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Escherichia coli Heptosyltransferase I: Investigation of Protein Dynamics of a GT-B Structural Enzyme

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Abstract

Heptosyltransferase I (HepI), the enzyme responsible for the transfer of L-glycero-D-manno-heptose to a 3-deoxy-α-D-oct-2-ulopyranosonic acid (Kdo) of the growing core region of lipopolysaccharide (LPS), is a member of the GT-B structural class of enzymes. Crystal structures have revealed open and closed conformations of apo and ligand-bound GT-B enzymes, implying that large-scale protein conformational dynamics play a role in their reaction mechanism. Here we report transient kinetic analysis of conformational changes in HepI reported by intrinsic tryptophan fluorescence, and present the first real time evidence for a GT-B enzyme undergoing substrate binding-induced transition from an open to closed state prior to catalysis.

The current widespread use and misuse of antimicrobials has led to the emergence of bacterial resistance to many commonly used antibiotics, necessitating development of new drug targets.[1] Lipopolysaccharides (LPS), a major constituent of the Gram-negative bacterial outer membrane, important for cell motility, intestinal colonization and bacterial biofilms formation, contribute substantively to antibiotic resistance by hampering antibiotic uptake.[2,3,4] These characteristics have spurred research into the development of inhibitors of the LPS biosynthetic pathway.[4] Heptosyltransferase I (HepI) catalyzes the first step in LPS biosynthesis following the lipid functionalization of Kdo2-LipidA (Scheme S1).[4,5] Blocking the addition of an L-*glycero*-D-*manno*-heptose to Kdo2-LipidA results in increased bacterial sensitivity to hydrophobic antibiotics and phagocytosis by microphages, thus, HepI is considered an excellent target for inhibitor design.[6]

HepI like all members of the GT-B glycosyltransferase structural family is characterized by having two βαβ Rossman-like domains connected by a linker region.[5,7] Several members of the GT-B family, including MshA, MurG, and GtfA, have been structurally characterized in open and closed conformations depending upon substrate occupancy.[8–11] Currently the crystal structure of apo HepI, as well as structures of HepI with ADP and an ADP-L-*glycero*-D-*manno*-Heptose (ADP-Hep) analogue in the donor binding site are available (PDB codes: 2H1F, 2H1H, and 2GT1); however, structures of HepI with LipidA or both

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ASSOCIATED CONTENT

Supporting Information

HepI structures showing Trp movement and distances, viscosity data, additional concentration-dependent traces and detailed experimental conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

substrates bound have not yet been determined.[5] In each of the three structures, HepI adopts an open conformation (Figure 1). The catalytic residue Asp13, which extracts a proton from LipidA to generate an oxyanionic nucleophile, is more than 8 Å away from the anomeric carbon of ADP-Hep (Figure S1).[5] Based upon these structural data, we hypothesized that HepI needs to adopt a closed conformation in order to bring the substrates into closer proximity, exclude water from the active site and bring Asp13 into a catalytically competent position (Figures 1 & S1). Here we report the first real time kinetic evidence for a GT-B enzyme undergoing conformational change on binding its substrate.

In order to assess whether large scale protein dynamics are important for catalysis, we investigated the impact of buffer viscosity on HepI steady state activity.[12] Specifically, the effects of microviscogens glycerol and ethylene glycol on HepI k_{cat} (0.3 s^{-1}) were compared with the effect of macroviscogen PEG 8000. The data, plotted as the log of reciprocal relative rates versus log of relative viscosity, yielded δ values of 0.93, 1.0, and 0.12 for glycerol and ethylene glycol and PEG 8000, respectively (Figure S2). According to Kramers' theory, a value of one indicates tight coupling of the active site to solvent while a value near zero predicts an enzyme active site that is largely uncoupled to solvent.[13] The high impact of the microviscogens glycerol and ethylene glycol on k_{cat} indicates that water reorganization is critical for catalysis, suggesting that HepI conformational dynamics are partially rate limiting in the reaction.[13,14] The small alteration in k_{cat} in an equally viscous PEG 8000 solution suggests that the observed effects are not due to solution vitrification.

Since HepI contains eight tryptophan residues (Figure S3), we explored the possibility of using intrinsic protein fluorescence to monitor conformational changes upon substrate binding to the enzyme. Based on our previous finding that deacylated analogues of Kdo2-LipidA improve catalytic efficiency and are also more soluble,[4] we utilized the O-deacylated Kdo2-LipidA (ODLA) analogue in this study instead of the more lipophilic native substrate. Fluorescence spectra were obtained for HepI in the absence and presence of one or both of its substrates (λ_{EX} = 290 nm). Apo HepI and HepI with ADP-Hep have identical fluorescence spectra under equilibrium conditions (Figure 2A). In contrast, the HepI spectra in the presence of ODLA exhibit a significant blue shift (with or without ADP-Hep), indicating movement of one or more of the tryptophans into a relatively nonpolar environment on binding of this substrate.[15] These tryptophans are likely to be among four surface exposed residues in the N-terminal domain (W47, W62, W66 or W116), all of which are predicted to undergo movements of greater than 7 Å from their locations in the open apo enzyme structure to those in the closed conformation, based upon our closed model structure (Figures 1 and S3). The relative independence of ODLA and ADP-Hep substrate binding effects on protein conformation is unsurprising since each βαβ Rossman-like domain binds to only one substrate (ODLA binds to the N-terminal domain and ADP-Hep binds to the C-terminal domain).[5]

We performed stopped-flow experiments next, in order to determine the kinetics of this conformational change in HepI and its place in the reaction mechanism. HepI was mixed rapidly with the substrates and the resulting change in tryptophan fluorescence was monitored over time (note: a 350 nm cut-on filter was used, hence there is an apparent decrease in fluorescence intensity on ODLA binding to HepI). Consistent with the results in Figure 2A, there is no detectable detectable change in signal when HepI is mixed with buffer or ADP-Hep alone (Figure 2B). With ODLA, there is a biphasic decrease in signal, which yields a fast rate of 21 s^{-1} and a slower rate of 5 s^{-1} at 1.5 μM ODLA, when fit to a double exponential function.

The concentration dependence of ODLA binding to HepI was then investigated over a 1 – 100 μM range of this substrate. The rate of the fast phase increased from 1 μM to about 5

μM, but then became independent of ODLA concentration (Figure 2C); note: at ODLA concentrations higher than 20 μM there is some signal interference likely due to light scattering caused by the lipid. The rate of the second slow phase remained almost constant at ~ 5 s^{-1}, and then increased slightly at > 10 μM ODLA. A plot of these rates versus ODLA concentration is shown in Figure 3A. The fast rate shows hyperbolic dependence on ODLA concentration, saturating at k_{fast} of 80 ± 7 s^{-1}, with a dissociation constant $K_{1/2}$ of 3 ± 0.7 μM. This result suggests a two-step binding mechanism in which formation of the initial collision complex between HepI and ODLA is followed by rate-limiting isomerization to form the final HepI-ODLA complex (Scheme 1). Since the $K_{1/2}$ value is comparable to our previously reported ODLA K_m of 0.9 μM,[4] we can make the assumption that ODLA binding to HepI is in rapid equilibrium in this two-step model. In this case, $K_{1/2}$ is equivalent to K_{D1}, the saturating rate is $k_2 + k_{-2}$, and the y-intercept is k_{-2} (estimated at < 5 s^{-1}).[16] The apparent bimolecular rate constant for ODLA binding can be estimated as k_{on} ~ 2.5×10^7 M^{-1} s^{-1} (k_2/K_{D1}); the slope of the initial linear phase yields the same value. The overall dissociation constant is estimated as 0.2 μM ($K_{D1}k_{-2}/k_2$), which is comparable to the K_D of 0.6 ± 0.1 μM obtained from hyperbolic dependence of the signal amplitude on ODLA concentration (Figure S4).

The second, slower change in intrinsic protein fluorescence cannot be interpreted unambiguously at present. Since the rate remains nearly constant (~ 5 s^{-1}) until higher ODLA concentrations (and potentially higher error in signal), this phase may reflect subsequent conformational change in the enzyme (Figure 3A). However, the signal may also originate from a HepI subpopulation or an off-pathway species. Further investigation of the reaction mechanism is needed to resolve this question.

Similar experiments performed in the presence of 50 μM ADP-Hep yielded the same hyperbolic dependence of the fast rate on ODLA concentration, and almost the same kinetic parameters for the binding mechanism (Figures 3B and S5A; $K_{1/2}$ of 3.9 ± 1 μM and k_{fast} of 87 ± 9^{-1}). This finding confirms that the lipid substrate is responsible for inducing the observed conformational change in the enzyme. Similar experiments performed with a catalytically inactive mutant, HepI D13A, with or without ADP-Hep, also yielded the same results (Figures 3C, S5B–S5C, S6; $K_{1/2}$ of 2.6 ± 0.7 μM and k_{fast} of 93 ± 8 s^{-1}, with ADP-Hep; $K_{1/2}$ of 3.6 ± 0.9 μM and k_{max} of 97 ± 9 s−1, without ADP-Hep), Thus, the ODLA-induced change in HepI occurs prior to chemistry. While limiting formation of the enzyme-substrate complex, this isomerization is fast relative to k_{cat} (0.3 s^{-1}), implying that other step(s), including conformational dynamics, before or after chemistry limit catalytic turnover. Further studies with a series of HepI mutants, including individual Trp to Phe substitutions, are underway to more precisely determine the origin of the observed fluorescence signal and the nature of the conformational changes in the enzyme.

In summary, we have demonstrated the utility of intrinsic protein fluorescence as an in-solution reporter of HepI conformation, by monitoring rapid substrate-dependent conformational changes that were predicted from crystal structures of the GT-B structural family members. This is the first kinetic analysis of substrate binding to a GT-B enzyme, as reported by the enzyme itself. The findings elucidate initial steps in the reaction, and reveal a slow step after substrate binding that may limit catalysis. The information is of particular significance for understanding protein dynamics in GT-B enzymes and for designing inhibitors against HepI and other related glycosyltransferases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

LPS	Lipopolysaccharides
HepI	HeptosyltransferaseI
ADP	adenosine diphosphate
Hep	L-*glycero*-D-*manno*-Heptose
Kdo	3-deoxy-α-D-oct-2-ulopyranosonic acid
NAD+	Nicotinamide adenine dinucleotide
ODLA	O-deacylated Kdo2-LipidA

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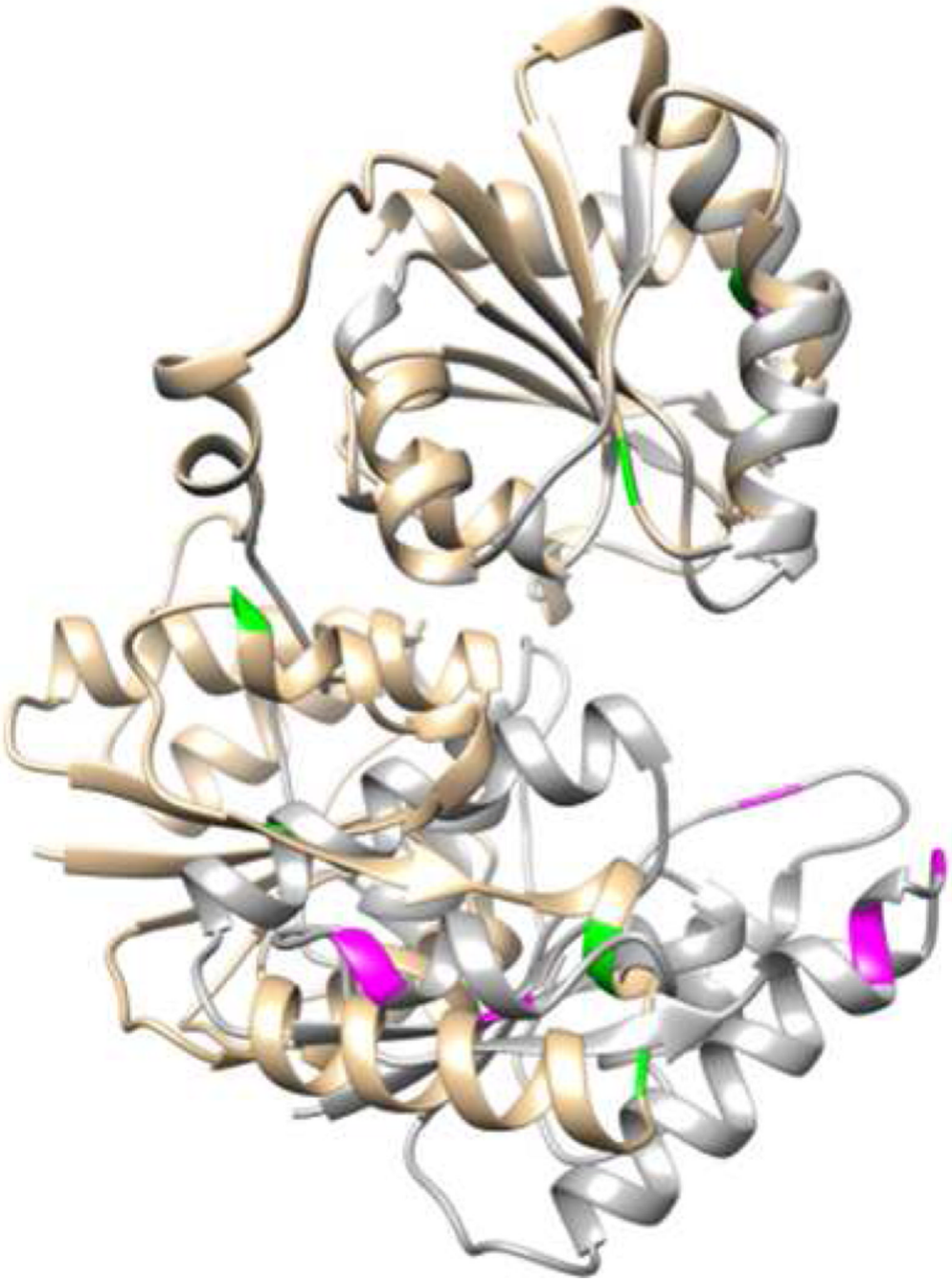

Figure 1.
HepI open structure in tan, with tryptophan residues denoted in green, superimposed with a structural model of closed HepI in grey, with tryptophans in magenta (methods for closed structural model building are found the supporting information).

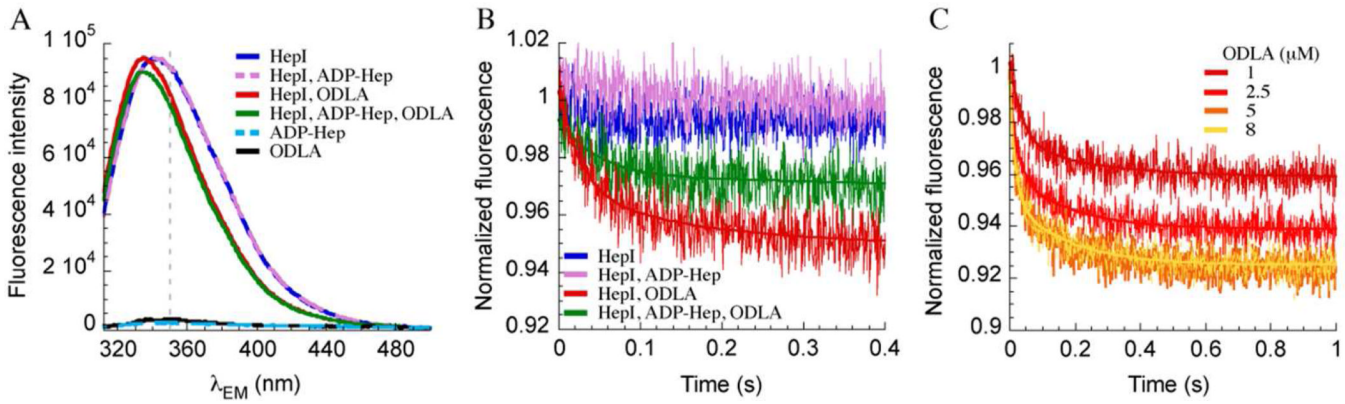

Figure 2.

Change in HepI intrinsic fluorescence induced by ODLA binding. (A) Emission spectra of HepI with ODLA ± ADP-Hep are blue shifted when compared with apo HepI (neither substrate contributes significantly to the signal). The gray dashed line indicates the 350 nm cut-on filter boundary. (B) Stopped-flow traces show rapid, biphasic decrease in HepI fluorescence when mixed with ODLA alone (1.5 μM) or with ODLA plus ADP-Hep (1.5 μM and 50 μM, respectively), but no change with ADP-Hep alone. (C) Representative traces from a titration of HepI with ODLA. The data were fit with a double exponential function to determine the rate constants for Figure 3.

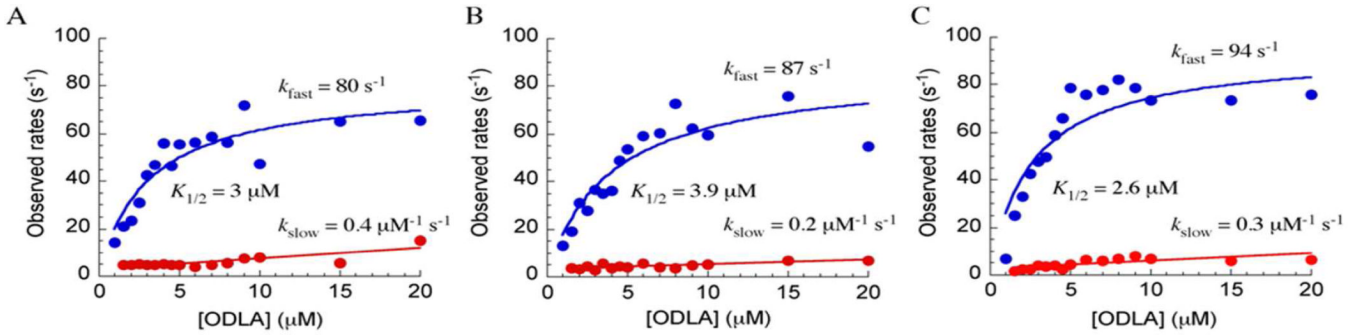

Figure 3.

Kinetics of ODLA binding and associated change in HepI conformation (fast phase: blue; slow phase: red). (A) Observed rates from WT HepI titrated with ODLA, (B) WT HepI pre-incubated with 50 μM ADP-Hep and titrated with ODLA, and (C) HepI D13A pre-incubated with 50 μM ADP-Hep titrated with ODLA (data for HepI D13A without ADP-Hep are shown in supplementary Figure S5).

Scheme 1.
Two step binding of ODLA with HepI