strong oxidizing agent (oxidized glutathione), however, only 0.43 cysteines were found to be free. When vitronectin was treated with varying concentrations of reducing agent, the maximum number of sulfhydryls observed was 5.35, with a saturation at approximately 4 mM DTT (see graph). Multimeric protein was analyzed in a similar manner, but only under native and denaturing conditions. Multimeric protein was found to have 0.57 sulfhydryls when untreated and 1.18 when denatured. This result clearly indicates the difference in the oxidation states of cysteine residues in monomeric and multimeric vitronectin.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of free Cysteines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Vitronectin</td>
<td>None</td>
</tr>
<tr>
<td>Reduced Vitronectin (10 mM DTT)</td>
<td>5.25</td>
</tr>
<tr>
<td>Denatured Vitronectin</td>
<td>1.88</td>
</tr>
<tr>
<td>Denatured Vitronectin with 10 mM Oxidized Glutathione</td>
<td>0.43</td>
</tr>
<tr>
<td>Untreated Multimeric Vitronectin</td>
<td>0.57</td>
</tr>
<tr>
<td>Denatured Multimeric Vitronectin</td>
<td>1.18</td>
</tr>
<tr>
<td>Reduced and Denatured Vitronectin</td>
<td>12.10</td>
</tr>
</tbody>
</table>
The fully reduced and denatured vitronectin gave an average of 12.1 sulphhydryls per molecule of the protein, in close agreement with the 14 cysteines expected from the known sequence of the protein, validating the use of DTNB for sulphhydryl quantitation.

**Varying Concentrations of Reducing Agents**

![Graph showing varying concentrations of reducing agents vs. number of reduced sulphhydryls.](image)

*Fluorescent Labeling*— Acrylodan was used for labeling on account of its sulphhydryl specificity and sensitivity towards environmental polarity (36). When the probe was reacted with untreated vitronectin no labeling was observed. This result was not surprising since it had been shown by the DTNB studies that native vitronectin contains no surface exposed free sulphhydryl groups. When vitronectin, however, was reduced with 0.2 mM DTT prior to the addition of the Acrylodan, two molecules of probe were incorporated per molecule of the protein.
Discussion:

*Cysteine Oxidation States*-- It is apparent from the native and denaturing DTNB reactions that vitronectin does indeed contain cysteines that are a mixture of reduced and oxidized forms as suggested in the literature (34). The native protein, as purified following the Bittorf *et al.* protocol (33), has no free sulfhydryls that are surface exposed, but does contain two that are buried within the core of the protein. These two sulfhydryls become surface exposed, and thus available for reaction with DTNB, only after chemical denaturant is added. It is presumably these two reduced cysteines that initiate the disulfide rearrangement upon denaturation as previously mentioned in the introduction of this paper. These cysteines are sequestered within the core of the protein under native conditions, but when the protein is denatured, they become labile. With the increased motility permitted by denaturation, the free sulfhydryls are able to move close enough to existing disulfides that a free sulfhydryl can attack a disulfide bond. This results in the previously reduced cysteine becoming oxidized, forming a new disulfide bond, and the disruption of the formerly existing disulfide. The severance of the disulfide also generates a new reduced cysteine that can go through the same attacking process or remain reduced. It has been reported that disulfide rearrangement can be prevented by blocking the free sulfhydryls contained within the core of vitronectin (31). The results obtained by reacting DTNB with protein that had been denatured in the presence of oxidized glutathione found that only 0.43 cysteines were reduced. This agrees with the observations made by analyzing SDS-polyacrylamide gels that the addition of oxidizing agent prevents disulfide rearrangement.
It was noted earlier that reduction of cysteines with increasing concentrations of reducing agent saturates at about 4 mM DTT. Only slightly over five cysteines could be reduced regardless of the concentration of the reducing agent. Vitronectin contains an even number of total cysteines and an even number of reduced cysteines in its native conformation. It may seem confusing then, that an odd number of cysteines are produced upon reduction, especially if one realizes that when one cysteine in a disulfide is reduced the second cysteine in the disulfide must be reduced as well. One explanation for the apparent reduction of an odd number of cysteines is a mixture of molecular species. It is possible that the reduced vitronectin existed as two discrete populations, one population with four free sulfhydryls and a second population with six free sulfhydryls. These two populations, if they contained an equal or slightly skewed distribution of molecules, would produce an average of five reduced cysteines when quantitated by the DTNB reaction. A second explanation would be that five is the correct value. It is possible that one of the two sulfhydryls buried within the protein became exposed when two disulfides on the surface were reduced. This would allow the sulfhydryl that was buried in the native protein to react with the DTNB. A final explanation for the apparent reduction of an odd number of cysteines is that of experimental error. It is possible that the 5.25 reduced cysteines should have been six, or even four. The DTNB reaction, although very precise, could simply not have been entirely accurate.

A final observation from the DTNB data regards the cysteine oxidation states of the monomer versus the multimer. It was found that the untreated monomer contains essentially no free sulfhydryls while the denatured monomer contains about two reduced
cysteines. The untreated multimer, however, contained about 0.6 and the denatured multimer about 1.2 free sulfhydryls per monomeric subunit of the multimer. Although nothing can be postulated about which cysteine residues are differentially reduced in these two forms of the protein, this data does help reinforce the observations that the monomer and the individual subunits of the multimer are different species (24,31).

**Fluorescent Labeling of Vitronectin**— Acrylodan was used as the fluorescent probe in the labeling reactions due to its sulfhydryl specificity and its environmental sensitivity (36). Acrylodan forms a thio-ether with the protein through a carbonyl group that stems from the central acryloyl rings. The probe is sensitive to the polarity of its environment, showing a blue shift in the emission spectrum when in hydrophobic environments and a red shift in hydrophilic environments. This may allow the probe to be a useful tool in folding and binding studies.

When the probe was reacted with the protein and excited with 390 nm light, the emission spectrum from 400 nm to 600 nm showed an increase in fluorescent intensity and an approximate 20 nm blue shift when compared to the same spectrum obtained from free probe (see graph). The labeling under mildly reducing conditions incorporated two molecules of the probe per molecule of vitronectin in agreement with the observation of two reduced cysteines under the same reaction conditions.
Conclusion:

Oxidation States of the Cysteines-- The DTNB reactions were useful in determining the number of free sulfhydryls in native and chemically altered vitronectin. It was found that native monomeric vitronectin contained no free surface exposed sulfhydryls, but had two free sulfhydryls buried within the core of the protein. These sulfhydryls become exposed upon denaturation and initiate the sulfhydryl rearrangement that is observed upon denaturation. These sulfhydryls, however, can be blocked by the addition of oxidizing agent and the disulfide rearrangement prevented.

DTNB experiments were also used to analyze and compare the oxidation states of the cysteines in monomeric and multimeric vitronectin. It was found that the oxidation states of the two proteins consistently differed under denaturing and non-denaturing conditions. This is in agreement with the observation that the monomeric unit in these two forms of the protein are structurally different (24,31).

Fluorescent Labeling-- It was found that vitronectin could be labeled with a sulfhydryl specific probe, Acrylodan, only after the protein was partially reduced. The reduction of two cysteines allowed for the incorporation of two molecules of probe. The bound probe showed an increase in fluorescent intensity with a 20 nm blue shift in probe emission wavelength.
Acknowledgments:

First and foremost I must thank Dr. Cynthia Peterson. She is a wonderful scientist and a wonderful person. I will always be indebted to her for giving me a chance and teaching me so much about what research is really like. I also must thank Dr. Ping Zhuang for "showing me the ropes" of protein purification and answering many questions. I also must acknowledge that the folding and unfolding curves, as well as some of the sulfhydryl protection work are Ping’s work. I would like to thank Dr. Baburaj Kunnumal for his help, his sense of humor, his editing skills, and for being around on weekends. I would also like to thank Angelia Gibson and Christine Scharr. These two are the best graduate students on campus even though they never explained to me why their molecular biology worked and mine didn’t. I would like to thank all of the people I have worked with in the lab. Marcus, Walt, Huo, Tonia, Jennifer, Mark, Herbert, and Colin...thanks. Finally, I would like to thank the Threshold Program and the Howard Hughes Medical Center for their support.
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