The Stories Tryptophans Tell: Exploring Protein Dynamics of Heptosyltransferase I from Escherichia coli

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Supporting Information

**ABSTRACT:** Heptosyltransferase I (HepI) catalyzes the addition of 1-glycero-β-D-manno-heptose to Kdo2-Lipid A, as part of the biosynthesis of the core region of lipopolysaccharide (LPS). Gram-negative bacteria with gene knockouts of HepI have reduced virulence and enhanced susceptibility to hydrophobic antibiotics, making the design of inhibitors of HepI of interest. Because HepI protein dynamics are partially rate-limiting, disruption of protein dynamics might provide a new strategy for inhibiting HepI. Discerning the global mechanism of HepI is anticipated to aid development of inhibitors of LPS biosynthesis. Herein, dynamic protein rearrangements involved in the HepI catalytic cycle were probed by combining mutagenesis with intrinsic tryptophan fluorescence and circular dichroism analyses. Using wild-type and mutant forms of HepI, multiple dynamic regions were identified via changes in Trp fluorescence. Interestingly, Trp residues (Trp199 and Trp217) in the C-terminal domain (which binds ADP-heptose) are in a more hydrophobic environment upon binding of ODLA to the N-terminal domain. These residues are adjacent to the ADP-heptose binding site (with Trp217 in van der Waals contact with the adenine ring of ADP-heptose), suggesting that the two binding sites interact to report on the occupancy state of the enzyme. ODLA binding was also accompanied by a significant stabilization of HepI (heating to 95 °C fails to denature the protein when it is in the presence of ODLA). These results suggest that conformational rearrangements, from an induced fit model of substrate binding to HepI, are important for catalysis, and the disruption of these conformational dynamics may serve as a novel mechanism for inhibiting this and other glycosyltransferase enzymes.

A rapid increase in antibiotic resistance in Gram-negative bacteria has arisen because of the overuse of many commonly used antibiotics, thus making them ineffective. Although antibiotic resistance is a global health concern and has the potential to reach pandemic proportions, new antibiotics are scarce. Glycosyltransferases (GTs) catalyze the addition of various sugars to other biomolecules, which are often essential to many biological processes (ranging from the synthesis of small molecules to allowing cell–lectin interactions). GTs encompass a large group of enzymes that are highly diverse, in both structure and mechanism. Despite their differences, most GTs catalyze the formation of a glycosidic bond, in which a high-energy nucleotide sugar donates a monosaccharide to an acceptor molecule. This acceptor can be a variety of molecules, such as oligosaccharides, monosaccharides, proteins, and lipids.

*Escherichia coli* heptosyltransferase I (HepI) is a glycosyltransferase, containing two βαβ Rossmann-like folds connected by an interdomain linker region, characteristic of the GT-B structural superfamily (Figure 1A). HepI catalyzes the addition of 1-glycero-β-D-manno-heptose (heptose) from ADP-heptose (ADPH) to the first 3-deoxy-α-D-manno-oct-2-ulopyranosonic acid (Kdo) of *E. coli* Kdo2-Lipid A, generating heptose-Kdo2-Lipid A. This is a crucial step for the formation of the lipopolysaccharide (LPS), which is located on the outer membrane of Gram-negative bacteria and contributes to bacterial pathogenicity and virulence. LPS was isolated from bacteria that are deficient in HepI and lack heptose and all additional sugars typically added afterward. Although formation of heptose-Kdo2-Lipid A is not necessary for cell viability, these HepI deficient mutants result in decreased bacterial pathogenicity in infected mice, have increased susceptibility to phagocytosis by macrophages, and lead to an increase in bacterial sensitivity to hydrophobic antibiotics. Efforts to design inhibitors of HepI have previously been reported; however, all have resulted in inhibitors with IC50 values of >1 μM. Enhancing the mechanistic understanding of HepI dynamics may provide a new strategy for inhibiting HepI.

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of HepI action would provide a molecular basis for the development of inhibitors for HepI as potential therapeutic agents for the treatment of Gram-negative bacterial infections.

While the overall reaction of HepI was discerned in the 1970s, little is known about the detailed chemical and kinetic mechanisms by which HepI catalyzes the addition of heptose to Kdo2-Lipid A. Recently, multiple enzymes of the GT-B structural class, including GfIA and MshA (the epivancosaminyl transferase from Amycolatopsis orientalis and the N-acetylglucosamine transferase from Corynebacterium glutamicum), have been crystallized in both open and closed conformations, dependent upon the ligation state of the proteins.6,7,13−15 Currently, structures are available for apo HepI and HepI complexed with Lipid A or both substrates bound; however, a model of a hypothesized closed structure was previously generated.6 Despite the lack of structural evidence supporting the hypothesis that HepI adopts a closed conformation, it is thought that HepI, like other GT-Bs, also interconverts between open and closed conformations to permit catalysis (Figure 1A−C). This hypothesis is supported by the observation from the apo crystal structure of HepI that the catalytic residue Asp13, which is needed to deprotonate Lipid A generating a nucleophile, is >8 Å from the anomeric carbon of ADP where the deprotonated Lipid A nucleophile attacks.15

In addition, our previous transient kinetic analysis of the intrinsic protein fluorescence revealed substrate-induced changes in fluorescence suggestive of multiple events occurring prior to chemistry, including conformational changes. Specifically, the fluorescence changes upon mixing of O-deacylated E. coli Kdo2-Lipid A (ODLA) with HepI report on the ODLA binding event and subsequent conformational change(s).6,8

In this work, the intrinsic tryptophan (Trp) fluorescence of wild-type HepI (HepI-WT) and mutants is used to further isolate the regions undergoing conformational changes to better understand the catalytically active structure of HepI. It has previously been shown that in the presence of ODLA, the fluorescence spectrum of HepI undergoes a blue shift. These results suggest that one or more Trp residues must move from a more solvated to a less solvated environment upon mixing with ODLA.6 To identify which Trp could be the source of this signal, each of the seven solvent-exposed Trp residues (Figure 1) was individually mutated to phenylalanine (Phe) to investigate their roles in the previously observed transient kinetic experiments. Kinetic analyses were performed to assess the magnitude of catalytic alterations induced by the current set of mutations. Circular dichroism (CD) measurements were used to determine the effect of the mutations on substrate binding and thermostability. Furthermore, to distinguish which Trp residues are responsible for the previously observed substrate-induced blue shift of HepI intrinsic fluorescence, WT and mutant HepI fluorescence spectra were obtained, revealing both N- and C-terminal domains are altered upon binding of ODLA. Ultimately, our results enhance our understanding of HepI dynamics and the conformational changes that occur in GT-B enzymes.

MATERIALS AND METHODS

Primers were designed using the BioMath Tm calculator (http://www.promega.com/a/apps/biomath/index.html?calc=tm). All primers and E. coli strains DH5α and BL-21-AI were obtained from Invitrogen (Carlsbad, CA). The Quikchange Lightning Site-Directed Mutagenesis Kit and E. coli strain XL10-gold were obtained from Stratagene (La Jolla, CA). The Zyppsy plasmid mini prep kit was attained from Zymo Research (Irvine, CA). Isopropyl β-D-1-thiogalactopyranoside was obtained from Gold Bio Technologies (St. Louis, MO). B-PER II Bacterial Protein Extraction Reagent, EDTA free protease inhibitor tablets, Sartorius Vivaspin 20 centrifugal concentrators with 10000 and 3000 molecular weight cutoffs (MWCOs), trypト酮, and yeast extract were obtained from Fisher Thermo Scientific (Pittsburgh, PA). Sodium chloride, sodium hydroxide, ampicillin (Amp), tetracycline (Tet), HEPES, imidazole, EDTA, cobalt sulfate, and l-arabinose were obtained from Sigma (St. Louis, MO); 30% acrylamide, a Bio-Scale Mini Bio-Gel P-6 desalting cartridge, and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad (Hercules, CA). The GenAmp 9700 PCR thermocycler was obtained from Applied Biosystems (Foster City, CA). All UV−vis measurements were taken using a Cary 100 Bio UV−vis spectrometer from Agilent (Santa Clara, CA). Fluorescence spectra were measured using a Fluoromax-4 spectrometer from Horiba Scientific (Edison, NJ). All cells were lysed using an EmulsiFlex-C5 homogenizer manufactured by Avestin Inc. (Ottawa, ON). The Toyopearl AF-Chelate-640 column was obtained from Tosoh (Grove City, OH) and used to purify HepI. All ESI-MS spectra were recorded using a Thermo Scientific (Waltham, MA) ESI spectrometer. Previously, HepI assays were performed at 37 °C; however, HepI thermal
Substrate Isolation and Decylation. Both ADP-H and Lipid A were extracted from E. coli WBB06 cells (HepI and HepII knockout E. coli strain), as previously described. In brief, from a 4 L growth of fresh WBB06 cells, ADP-H was extracted with ethanol and placed over a DAEAE column to purify (yield of ~10 mg). KDO₂-Lipid A was extracted from 4 g of frozen dried down (cells were dried with acetone followed by diethyl ether) WBB06 cells using a 2:5:8 phenol/chloroform/petroleum ether solution (yield of ~50 mg). O-Deacylation of Lipid A was performed by stirring 60 mg of Lipid A with 6 mL of hydrazine for 1 h at 37 °C. The solution was then placed on ice, and 60 mL of cold acetone was added to precipitate ODLA followed by centrifugation for 30 min at 11000 rpm. The pellet was washed twice with cold acetone and once with diethyl ether, dissolved in water, and placed in a −80 °C freezer for 20 min to freeze the solution, followed by lyophilization to dry it. Decylation was confirmed by ESI mass spectroscopy in a 50:50 acetonitrile/water solution by observation of the half-mass (m/z 696 corresponding to the M⁻¹ – H complex).

Mutagenesis and Solubility Expression Optimization. E. coli pTOM HepI vector DNA was extracted from DH5α cells using the Zyppsy plasmid mini prep kit. Hep I tryptophan single mutants Trp217Phe, Trp199Phe, Trp194Phe, Trp116Phe, Trp62Phe, and Trp47Phe were prepared using the Zyppy plasmid mini prep kit. Hep I tryptophan single mutants except HepI Trp116Phe were the same as those of wild-type HepI previously described by Czyzyk et al. The Trp116Phe mutant was expressed in artic express cells at 10 °C and left to shake for 48 h. The rest of the purification procedure for Trp116Phe was the same as that for the wild type. Protein was stored at 4 °C, and assays were performed within 2 weeks of purification.

HepI Activity Assay. As previously reported, an ADP/NADH coupled assay was used to monitor Hep I activity by monitoring the absorbance change at 340 nm at 37 °C on a Cary spectrometer. Under normal conditions, the assay buffer consisted of 50 mM HEPES, 50 mM KCl, and 10 mM MgCl₂ (pH 7.5). High-salt assays were performed with 50 mM HEPES, 2 M KCl, and 10 mM MgCl₂ (pH 7.5). The coupled enzyme reaction mixture additionally contained 100 μM phosphoenolpyruvate, 100 μM NADH, 100 μM ADP-heptose, 100 μM ODLA, and both pyruvate kinase and lactate dehydrogenase at 0.005 unit/μL. Once a stable baseline was established, the reaction was initiated by addition of 50 nM enzyme, and all reported reaction rates are after background subtraction.

Circular Dichroism (CD). Protein thermal stability was assessed via CD melt analysis. Spectra were recorded in triplicate at 20 °C or as a function of increasing temperature (5–95 °C, in 5 °C increments) using quartz cuvettes (0.2 cm × 1 cm) containing 5 μM HepI and 100 μM ODLA in 10 mM Tris-HCl and 100 mM KCl (pH 7.5) buffer; melts were performed in the presence and absence of ODLA. For salt titration experiments, the KCl concentration was increased to 250 mM to 500 mM to 1 M to 2 M while keeping other components constant. All measurements were taken using a Jasco J-810 spectropolarimeter. Thermodynamic stability parameters of HepI-WT and mutants were calculated as previously reported by Mehl et al., using ellipticities at 211 and 222 nm (Table S3). The percent composition of secondary structure (α-helix and β-strand percentages) was determined using the K2D3 CD spectral analysis tool (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3//info/about.html).

Intrinsic Tryptophan Fluorescence Spectral Measurements. Fluorescence spectra were measured in triplicate at room temperature using 200 μL samples containing 1 μM HepI, in a 10 mM HEPES, 50 mM KCl, and 10 mM MgCl₂ (pH 7.5) buffer, using a 0.3 cm × 0.3 cm cuvette. Substrate concentrations were 100 μM ODLA and 100 μM ADP-Hep. All measurements were taken using a Fluoromax-4 fluorimeter with an excitation slit bandpass of 2 nm and an emission slit bandpass of 4 nm (λem = 290 nm, and λex = 310–450 nm). Spekwin32 (version 1.71.6.1) spectroscopy software was used to convert spectra to csv files, subtract spectra, and normalize data. Peak emission wavelengths were determined by taking the first derivative of each fluorescence spectrum using MATLAB (R2012B) with the λmax wavelength being determined from a linear fit of five points about zero.

Molecular Dynamics (MD) Simulation. The apo form HepI protein system (PDB entry 2GT1, chain A) was prepared for MD using the Schrodinger modeling package (version 2015.3, Schrodinger, LLC, New York, NY) and related utilities. Unless noted otherwise, all procedures were performed with Schrodinger’s Maestro GUI. Addition of unresolved missing atoms and hydrogens were modeled using Prime. Proper protonation of charged residues at pH 7.0 was assessed via PROPKA, ensuring an optimal hydrogen bonding network. The system was solvated in an orthorhombic TIP3 explicit water box with a 15 Å buffer region, followed by addition of sodium and chloride counterions to ensure electrostatic neutrality. The final salt concentration of the solvated, neutralized system was 0.15 M NaCl. Both system initialization (restrained coordinates) and subsequent MD production (100 ns, unrestrained coordinates) employed default settings and were conducted using the Desmond MD software (D. E. Shaw Research, New York, NY) with the OPLS-AA 2005 force field. Production stage dynamics were propagated using the RESP integrator with a time step of 2 fs. The isothermal–isobaric (NPT) ensemble at 300 K with Nose–Hoover chain thermostat and Martyna–Tobias–Klein barostat (isotropic) was used. Particle mesh Ewald accounted for long-range electrostatic interactions under periodic boundary conditions. A production run was performed on the Minnesota Supercomputing Institutes (MSI) Mesabi supercomputer resource with system coordinates collected every 4.8 ps.

Solvent Accessible Surface Area (SASA) Calculations. SASA values (square angstroms) were computed for the HepI reference apo form crystal structure (PDB entry 2GT1, chain A) and for the 20833 protein coordinates derived from the 100 ns HepI MD simulation using the Shake–Rupley algorithm with a probe radius of 1.4 Å. Only the aromatic Trp side-chain atoms (heavy and hydrogen) were considered in the calculation.

RESULTS AND DISCUSSION

Previously, our equilibrium and transient kinetic analyses of Trp fluorescence of HepI suggested that protein conformational changes were induced upon binding of ODLA. Biphase pre-steady state kinetics were observed over a range of
concentrations of ODLA, with the fast rate exhibiting a hyperbolic dependence on ODLA concentration (saturating to a concentration-independent process occurring at ~80 s^{-1}) and a concentration-independent slow rate of ~5 s^{-1}. Because a catalytically impaired mutant (D13A) of HepI yielded the same pre-steady state kinetics, we hypothesized that ODLA induces two conformational changes prior to catalysis: a grasping of the substrate likely caused by loop movements followed by a slower, larger conformational change.6 To better understand the protein configuration of the catalytic HepI Michaelis complex, we examined single-Trp mutants using fluorescence and CD spectroscopy to identify the regions undergoing these conformational changes.

Secondary Structural Changes and Thermal Unfolding of HepI. CD spectroscopy is a powerful tool for assessing the secondary structural elements of a protein under a variety of conditions.21-23 To assess the conformational states of HepI, CD spectra were obtained for apo HepI and HepI in the presence of either ODLA or ADPH (Figure 2). Apo HepI exhibits a double minimum with unequal intensity, with a sharp peak at ~222 nm and a small peak at 211 nm, characteristic of a protein with significant α-helical content. The CD spectrum obtained for HepI with ADPH bound resembles that of apo HepI; however, the spectrum for the HepI-ODLA complex has an approximately 12-fold increase in ellipticity at 211 nm. Analysis of the spectra of apo HepI and the HepI-ADPH complex with the K2D3 CD analysis Web server shows little change in the overall calculated α-helical or β-strand percentages upon ADPH binding (with the apo protein having 43.4% α-helical and 12.5% β-strand, as compared to 39.4% α-helical and 18.5% β-strand for the ADPH-bound enzyme).24 Consistent with the increase in the intensity of the peak minimum, the HepI-ODLA complex was calculated to have an approximately 14% increase in α-helical as compared to that of apo HepI [with the HepI-ODLA complex having 57.2% α-helical and 3.3% β-strand (see Table S3)].

Because the crystal structures of apo HepI and HepI with a fluorinated ADPH analogue (PDB entries 2GT1 and 2H1H, respectively) bound are virtually identical (root-mean-square deviation of 0.19 Å), no change in the CD spectra was expected. The similar CD spectra obtained for HepI and the HepI-ADPH complex are consistent with this expectation, support an “open form” structure for both species, and align with our earlier conclusion from fluorescence experiments that ADPH binding minimally changes HepI conformation.6 The increase in ellipticity at 211 nm in the presence of ODLA is also in keeping with our previously observed changes in the intrinsic fluorescence of HepI upon binding of ODLA, which suggested that the protein conformation changes upon formation of the HepI-ODLA complex. The CD results presented here suggest that binding of ODLA induces folding of loop regions into an α-helical structure to enhance binding interactions with ODLA. A similar substrate-induced increase in α-helicity has been observed previously for the glycosyltransferase N-acetylglucosaminyltransferase V, suggesting that an induced fit mechanism might be a general phenomenon for binding of a substrate to a Rossman-like domain.25 The observed increases in helicity upon ODLA binding suggest that the active site only fully assembles upon substrate binding; therefore, the use of the crystal structures presented here to design inhibitors is limited as the structures are static and do not encapsulate the specific conformational changes that are occurring.

To explore the relative stability of the ligand-bound forms, the thermodynamic stability of HepI in the presence and absence of substrates was assessed via a series of thermal denaturation CD experiments [performed from 5 to 95 °C, in 5 °C intervals (see Figure 3)]. As the protein unfolds, a loss of ellipticity is observed, signifying a loss of secondary structure; as the experiment is performed under equilibrium conditions, determination of the melting temperature (T_m) (Figure S1)26 allows us to assess whether the stability of the protein is altered by the presence of a ligand. From the CD melt experiment, apo HepI was shown to have a T_m of 40 °C and the HepI-ADPH complex a T_m of 47 °C (Figure 3A and Table S3). Interestingly, when ODLA was bound, it appears that HepI remains mostly folded (with a minimal change in ellipticity) even at 95 °C (Figure 3B). This suggests that ODLA is significantly stabilizing HepI, and because of the lack of unfolding, a T_m cannot be calculated for the HepI-ODLA complex and must be >100 °C. Surprisingly, heating of HepI to 95 °C in the presence of ODLA also did not lead to a significant loss of activity, underscoring that binding of ODLA maintains the protein in an active conformation at temperatures >50 °C above the apo T_m. We hypothesize that the stabilization of HepI is most likely caused by a conformational change upon ODLA binding that leads to the formation of hydrogen bonds and/or salt bridges (ionic interactions) within HepI and between HepI and ODLA. In contrast, ADPH alone does not have a major effect on protein stability. This finding is consistent with previously reported stopped-flow experiments and current CD results in which ADPH binding does not significantly alter the HepI conformation.6 Because the two substrates bind to different Rossmann-like domains of HepI (with ODLA binding in the N-terminal domain and ADPH binding in the C-terminal domain), this suggests that HepI stabilization arises predominantly from conformational changes in the N-terminal domain.

Without a priori information about where ODLA binds, examination of the ODLA structure suggests phosphates and carboxylate moieties in ODLA might coordinate to positively charged residues within HepI. To test for the presence of these hypothetical electrostatic binding interactions between HepI and ODLA, CD melt experiments were performed in the
If an increase in salt concentration disrupts these interactions formed between ODLA and HepI, we would anticipate an enhancement of melting of the HepI-ODLA complex. It was observed that at a high salt concentration (1 M KCl) there is a 70% change in the overall ellipticity at 222 nm over the temperature range monitored, as compared to the 30% change at 100 mM KCl. At 1 M KCl, there is sufficient melting of HepI to allow determination of a value of 64.7 ± 0.5 °C. To ensure that a higher salt concentration was not causing the protein to adopt a catalytically impaired or non-native conformation, reaction kinetics were determined at both 100 mM and 2 M KCl (the extremes described herein). Under the high-salt conditions, there was only a 10-fold decrease in the ODLA-dependent $K_m$ and a small increase in $k_{cat}$ relative to the values seen under the lower-salt conditions (Table S1). A slight increase in HepI stability was also observed in CD melts performed with no substrate present at high salt concentrations (Table S3). We suggest therefore that HepI is stabilized by ionic interactions (from either ODLA or salt) and ODLA affinity is impacted by the high salt concentration. The nearly 2-fold increase in the level of observable melting at 250 mM KCl suggests that a high-salt environment reduces HepI-ODLA stability and facilitates unfolding, which suggests that ionic interactions between ODLA and HepI must have been at least partially disrupted. Additionally, it has previously been shown that salts can have a stabilizing effect on $\alpha$-helices and proteins. We therefore examined the $\alpha$-helicity at a high salt concentration (1 M KCl) and observed that it was relatively unchanged from the salt concentration of 100 mM described above for the apo protein (40.2% $\alpha$-helical and 17.0% $\beta$-strand). This suggested that the changes in behavior are the result of substrate-induced changes rather than from a change in protein conformation induced by salt. Furthermore, the HepI stability ($T_m$) did not revert to what was observed for the apo protein at high salt concentrations, as HepI coordination of ODLA probably also involves other interactions, such as hydrogen bonds and hydrophobic interactions, in addition to the electrostatic interactions (based upon its structure). Consequently, the high salt concentration can only partially disrupt the stabilization effects caused by ODLA binding (Table S3). Isolating the Protein Conformational Changes Causing HepI Stabilization. Building upon an extensive literature describing the use of tryptophan (Trp) residues to report on conformational changes upon substrate binding, we saw HepI, with its eight Trp residues, as an ideal candidate for exploring the protein dynamics of a GT-B protein. Previously, we observed a blue shift in the intrinsic tryptophan (Trp) fluorescence spectrum of HepI upon binding ODLA, which suggests that one or more Trp residues move from a more polar to a more nonpolar or hydrophobic environment upon formation of the HepI-ODLA complex. While there are many factors that can affect tryptophan fluorescence such as solvent polarity, conformational changes, hydrogen bonding, excited state reactions, and tryptophan–tryptophan interactions, we hypothesized that this spectral change could allow us to understand the HepI protein dynamics that occur prior to catalysis. We noted that HepI contains eight Trp residues at positions 35, 47, 62, 66, 116, 194, 199, and 217 and elected to study how each Trp residue contributes to the fluorescence and, by inference, protein dynamics (Figure 1). In advance of mutagenesis, we performed a solvent accessibility surface area (SASA) analysis of the apo crystal structure, as well as of the
ensemble of spatial orientations adopted throughout a 100 ns MD simulation to identify the Trp residues most likely to contribute to the observed fluorescence change (Table S4 and Figure 5). Figure 5 shows that most Trp residues have small deviations in their geometries as compared to the crystal structure with the exception of Trp66 and Trp194, both of which have large changes and therefore are very dynamic and/or more solvent accessible than would be predicted by the crystal structure (Table S4). Trp35 was observed to have zero or near-zero solvent accessibility either in the crystal structure or in the simulation. Because Trp35 is already fully in a hydrophobic environment, we judged it unlikely to contribute to the observed changes in intrinsic HepI fluorescence, and therefore, we chose not to mutate this residue.

Driven by concern about protein alterations that might result from removing all of the Trp residues simultaneously and building upon precedent in the literature for more conservative approaches for studying proteins with many Trp residues,30,38,40−44 we individually mutated each of the remaining seven Trp residues to phenylalanine (Phe). All of the point mutants were tested for alterations in kinetics, thermal stability, and fluorescence spectra relative to those of HepI-WT. Reaction kinetics for each of the Trp mutants showed little change, with only slightly diminished $k_{cat}$ and slightly increased $K_m$ (Table S1). Given that none of the Trp residues are near the active site, it was anticipated that catalysis would not be dramatically impaired. Additionally, CD spectra for all mutants with and without ODLA were recorded and resulted in spectra similar to those of the wild type, implying that all mutants are folded and bind the substrates normally. Interestingly, the CD melts revealed that the thermal stabilities of all apo mutants, except W47F and W194F, were increased relative to that of the wild type (Table S3), perhaps suggesting that the remaining Trp residues are dynamic in the protein. Because of the minimal impact of mutagenesis on the kinetics and thermodynamics, we reasoned therefore that any changes observed in the fluorescence of these mutants would be due specifically to the removal of that tryptophan and not because of changes in the general behavior or folding of the mutant enzymes.

To assess the contribution of each Trp to the overall fluorescence spectrum of HepI, individual Trp fluorescence spectra were generated by subtracting those obtained for each mutant protein from that of HepI-WT (Figure 6). This analysis of Trp fluorescence has widely been used to approximate the contribution of a single Trp to the spectra of a protein.40,41,45,46 However, it is important to note that this analysis may not account for any of the complex interactions that may occur between each Trp residue and other residues within the protein, including Trp residues being quenched and/or having spectral shifts caused by neighboring phenylalanine, tryptophan, or charged residues.47,48 As shown in the case of the fluorescence spectra of the bacterial ribonuclease Barnase, energy transfer between proximal tryptophan residues was observed; additionally, a Trp residue was quenched by a nearby charged His residue.45,48 Because there are multiple Trp residues proximal to each other in HepI, as well as lysine and arginine residues adjacent to some Trps, similar complex interactions could impact our analysis. As shown in Figure 6, Trp62, Trp116, and Trp217 contribute most significantly to HepI's fluorescence,48 with Trp199 making a smaller but non-zero contribution. The three remaining residues (Trp47, Trp66, and Trp194) all make either a very slight contribution or a negative contribution to the total protein fluorescence. Trp66 and Trp194 are both in the proximity of other Trp residues (Trp62 and Trp199, respectively), which could result in some fluorescence quenching from the interactions with these neighboring tryptophans.45

To examine how each Trp mutation contributed to the overall ODLA-induced fluorescence spectral changes of HepI,
\( \lambda_{\text{max}} \) values were determined for all mutants in the apo form and with ODLA, ADPH, and both substrates bound (Table 1 and Figure S3). On the basis of our prior results and those of others, Trp residues located in environments that are more nonpolar are blue-shifted relative to those in more polar and/or solvated environments.3949 Observing the changes in fluorescence wavelengths (or lack of changes) for a given mutant would allow us to determine the specific impact of substrate binding on the solvation state of that Trp residue. While the fitting of the data allowed the determination of \( \lambda_{\text{max}} \) with high precision, all values were rounded to the nearest integer value to account for the intrinsic experimental error introduced by our acquisition parameters. For all mutants, when ADPH was bound, the fluorescence spectra were virtually identical to that of the apo enzyme, as was observed for HepI-WT. In contrast, the binding of ODLA resulted in much larger changes to the peak emission wavelengths (Figure 7, Figure S3, and Table 1). Removal of Trp47 resulted in \( \lambda_{\text{max}} \) values nearly identical to that of the wild type under all conditions. The mutation of this residue resulted in an increase in the overall fluorescence intensity relative to that of HepI-WT, and the \( \lambda_{\text{max}} \) wavelengths for Trp47Phe (for the apo form or the ODLA complex) are also virtually unchanged (any \( \lambda_{\text{max}} \) with a change of \( \pm 1 \) nm relative to that of HepI-WT was considered identical). This result suggests that Trp47 has little or no role in the overall ODLA-induced blue shift.

Trp66Phe and Trp194Phe exhibited \( \lambda_{\text{max}} \) changes in the apo form relative to that of HepI-WT, even though the HepI-ODLA complex peak wavelengths were unchanged relative to HepI-WT. Both of these mutants exhibit a 3 nm blue shift in \( \lambda_{\text{max}} \), compared to that of the wild type, which suggests these residues reside in a highly polar environment in apo HepI-WT and contribute to the overall emission maximum of the WT protein. For the Trp116Phe and Trp199Phe mutants, both exhibit peak wavelengths similar to that of apo HepI-WT in their apo forms; however, neither of these proteins demonstrates wild-type-like blue shifts upon ODLA binding. The partial blue shifts exhibited by these two mutants suggest that these Trp residues are minor contributors of the ODLA-induced conformational changes. In the apo forms, Trp62Phe, Trp66Phe, and Trp194Phe exhibit a blue shift in peak emission resulting in a smaller wavelength shift relative to that of HepI-WT, like the Trp116Phe and Trp199Phe mutants. Interestingly, Trp62Phe, Trp116Phe, and Trp199Phe all exhibit peak \( \lambda_{\text{max}} \) values that are red-shifted relative to that of HepI-WT upon ODLA binding, suggesting that all three residues contribute to the observed blue shift. These results indicate that apo HepI adopts a conformation in which Trp62 is in a polar environment and moves to a more hydrophilic environment upon ODLA binding, making Trp62 a contributor to the overall fluorescence change. The remaining mutant, Trp217Phe, has a \( \lambda_{\text{max}} \) value that is unchanged relative to that of HepI-WT as the apo protein and, surprisingly, upon ODLA binding exhibits a red shift (4.7 nm) relative to that of HepI-WT. This is the most significant effect observed from mutagenesis and suggests that Trp217 is the major contributor to the ODLA-induced blue shift because its removal eliminates the ability to observe ODLA binding (Figure 7 and Figure S3).

ODLA binding-induced changes in the behavior of tryptophan residues in the N-terminus (Trp62, Trp66, and Trp116) were anticipated a priori because the sugar acceptor substrates have been shown in other systems to bind to the N-terminal domain.15,50,51 Mutation of Trp62 and Trp116 produced no change in their apo fluorescence spectra from that of the wild type but yields a red shift in \( \lambda_{\text{max}} \) relative to that of HepI-WT upon binding of ODLA; thus, we consider Trp62 and Trp116 to be local reporters of ODLA binding. These residues are adjacent to positively charged amino acids that might be interacting with the phosphates and carboxylates of ODLA. This would then likely result in an internalization of these residues upon binding. Taken together, these spectral changes provide insights into the regions that may drive the increase in \( \alpha \)-helicity observed in our CD experiments triggered by formation of the HepI-ODLA complex. Further investigations to discern the residues important for binding and the associated conformational changes are ongoing.

What is most unexpected is that both Trp199 and Trp217 are in a more nonpolar environment upon binding of ODLA, as determined from the peak emission of the point mutants. Mutagenesis of Trp217 (and Trp199) was not expected to impact the fluorescence spectra on the basis of their location in the C-terminal domain because ODLA binds to the N-terminal domain of HepI. Interestingly, these residues in the C-terminal domain are located proximal to the ADPH binding site. While it is possible that the mutation of Trp217 to a hydrophobic residue (Phe) resulted in small structural changes to the

**Table 1. Trp Fluorescence Emission Maxima (\( \lambda_{\text{max}} \)) of HepI-WT and Trp Mutants**

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<td>HepI</td>
<td>340</td>
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<tr>
<td>W47F</td>
<td>340</td>
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<tr>
<td>W62F</td>
<td>337</td>
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<tr>
<td>W66F</td>
<td>337</td>
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<td>W116F</td>
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<td>W194F</td>
<td>337</td>
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<tr>
<td>W199F</td>
<td>341</td>
</tr>
<tr>
<td>W217F</td>
<td>340</td>
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</table>

*Spectra were recorded with 1 mM HepI at 25 °C, and samples were excited at 290 nm. The excitation slit width was 2 nm, and the emission slit width was 4 nm. All values have an associated error ±1 nm.*

**Figure 7.** Degree of blue shift upon ODLA binding by calculating the change between apo and ODLA spectra. The fractional blue shift was determined to be [(apo mutant – ODLA mutant)/(apo WT – ODLA WT)]. Note that Trp217Phe has a red shift, and therefore, no blue shift is indicated.

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adjacent amino acids ultimately altering the intrinsic fluorescence, because the apo protein behaved like HepI-WT, this seems unlikely. The observation that mutagenesis of Trp217, which is located directly adjacent to ADPH but is >21 Å from the catalytic Asp13 residue that deprotonates the nucleophile, has the most pronounced impact on the blue shift shows that the C-terminal domain residues are altered by the binding of a substrate to the N-terminal domain. This indicates that these two binding sites might be communicating their occupancy to each other via conformational changes. Perhaps binding of ODLA leads to the closing of HepI, ultimately by burying Trp217 through loop–loop interactions between the two domains as they become proximal. Dramatic changes observed in the Trp217Phe mutant suggest that the true closed structure may even be more “closed” then our model suggests (Figure 1B). Because crystal structures of GT-B structural enzymes that are unliganded (apo enzymes) uniformly adopt an open structural conformation, while the few closed GT-B structures are of liganded complexes, it is clear that the occupancy of the ligand binding sites impacts the overall conformational state of the enzyme.6–11 These results provide the first evidence of GT-B enzymes having interdomain alterations related to the occupancy of a substrate binding domain. Further investigations examining the conformational dynamics (both in silico and in vitro) are ongoing, and we hope to elucidate the origin of the scarcity of GT-B protein structures that adopt the closed conformation.

CONCLUSIONS

To effectively inhibit an enzyme, understanding not only its reaction but also its dynamics is essential.51 Often, crystal structures allow us to deduce static changes in an enzyme upon substrate binding. Unfortunately, these structures are not always available. Here, we were able to use circular dichroism and intrinsic tryptophan fluorescence spectra to observe conformational changes in HepI. CD experiments in the presence and absence of ODLA suggest that the HepI active site might not be fully formed in the apo protein, and that conformational changes that increase the overall α-helicity of the protein are necessary for formation of the Michaelis complex. This is an important insight that can help rationalize the dearth of potent inhibitors for glycosyltransferase enzymes, despite prior in silico drug design efforts.50,52,53

Additionally, seven Trp mutants were examined to identify the regions of HepI that are altered upon ligand binding. W62F and W116F, which are located in the N-terminal ODLA binding domain, exhibited smaller changes in peak fluorescence upon ODLA binding, compared to that of WT-HepI. These residues are located on dynamic loops (N-3 and N-7), and it is hypothesized that binding of ODLA results in a conformational change that stabilizes these loops, which potentially changes the local environment of these residues. We hypothesize on the basis of the investigations of the HepI stability changes in conjunction with the fluorescence behavior of these mutants, that the dynamic N-3 and N-7 loops bring positively charged amino acid residues proximal to the negatively charged regions of ODLA (phosphates and carboxylates) to form salt bridges that can be destabilized with a high salt concentration. The fluorescence and CD melt experiments perhaps suggest loop movements that are consistent with our previously observed ∼80 s−1, concentration-independent, blue shift from our pre-steady state kinetic experiments.5 Additionally, these experiments suggest that HepI functions through either an ordered mechanism (ODLA must bind second for chemistry to occur) or a dynamic mechanism where the enzyme is converting between open and closed states, where binding of ODLA shifts the thermodynamic landscape to favor the closed conformational state.

Surprisingly, mutation of Trp217 resulted in a complete loss of the blue shift upon substrate binding. We hypothesize that Trp217 could be making contact with the N-terminal loops directly below it (where both Trp62 and Trp66 are located) or could be experiencing changes in its dynamic motions (propagated throughout the protein) as a result of ODLA binding; additional studies would be necessary to clearly understand how ODLA binding is impacting Trp217. While the available crystal structures suggest that the C-terminal domain is relatively static regardless of whether the sugar donor substrate is bound, these data suggest that ODLA binding leads to changes in the protein that are widespread and not solely confined to the N-terminal domain. This study has helped to isolate regions important for formation of the enzyme-substrate complex. In addition, there is now evidence that heptosyltransferase I utilizes an induced fit model for ligand binding that involves rearrangements of both N- and C-terminal domains and may shed light on regions of the protein to target for development of an inhibitor of heptosyltransferase I.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00850.

Primer sequences used for site-directed mutagenesis and additional kinetic, circular dichroism, and fluorescence data (PDF)

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Notes

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ABBREVIATIONS

GT, glycosyltransferase; HepI, heptosyltransferase I; heptose, 1-glycero-3-manno-heptose; ADP-heptose or ADPH, ADP-1-glycero-3-manno-heptose; LPS, lipopolysaccharide; ODLA, O-deacylated E. coli Kdo2-Lipid A; CD, circular dichroism; MD,
molecular dynamics; SASA, solvent accessible surface area; PDB, Protein Data Bank.

**REFERENCES**


