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Investigating the Metal Binding Properties of Plasminogen Activator Inhibitor Type 1 (PAI-1) with Intrinsic Tryptophan Fluorescence

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Senior Honors Thesis
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Abstract

Human plasminogen activator inhibitor type 1 (PAI-1) is an important serpin that plays a role in the delicate balance between blood clot formation and fibrinolysis. Elevated PAI-1 levels correlate with many diseased states such as diabetes, heart disease, and arthrosclerosis. PAI-1 functions to regulate plasminogen activation by inhibiting tissue-type and urokinase-type plasminogen activators, which are serine proteases that cleave the zymogen form to produce active plasmin. Its reactive center loop (RCL) binds the active sites of the target protease and inactivates its enzymatic function. From immobilized metal affinity chromatography, it was known that recombinant PAI-1 binds metals. This is relevant because metals have been demonstrated to play roles in inflammatory response, tumor progression, diabetes and other diseased states. Previous work has shown the effects of alkaline earth metals and transition metals in the absence and presence of cofactor Vitronectin. We are currently exploring the use of a lanthanide called terbium (Tb) that has been used with calcium binding proteins to accept energy transferred from neighboring Tryptophan (Trp) residues. Native PAI-1 has four Trp residues located at positions 86, 139, 175, and 262 in the functional protein sequence. Previous investigation has shown the importance of the Trp side chains in kinetic stability of active PAI-1 and has shown that the high quantum yield of W262 gives the greatest contribution to PAI-1 fluorescence. Studies were conducted making steady state fluorescence measurements pertaining to both L-Trp and wild-type/mutant PAI-1 experimentation using Trp light emission as a donor for excitation of Tb as a Förster resonance energy transfer (FRET) acceptor in effort to obtain donor-acceptor interdistance information. The increase of fluorescence emission at 544 nm suggests that the metal and protein are indeed binding, and signal differences among the four Trp sites, of which the brightest W262 site is highlighted in this work, will be used to elucidate the approximate location of the transition metal binding site.

Introduction

Human plasminogen activator inhibitor type 1 (PAI-1) is a <u>ser</u>ine <u>p</u>rotease <u>in</u>hibitor, or serpin, that plays an important role in hemostasis, the delicate balance between blood clot formation and fibrinolysis. PAI-1 enters the circulation by release from platelets, hepatocytes, and adipocytes as well as other kinds of cells. Diseased states like arthrosclerosis and severe sepsis exhibit marked inflammation with a dramatic increase in plasma concentrations of PAI-1, and there is positive correlation between high levels of PAI-1 and invasiveness of certain types of cancer.[1] The PAI-1 serpin is comprised of 379 amino acids to produce a molecular weight of 43 kilodaltons (kDa) in its recombinant form, or approximately 50 kDa in its glycosylated native form. This plasma glycoprotein regulates plasminogen activation by inhibiting tissue-type and urokinase-type serine proteases, through presentation of a solvent-exposed pseudosubstrate loop
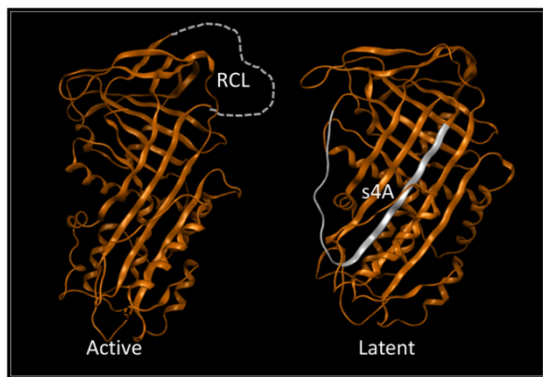

Figure 1: The transition of PAI-1 from active to latent conformation.

that effectively traps its bait protein. This reactive center loop (RCL) of PAI-1 initiates the suicidal behavior of the serpin as it binds the active site of the target protease, effectively inactivating the enzymatic function of the protease to generate active plasmin by cleaving plasminogen. This serpin is considered metastable, as PAI-1 spontaneously transitions from an active to a latent conformation (Fig. 1) when the RCL inserts as a beta strand into the central beta sheet. The RCL's mobility drives the conformational transitions. The activity of PAI-1 is typically measured by its ability to inhibit its target protease tPA over time. As PAI-1 transitions to the latent confirmation, there is less inhibition of target proteases and thus greater activity of tPA. The half-life of active PAI-1 under physiological conditions (pH 7.4 and 37°C) is about 1-2 hours. When associated with cofactor extracellular matrix protein vitronectin (VN) there is roughly a 50% increase in half-life.

PAI-1 and VN exhibit low nanomolar affinity for complex formation (Fig. 2), and are both found in circulation and the extracellular matrix. Aside from stabilizing the active PAI-1
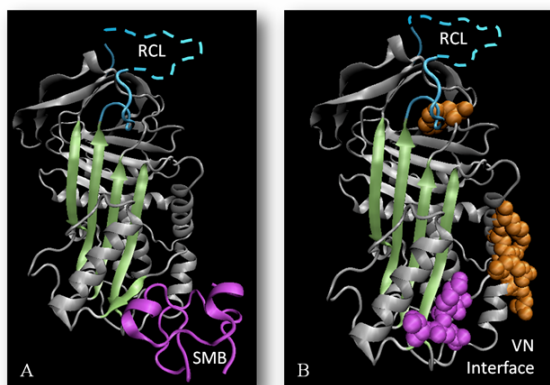
Figure 2: PAI-1 and VN complex

conformation by binding at its Somatomedin B (SMB) interface and across an extended binding interface,[2] VN appears to localize the complex to sites where inhibition of plasminogen activation is needed, such as the fibrin matrix of nascent blood clots. These proteins reside in the plasma and extracellular space where they function to inhibit fibrinolysis or extracellular proteolysis, control cell adhesion and migration,

and assist in inflammatory response.

From immobilized metal affinity chromatography (IMAC) purification it was known that recombinant PAI-1 binds metals, and the role of metals in PAI-1 is relevant because metals have been demonstrated to play a role in the severity of inflammatory response, tumor progression, diabetes, atherosclerosis, and neurological disease. According to the Protein Data Bank (PDB), about 40% of proteins contain metal cofactors that serve structural and/or functional capacities. Previous work in the Peterson Laboratory examined the effect of both alkaline earth metals (Mg, Ca, Mn) and some transition metals (Fe, Co, Ni, Cu) on the stability of PAI-1 in the absence and presence of cofactor VN.[3] Metals were classified as oxygen seeking versus nitrogen/sulfur seeking. This facilitated division into Type I alkaline earth metals which tend to form ionic
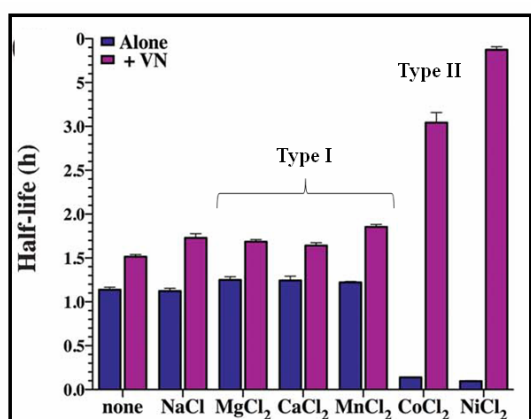

Figure 3: PAI-1 half-life in presence of metals (taken from (2): Thompson *et al*, 2011).

bonds and Type II transition metals which tend to have partially covalent bond character. As illustrated in Fig. 3, Type I metals (Mg, Ca, Mn) promoted a 10-20 percent increase in PAI-1 stability, but no additional stabilization when PAI-1 was complexed with VN. In contrast, Type II (Cu, Ni, Co) metals had a dramatic destabilizing effect on PAI-1 alone and a stabilizing effect on the PAI-1: VN complex. For example, nickel produced a 40-fold change in the half-life of PAI-1 when assayed in the absence and presence of VN.

This investigation also established binding affinities of PAI-1 for the Type II metals [4] using kinetic approach-to-equilibrium measurements of intrinsic tryptophan (Trp or W) fluorescence shifts upon metal binding to PAI-1, which contains four Trp residues. These included a first exponential corresponding to the meta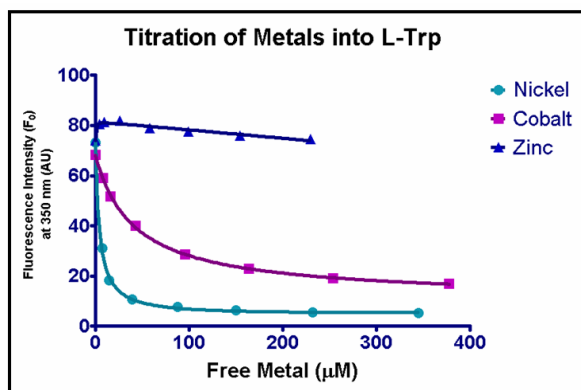l-binding event, along with two more exponentials thought to represent ensuing conformational change of the metal-bound protein. However, more recent work in the Peterson Lab revealed that some of the Type II metals interact directly with tryptophan (L-Trp) in its free amino acid form (Fig. 4), resulting in quenched fluorescence of the indole side chain. The study now includes zinc, which competes with the interfering metals to restore quenched L-Trp fluorescence. This post-transition metal

Figure 4: Some Type II metals quench intrinsic Trp fluorescence.

also causes rapid destabilization of active PAI-1, consistent with results from copper, the highest affinity ligand among the Type II metals tested. Due to the low solubility of zinc, glycine has been employed as a bioavailable chelator to assist dissolution. The presence of this additional component creates uncertainty for actual free metal calculations and produces some fluorescence measurement artifacts. Therefore, we are currently exploring the use of an optically active lanthanide called terbium (Tb) that has historically been used with calcium binding proteins to accept energy transferred from neighboring Trp residues using excitation light (295 nm) outside the Tyr range and exclusive to the Trp range.[5] This phenomenon is called Förster resonance energy transfer (FRET), and if pilot studies with wild-type PAI-1 protein prove fruitful, then we will expand our work with Tb to include the four mutant PAI-1 proteins that each contain only a single Trp residue in effort to determine their individual proximities to the putative metal binding site.

Native PAI-1 has four Trp residues located at positions 86, 139, 175, and 262 (Fig. 5). Based on work by another group,[6] the Peterson Lab has generated mutant PAI-1 proteins that contain a single Trp site, wherein the other three residues are conservatively mutated to phenylalanine (Phe or F). Phe was chosen to replace Trp because both are aromatic, and the remaining aromatic residue, tyrosine (Tyr or Y), could interfere with fluorescence measurements
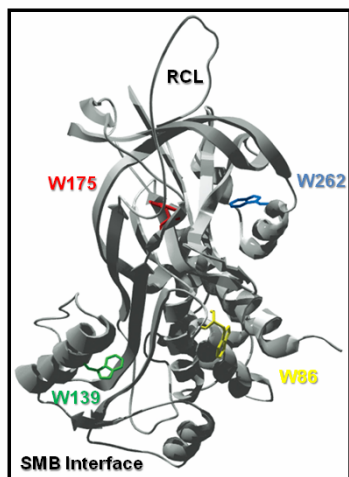
Figure 5: Trp sites in PAI-1 (structure image taken from Verheyden _et al,_

despite using excitation wavelength of 295 nm. Their investigation showed the importance of the Trp side chains in kinetic stability of active PAI-1 and showed that the high quantum yield of W262 gives the greatest contribution to PAI-1 fluorescence. In the wild-type protein, fluorescence of this residue is likely quenched by energy transfer to residue 175, as its overall signal is lower than that of the "W262-Only" (W86-139-175F) mutant. In general, the three W262F mutants (W86-Only, W139-Only, and W175-Only) have lower quantum yields relative to wild-type PAI-1. Compared to wild-type PAI-1, all the triple mutants containing W262F have ~20 min half-lives, while the W262-Only mutant has 5 hour half-life reminiscent of the W175F

mutant.[7] Manipulation of the W175 site profoundly influences RCL movement, including the insertion of the N-terminal portion of the RCL into beta-sheet A during latency transition.

Spectral isolation of the individual Trp sites should allow us to gain perspective on the metal binding conformational changes reported by each location. Therefore, studies were conducted on four Trp mutants, with an emphasis on investigation of the W262-Only mutant, using Tb as a FRET acceptor for selectively excited Trp fluorescence emissions.

Materials and Methods

These experiments focused on the interaction of PAI-1 with Terbium metal. To facilitate the experiments, mutant strains of PAI-1 isolating 86, 139, 175, and 262 were needed. _E. coli_ Rosetta 2 (DE3) pLysS cells (Novagen) were used in this study because they have been optimized for recombinant protein expression and have a quick doubling time of 45 minutes. Competent cells were stored in $CaCl_2$ solution inside the -80°C freezer. The standard transformation reaction required 20 µL cells. Cells were removed from freezer and allowed to thaw while immersed in ice for a few minutes. Visual examination was used to make sure they had thawed, and tubes were finger-tapped to resuspend cells. Plasmid DNA amounting to 100 nanograms was added to 50 µL of the competent cell tubes, and the tubes were allowed to sit on ice for 30 minutes. To trap the plasmids inside the cells, the tubes were heated for 30 to 90 seconds at 42°C in a water bath to activate housekeeping genes. This is known as the heat shock

step. Then the tubes were cooled on ice for another two minutes. Then 500 µL of super optimal broth with catabolite repression (SOC) media was added to each tube. This was followed by growth in optimal conditions (37°C and shaking at 250 rpm) for two hours. Tubes were then centrifuged for five minutes at 14,000 x g, and 400 µL of supernatant was removed leaving 100 µL for plating. Cell suspensions were then smeared on plates with antibiotics to select for the transformed cells. Plating involved immersing the plate spreader (bent glass rod) into ethanol and flaming for sterilization. The spreader was allowed to cool, and then cells were spread evenly across the plate with making sure to use gentle contact with plate. Plates were then incubated at 37°C overnight with the cover-side down.

Starter cultures were formed either from plates or from glycerol stocks that were kept in the -80°C freezer. If a glycerol stock was being used, then a sterile pipet tip was simply inserted into the frozen stock and some of the stock would adhere to the tip. The tip would then be ejected into a sterile conical flask with 15 mL of terrific broth. If the cells from the plates were being used, then an inoculation loop was used to pick off a colony from the plate, and the rod could then be submerged into the broth. The broth was always treated with antibiotics, specifically 15 µL of Chloramphenicol (CAM) and 15 µL of Kanamycin (KAN).

After 1 hour of incubation at optimal conditions, the starter culture was subcultured into one liter flasks and brought down to an optical density (OD) at 600 nm of 0.02. Growth in the one liter flasks was controlled in an incubator shaking at 250 rpm. When an OD_{600} of 1.2-1.5 was reached, cells were cooled to 15°C for equilibration. After approximately one hour, OD_{600} would reach 2-2.3 and expression could be induced. Induction was accomplished by adding 10 µL of IPTG to each one liter flask. Cells were then left to incubate overnight at 15°C (approximately 16-18 hours) and were harvested the following morning. Cell harvest was completed by 30 minutes of centrifugation at 4°C and 10,000 x g, followed by freezing of cell pellets at -80°C.

To make sure that PAI-1 expression was being induced (Fig. 6), samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were
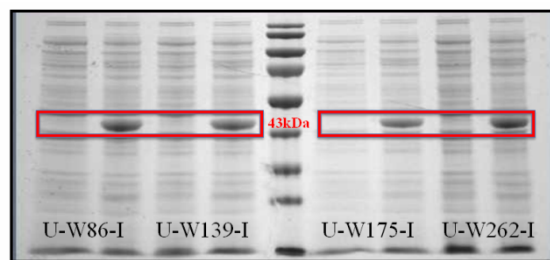

Figure 6: Induction of PAI-1 protein expression (image credit: REU student
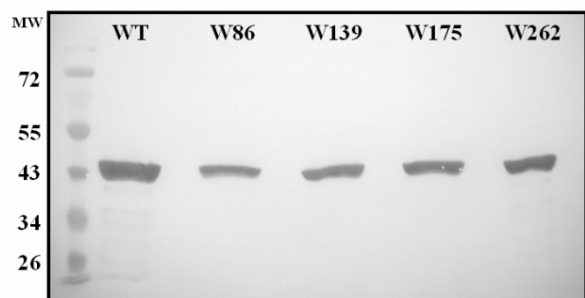
Figure 7: Purified PAI-1 proteins analyzed by SDS-PAGE and Western immunoblot.

denatured by boiling with SDS and reducing sample dye. Wells were loaded with ladder in the first lane and samples in subsequent lanes, and gels were run at 150 volts for 70 minutes. Transfer to nitrocellulose membrane for "Western blotting" was begun by placing the gel and nitrocellulose sheet in between two foam pieces wet with conductive transfer buffer and running for 10 minutes at 15 volts. To block the membrane from nonspecific binding, it was incubated for one hour at room temperature shaking in 10% milk. This was followed by addition of primary antibody (usually rabbit antihuman PAI-1), and the membrane was allowed to incubate for two hours. Then the secondary antibody was added (usually peroxidase labeled anti-rabbit), and the membrane was incubated for one hour shaking at room temperature. The membrane was washed three times with phosphate buffered saline (PBS) containing Tween in between blocking, primary antibody addition, and secondary antibody addition. To develop the Western: 30 mL of 1x Pbs, 5 mL of 4-chloro-1-napthol, and 50 µL of 30% hydrogen peroxide were added to the membrane. The partially-transferred gel was stained in Coomassie Blue and then destained overnight.

The harvested cells were lysed (Fisher Scientific 550 Sonicator Dismembrator), and PAI-1 was purified using an EDTA-free purification protocol that included Chelex-treated buffers. The lysate was centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was then loaded on SP-Sepharose cation exchange column at 10 mL/min and flow thru was collected. Elution was done by using a linear 80-500 mM $(NH_4)_2SO_4$ gradient to gradually disrupt ionic interactions. During the protein elution, we collected one minute fractions at 10 mL/min, and read the absorbance at 280 nm (A280) fractions and plotted elution versus absorbance to make the elution profile. The samples collected from supernatant, flow thru, and wash were analyzed by SDS-PAGE and Western blot. The pool was then transferred to dialysis membrane tubing (molecular weight cutoff 12-14,000 Da) and dialyzed against 4 L of imidazole buffer overnight at 4°C. The next day IMAC was utilized by charging a Chelating Sepharose column with 0.2 M $NiSO_4$ flowing long enough to allow nickel to bind the column, and excess nickel was removed with the wash buffer. Dialyzed protein was loaded onto Ni^{2+} Sepharose column. Then we washed with

imidazole buffer and applied an imidazole gradient to compete with histidine binding for release of bound proteins. The fractions were read to create and elution profile, and we analyzed by SDS-PAGE and Western blot. The protein was then concentrated to 2-3 milliliters volume and loaded on S-100 filtration column for fraction collection overnight. This was followed by reading fractions and making an elution profile. The PAI-1 peak fractions were pooled and 100 µL aliquots were stored in -80°C freezer until use. Proteins were analyzed for purity by SDS-PAGE and identity of PAI-1 proteins was confirmed by Western blot. Mutants were assayed for tPA-binding activity.

To measure protein concentration we used the bicinchoninic acid (BCA) protein assay. We prepared the BCA Working Reagent (WR) by using a formulate to determine the total of volume of WR needed in order to add 2 mL to each sample, and then preparing the WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. We used the standard protocol for the test-tube procedure. This begins with pipetting 0.1 mL of each standard and unknown sample replicate to each tube, then adding 2.0 mL of the WR to each tube and mixing. The tubes are then incubated at 37°C for 30 minutes in a water bath, and then cooled to room temperature. Then we measured absorbance at 562 nm, and we were able to the make a standard curve by plotting the measurements against concentration (µg/mL) after subtracting the absorbance measurement of the blank.

To investigate metal binding to our protein and detect changes in affinity and enthalpy, we used a technique called isothermal titration calorimetry (ITC). We ran our protein through a desalting PD-10 column to remove any EDTA and phosphate. We began by running 40 mL of 50 mM MOPS, 100 mM $(NH_4)_2SO_4$ buffer through the PD-10 column, and then we ran the protein through the column in 2.5 mL of buffer. We then placed the protein in a dialysis cassette and placed the cassette in the ITC buffer, and we allowed it to stir in the cold room overnight. After extracting the protein solution and adding the metal to it, we made sure to keep our solution at a pH of 7.4. We could now take it to the calorimeter. The calorimeter contains a reference cell and an experimental cell, and we began by degassing all solutions and placing degassed deionized water in the experimental cell. We rinsed the cell several times with water and then buffer, and we added our ligand and protein solutions into the instrument. Finally, the experimental titration was started and the data was collected at 10°C

Using a PTI fluorometer, we tested the transmission of single Trp PAI-1 mutants W86-Only, W139-Only, W175-Only, and W262-Only to the FRET donor Tb(III). With excitation slit width set to 8 nm, emission scans were completed with the wavelength range of 310 to 360 nm (range of FRET donor Trp) using a 2 nm slit width, and 520 to 570 nm (range of FRET acceptor Tb) using a 12 nm slit width. Using quartz cuvettes we produced 2 mL solution with the concentrations of 100 mM MOPS, 50 µM TbCl₃, and a relevant concentration of the PAI-1 mutant. Before taking measurements, we inserted a glass panel between the sample and detector to eliminate the transmission of excitation wavelength scattering. Our first measurement was without Tb, followed by a zero time point with 50 µM Tb, followed by measurements at 5-10 minute intervals until 30 minutes were complete. With each mutant we obtained thirty minutes of data for each of four increasing concentrations (250, 500, 750, 1000 nM) of protein.

Results and Discussion

These studies were conducted with the goal of obtaining more information about the metal binding location within PAI-1 as reported by the four Trp sites shown in Fig. 5. To obtain site-specific results, mutants were generated to isolate the spectral properties of each Trp residue. Plasmids were successfully produced for each mutant such that the other three Trp sites were genetically altered to encode Phe, leaving only a single Trp in the mutants known as W86-Only,
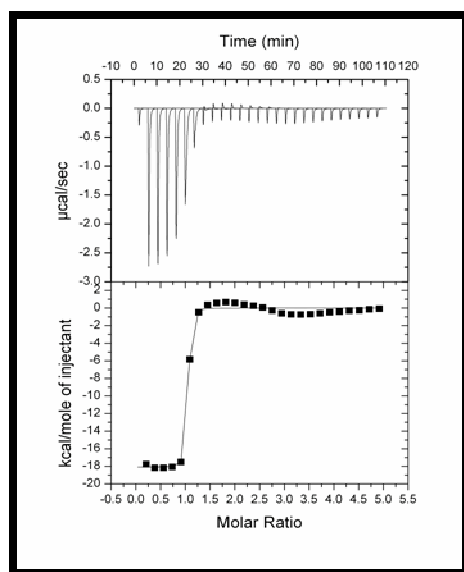

Figure 8: ITC plot for W262-Only PAI-1 titrating copper sulfate.

W139-Only, W175-Only, and W262-Only. We can see positive mutant expression in Fig. 6 as indicated by the bands by SDS-PAGE, and after protein purification the identities were confirmed as PAI-1 by Western blot (Fig. 7). Using ITC we tested the mutant PAI-1 protein W262-Only (Fig. 8) to confirm that mutagenesis did not alter metal-binding properties. According to a one-site binding fit of the data, the W262-Only PAI-1 protein ($K_d \sim 10$ nM) showed a modest increase in the apparent affinity for copper relative to results obtained for wild-type PAI-1 ($K_d \sim 22$ nM). This may be within experimental variation for ITC measurements, especially given the sensitivity of the technique to any change in protein concentration. Because

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the concentration of the Trp mutants cannot be reliably determined by spectrophotometric absorbance at 280 nm due to differences in quantum yield among individual sites,[6] they must be BCA assayed for measurement accuracy. Since the protein solution was subjected to dialysis to exactly match the buffer used for titrating copper, a slight dilution could have occurred that would alter the numbers generated while fitting the ITC data points. Additionally, there is an artifact in the ITC data (after the sigmoidal enthalpic shift) as the instrument overshot the baseline while regaining temperature equilibrium, so a repeat measurement is warranted to verify the result. Regardless of the specific details, the plot shows that the mutant binds copper, which was the verifiable goal of the measurement.

Terbium has been used to map calcium binding sites because of its similar ionic radius and metal coordination chemistry.[5] While transition metals display variable physical features, it was confirmed with stopped-flow fluorometry that the conformational change reported by W262-Only looks similar to the response evoked by zinc (data not shown). Fluorescence resonance energy transfer can be utilized to determine distance of the transition metal binding site from individual Trp

Figure 9: Terbium fluorescence changes with increasing concentration of W262-

sites in PAI-1 because of energy transfer from the aromatic residues to the bound terbium ions. Signal differences among the four Trp sites can be used to elucidate the location of the transition metal binding site. One caveat to this assertion is the aforementioned fact that each Trp residue contributes a different amount of light, so the resulting changes in terbium fluorescence should be viewed through this prism. As shown in Fig. 9, a 30 µM Tb(III) solution was used with increasing W262-Only concentrations from 250 nM to 1000 nM. Excitation wavelength was set to 295 nm to selectively activate Trp sites and avoid confounding data with Tyr signals. Each data point in the figure is an average of the fluorescence measurements taken over 30 minutes. The increase of fluorescence emission at 544 nm suggests that the metal is indeed binding to the protein, with the nearby Trp residue transmitting light to increase the Tb signal. It is important to note that the W262-Only site is the most buried from solvent within the core of the folded protein, yet it exhibits the highest quantum yield of emitted light relative to the other three sites.
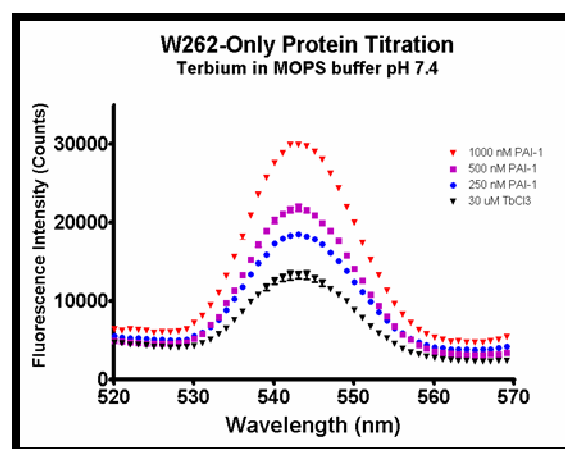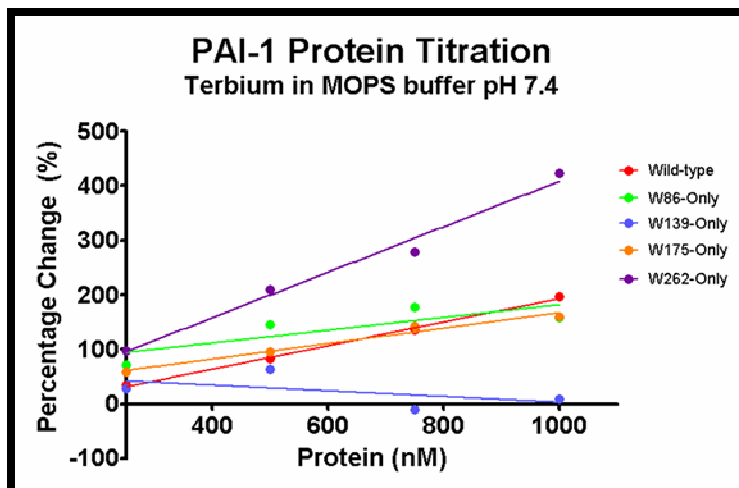
Figure 10: Linear changes in terbium fluorescence with increasing concentration of PAI-1 protein constructs.

For comparison, Fig. 10 shows relative changes for all of the PAI-1 protein constructs after an hour-long incubation with 50 µM Tb(III). At first glance, the W262-Only mutant looks most interesting as the protein undergoing the biggest change in fluorescence when more protein is available to bind the terbium. However, it is unclear whether this trend will be upheld after data correction to include provision for differences in quantum yield. For instance, the slope of the W175-Only mutant data is comparable to that of the W262-Only mutant, and factoring in the lower brightness of the W175 site, the overall trend may become more dramatic after correction treatment. Interestingly, the W175 and W262 sites are located near the same "top" pole of the protein close to the RCL, while the W139 site is located near the "bottom" pole within the SMB domain and the highly solvent-exposed W86 site is situated in between the two extremes. Perhaps this data is suggesting the metal binding site is close to the top end of PAI-1, but further investigation will certainly be required to answer that hypothesis.

Conclusion

We effectively generated and investigated the W262-Only PAI-1 mutant using a combination of techniques from molecular biology and protein biochemistry to isothermal binding kinetics and intrinsic tryptophan fluorescence in order to better understand its metal interaction properties. Our focus on the W262-Only mutant is only one piece of the puzzle; however, more work remains to be completed in order to successfully elucidate the location of the transition metal binding site on the PAI-1 structure. When further analysis is completed on the other three mutants, more critical steps will have been taken toward the characterization of the putative metal binding site within this important therapeutic target for inflammatory disease.

Acknowledgments

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