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The Perturbation of Tryptophan Fluorescence by Phenylalanine to Alanine Mutations Identifies the Hydrophobic Core in a Subset of Bacterial Ig-like Domains

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Supporting Information

ABSTRACT: Many host−parasite interactions are mediated via surface-exposed proteins containing bacterial immunoglobulin-like (Big) domains. Here, we utilize the spectral properties of a conserved Trp to provide evidence that, along with a Phe, these residues are positioned within the hydrophobic core of a subset of Big_2 domains. The mutation of the Phe to Ala decreases Big_2 domain stability and impairs the ability of LigBCen2 to bind to the host protein, fibronectin.

The bacterial immunoglobulin-like (Big) domain, group 2 family (Big_2) (Pfam ID PF02368) is found in a variety of bacterial and phage surface-exposed proteins. Big_2 domain-containing proteins are involved in recognition, binding, and adhesion, which are properties that can confer an increase in pathogenicity.[1] Big_2 domains are small modules (~79 residues) that are often present in multiple copies. Leptospiral immunoglobulin-like (Ligs) proteins make up a family of proteins on the surface of the pathogenic bacteria *Leptospira*. LigA and LigB contain 13 and 12 Big_2 domains, respectively.[2] Lig proteins interact with various extracellular matrix proteins, such as fibronectin, elastin, collagen, and fibrinogen.[3,4] Developing a better understanding of Lig proteins and the molecular mechanisms of Lig-mediated adhesion could provide insight into the initial steps involved in leptospiral infection.

Previous Trp fluorescence studies of isolated Big_2 domains from Ligs revealed that the domain's lone Trp has a blue-shifted spectrum composed of two emission peaks.[3,5] The single Trp (W1073) from the 12th Big_2 domain of LigB [LigBCen2 (Figure S1A)] has an emission spectrum with a major peak at 317 nm and a minor peak at 330 nm (Figure 1A) that can more easily be observed in the second-derivative spectra (Figure 1D). A blue shift (<340 nm) in Trp emission fluorescence has been shown to occur when the Trp is located in environments less polar than water, which typically corresponds with the interior of folded proteins.[6,7] The Trp found in LigBCen2 is one of the most conserved residues within Big_2 domains (Figures S1B and S2). The small size of Ig-like domains combined with the blue-shifted fluorescence spectrum suggests that the Trp is a key residue in forming the main hydrophobic core of Big_2 domains; however, because of a lack of structural information about the Lig subset of Big_2 domains, the specific residues that surround the Trp are speculative. The wavelength of the emission peak is sensitive to small changes in the packing density and can therefore be indicative of subtle differences in the positioning of surrounding residues. Here, we explore the molecular basis for the blue-shifted emission spectrum by examining the effect of differences in residues that are likely to be contributing to the hydrophobic environment around the Trp in Big_2 domains. In addition, the potential for the Big_2 emission spectrum to be a structural fingerprint for its hydrophobic core and to provide opportunities to study the core's functional importance is also examined.

Blue-shifted Trp emission spectra have been characterized in a few proteins of known structure, including δ-crystallin[8] and the C5 protein of RNase P.[9] The Trp residues corresponding to the blue shift are packed within the core of these proteins. The most extreme blue shifts have been correlated with increasingly nonpolar and densely packed local Trp environments. Aromatic residues play a key role in creating the hydrophobic environment that surrounds the Trp residues and shifts the Trp emission wavelength. LigBCen2 contains seven aromatic residues (Y1017, F1053, F1054, Y1059, Y1107, Y1143, and Y1158), all of which are positioned at least 10 residues from W1073 in the primary sequence. Because Phe residues are slightly more hydrophobic and should offer a more nonpolar packing environment than Tyr, F1053 and F1054 were targeted using Ala mutagenesis (see Table S1 for all mutations). The Trp emission spectra (Figure 1A) and the second derivatives (Figure 1D) for the F1053A mutant and the F1053A/F1054A double mutant show a shift in the major peak from 317 to 330 nm. The loss of the peak at 317 nm identifies F1053 as a residue that is important in LigBCen2 forming the hydrophobic core that shields W1073 from water molecules. Because surrounding aromatic residues offer the densely packed hydrophobic Trp environment that is required to obtain the blue-shifted spectra of LigBCen2, F1053 is a good candidate to be a Trp neighboring residue. The F1054A mutant generates a Trp emission spectrum that is similar to that of wild-type LigBCen2 (Figure 1A,D). While F1053 is potentially located near W1073 in the folded protein, the neighboring F1054 is likely to be positioned away from W1073 because there is little effect of the F1054A mutation.

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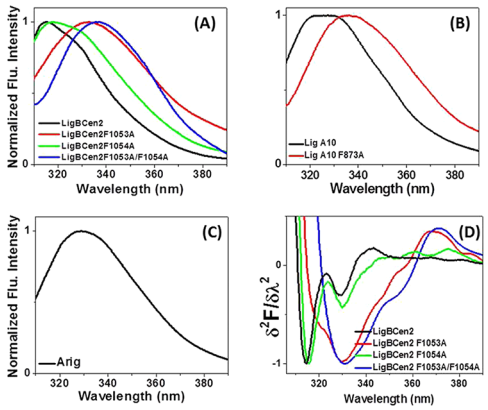

Figure 1. Identification of the Phe residue responsible for the blue-shifted Trp emission spectra of Big_2 domains from Lig and other proteins. Normalized Trp emission spectra of (A) LigBCen2, F1053A, F1054A, and F1053A/F1054A, (B) LigA10 and F873A, and (C) *Arthrobacter* immunoglobulin-like protein and (D) second-derivative spectra of the Trp emission spectra from panel A.

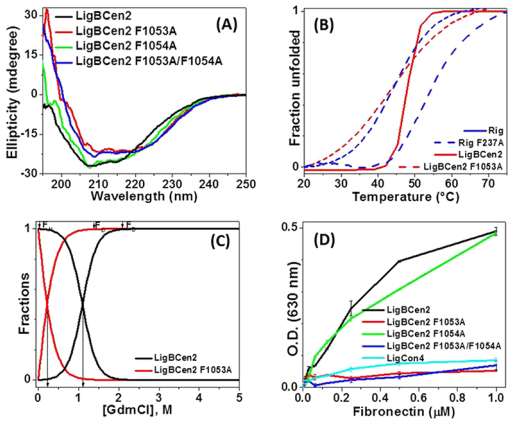

Figure 2. Implications of Phe to Ala mutations on fold stability and fibronectin (Fn) binding. (A) Far-UV CD spectra for LigBCen2 and Phe mutants. (B) Thermal denaturation curves for LigBCen2 and Rigs and Phe to Ala mutants. (C) Denaturant-dependent equilibrium unfolding for wild-type (WT) LigBCen2 and F1053A. F_N is the fraction of native protein and F_D the fraction of denatured protein for the unfolding transition. (D) Enzyme-linked immunosorbent assay for binding of LigBCen2 and the Phe mutant to full-length Fn. LigBCen2 WT and F1054A bind to Fn, while F1053A, F1053A/F1054A, and LigCon4 (negative control) do not bind to Fn.

Trp emission spectra containing peaks at 317 and 330 nm have also been observed for the 9th and 10th Big_2 domains of LigA [LigA9 and LigA10, respectively (Figure 1B and Figure S3A)].[5] A sequence alignment of the 18 unique Big_2 domains from LigA and LigB proteins shows that the Phe and Trp residues corresponding to F1053 and W1073 in LigB are well conserved and that all but two maintain an interval of 19 residues (Figure S2). To better describe how the Phe and Trp residues are positioned within the broader class of Big_2 domains, the relative aligned location of F1053 is defined as position A (A_{pos}) and the relative aligned location of W1073 is defined as position B (B_{pos}) (Figures S1B and S2). In LigA10, mutating the A_{pos} Phe (LigA10 F873A) to Ala largely eliminates the peak at 317 nm (Figure 1B and Figure S3A) and implies that the Phe at A_{pos} is important for the formation of the native microenvironment that surrounds the Trp within the hydrophobic core of Lig Big_2 domains.

To explore the contribution of the A_{pos} Phe to the hydrophobic cores of other Big_2 domains, Rig (*Rhodoferax* immunoglobulin-like protein), a A_{pos} Phe-containing Big_2 domain from a predicted protein (NCBI entry YP_523138) of the bacterium *Rhodoferax ferrireducens* T118, was cloned and purified (Figure S1 and Table S1). The Trp emission spectrum of Rig and its second-order derivation resulted in two emission peaks at 322 and 338 nm (Figure S3B,C). The mutation of the A_{pos} Phe in Rig to Ala (Rig F237A) altered the second derivative of the Trp emission spectra such that peak resolution within the 320−340 nm range is lost but the peak at 322 nm does not disappear. Although the F237A mutation results in a change to the spectrum slightly different from that for the corresponding A_{pos} Ala mutations in LigBCen2 and LigA10, the spectral changes are still consistent with a disruption of the core hydrophobic environment that includes the B_{pos} Trp (Figure S3B,C). For the Big_2 domains studied, mutating the A_{pos} Phe also influences the steady-state anisotropy and fluorescence lifetimes of Trp (Figure S4 and Table S2). Trp bands from 280 to 296 nm in near-UV circular dichroism (CD) spectra are affected when any Phe residue is mutated to Ala, although a larger change is observed by the mutation of Phe at A_{pos} (Figure S5C−E).

A Pfam analysis of the Big_2 family (PF02368) based on probability profile Hidden Markov Models suggests that amino acid size and hydrophobicity are highly conserved at A_{pos}. For all Big_2 domains in Pfam, Leu is found slightly more often than Phe at A_{pos}, but Tyr and Val are also not uncommon at this position. The Trp emission spectrum of a Big_2 domain with a A_{pos} Leu was studied using Arig (*Arthrobacter* immunoglobulin-like protein), the first Big_2 domain from a two-Big_2 domain-containing *Arthrobacter aurescens* TC1 protein (NCBI entry YP_949309) (Figure S1). In the second derivative of the Trp emission spectrum, Arig shows only a single broad blue-shifted peak at 322 nm similar to that in the Rig F237A spectrum (Figure 1C and Figure S3D). The resolution of two peaks in the second-derivative Trp emission spectrum is likely to be related to the shape and hydrophobic character of the residue at A_{pos}. If the A_{pos} residue neighbors the Trp, a flexible Leu side chain at A_{pos} would allow for an increase in heterogeneity leading to a broader emission peak. Our observations have identified only Big_2 domains with a A_{pos} Phe as having two resolved peaks in the second-derivative Trp emission spectrum. The two peaks may be a characteristic spectral feature for a specific packing arrangement of a Big_2 hydrophobic core containing both a Phe and a Trp residue.

Structures of homologous Big domains were analyzed to gain further insight into the hydrophobic core of Big domains. Determined Big_2 domain structures from two virulence factors, invasin [Protein Data Bank (PDB) entry 1CWV; domain 4] and intimin (PDB entry 1F00; domain 2), and a phage tail tube protein (PDB entry 2L04) consist of two β-sheets, one on either side of a hydrophobic core in a sandwich arrangement.[10−12] In an alignment with the Big_2 domain consensus sequence, B_{pos} is represented by the conserved Trp residue for both invasin and intimin Big_2 domains but is represented by an Ala in the phage protein (Figure S6A). For all three proteins, Leu was found at A_{pos} in agreement with the Big_2 consensus sequence. An obvious difference between the A_{pos} Phe-containing Big_2 domains studied in this work and the reported structures of Big_2 domains is the large variable alignment gap between A_{pos} and B_{pos}, which are on β-strands B and C, respectively. The variation from a 19-residue separation to a 10−15-residue separation can be explained by structural differences in the loop between β-strands B and C (Figure S6B,C). Although the experimental evidence does not exclude the possibility that the A_{pos} Phe is outside of the LigBCen2

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hydrophobic core, the mutational effects on the blue-shifted spectra are consistent with the structure of other Big_2 domain cores, where the residue at A_{pos} from β-strand B and the residue at B_{pos} from β-strand C extend from opposite sheets to form a conserved hydrophobic nucleus.

On the basis of changes in the Trp emission spectra and support from homology studies, mutating the LigBCen2 A_{pos} Phe to Ala decreases the packing density around the B_{pos} Trp. For Big_2 domains, reducing the size and hydrophobic nature of the A_{pos} residue should weaken the overall forces driving together the two β-sheets that form the sandwich structure. The far-UV CD spectrum of LigBCen2 has a broad negative peak at 208−218 nm with a crossover point at ∼195 nm, which is typical of β-sheet proteins (Figure 2A). In the F1053A mutant of LigBCen2, the negative CD peak is both shifted to higher wavelengths and reduced in intensity, while the ellipticity crossover point is lower. The changes in the CD spectra are consistent with a small increase in the random-coil content for the F1053A mutant. The far-UV CD spectra of LigA10 and Rig also show a small decrease in the β-sheet character for the A_{pos} mutants (Figure S5A,B).

Both heat and denaturants were used to assess the relative secondary structure stability of the wild type (WT) and A_{pos} Phe to Ala mutants. Using CD measurements, thermal unfolding of LigBCen2 and Rig demonstrates decreases in the A_{pos} Phe to Ala mutant's melting temperature (T_m) versus that of the WT of 6.5 and 3 °C, respectively (Figure 2B). The midpoint ($D_{1/2}$) of the denaturant, guanidinium chloride (GdmCl), required for secondary structure unfolding was determined using the fluorescence emission intensity at 330 nm. A substantial decrease in $D_{1/2}$ from 1.1 M (WT) to 0.22 M (F1053A) was observed (Figure 2C). In previous work, the dependence of $\Delta G°$ (free energy change from the native to the unfolded state) on denaturant concentration, the m value, was measured to be 3.344 kcal mol^{-1} M^{-1} for LigBCen2.[5,13] The m value of the LigBCen2 F1053A mutant was lower than that of the wild type (2.922 kcal mol^{-1} M^{-1}). Disruption of the Trp's hydrophobic environment in the A_{pos} Phe to Ala mutant drastically attenuates the stability of the Big_2 domain fold. The importance of A_{pos} to Big_2 stability suggests a basis for conservation of both the A_{pos} amino acid's size and hydrophobicity.

An enzyme-linked immunosorbent assay-based fibronectin (Fn) binding assay[3] was used to test changes in the functional adhesion of LigBCen2Phe to Ala mutants. LigBCen2 binds to Fn with a dissociation constant (K_D) of 0.53 ± 0.08 μM (Figure 2D). LigBCen2 F1053A fails to interact with Fn, even though the A_{pos} Phe is likely to be buried within the hydrophobic core of LigBCen2 and away from potential binding surfaces (Figure S6D). Differences in the core of LigBCen2 F1053A (including collapse or loosening) could translate into changes on the protein's surface. The specific three-dimensional fold of LigBCen2 (not just a linear peptide epitope) may be necessary for Fn recognition. The Ala mutation of the neighboring Phe (F1054A) does not prevent LigBCen2 from binding to Fn (K_D = 0.62 ± 0.11 μM) (Figure 2D). On the basis of homology, F1054 is probably a surface-exposed residue within β-strand B. The lack of an effect on Fn binding provides evidence that the hydrophobic surface of F1054 is not involved in a direct interaction with Fn.

Improving our understanding of Big_2 domains offers the potential for future improvements in pathogen detection as well as in vaccine development. Antigens derived from Lig proteins are currently being used for the development of vaccines.[14] Assessment of antigen stability is necessary as a predictor of pathogen protection efficiency, and the Trp emission signature of Lig proteins offers a potentially quick method for determining vaccine expiration. Low-cost, Trp fluorescence spectroscopy-based, quality assessment methods are currently being utilized in the screening of formulations for monoclonal antibody stability.[15] The potential exists for fluorescence spectroscopy to make a large contribution to infectious disease prevention efforts particularly in underdeveloped nations.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, Figures S1−S6, Tables S1 and S2, and a discussion of these results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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