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

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Supporting Information

ABSTRACT: Heptosyltransferase I (HepI), the enzyme responsible for the transfer of L-*glycero*-D-*manno*-heptose to a 3-deoxy-α-D-*manno*-oct-2-ulopyranosonic acid (Kdo) of the growing core region of lipopolysaccharide, 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 of a GT-B enzyme undergoing a 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 biofilm formation, contribute substantively to antibiotic resistance by hampering antibiotic uptake.[2−4] These characteristics have spurred research on inhibitors of the LPS biosynthetic pathway.[4] Heptosyltransferase I (HepI) catalyzes the first step in LPS biosynthesis following the lipid functionalization that produces Kdo_2-LipidA (Scheme S1, Supporting Information).[4,5] Blocking the addition of an L-*glycero*-D-*manno*-heptose to Kdo_2-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 two $\beta\alpha\beta$ 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 and structures of HepI with ADP and an ADP-L-*glycero*-D-*manno*-heptose (ADP-Hep) analogue in the donor binding site are available (Protein Data Bank entries 2H1F, 2H1H, and 2GT1, respectively); however, structures of HepI with Kdo_2-LipidA or both 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 Kdo_2-LipidA to generate an oxyanionic nucleophile, is more than 8 Å from the anomeric carbon of ADP-Hep (Figure S1).[5] On the basis of these structural data, we hypothesized that HepI needs to adopt a closed conformation to bring the substrates closer together, exclude water from the active site, and bring Asp13 into a catalytically competent position (Figure 1 and Figure S1). Here we report the first real-time kinetic evidence of a GT-B enzyme undergoing conformational change upon binding its substrate.

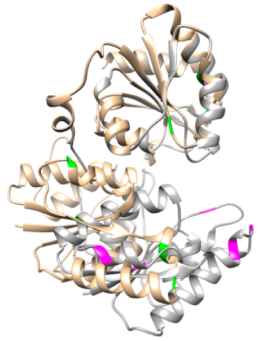

Figure 1. HepI open structure colored tan, with tryptophan residues colored green, superimposed with a structural model of closed HepI colored gray, with tryptophans colored magenta (methods for closed structural model building are found in the Supporting Information).

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 the HepI k_{cat} were compared with the effect of macroviscogen PEG 8000. The data, plotted as the log of reciprocal relative rates versus the log of relative viscosity, yielded δ values of 0.93, 1.0, and 0.12 for glycerol, ethylene glycol, and PEG 8000, respectively (Figure S2). According to Kramers' theory, a value of 1 indicates tight coupling of the active site to solvent while a value near zero

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predicts an enzyme active site that is largely uncoupled from solvent.[13] The strong 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 binding of the substrate to the enzyme. On the basis of our previous finding that deacylated analogues of Kdo_2-LipidA improve catalytic efficiency and are also more soluble,[4] we utilized the O-deacylated Kdo_2-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 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, and W116), all of which are predicted to undergo movements of >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 the effects of ODLA and ADP-Hep substrate binding on protein conformation is unsurprising because each $\beta\alpha\beta$ Rossman-like domain binds to only one substrate (ODLA binds to the N-terminal domain and ADP-Hep to the C-terminal domain).[5]

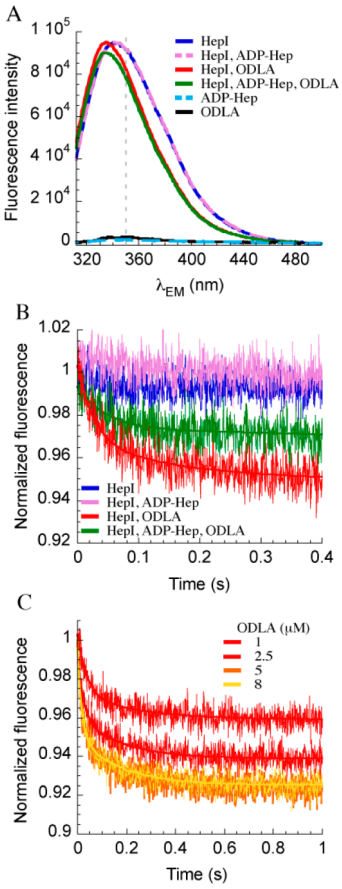

Figure 2. Change in HepI intrinsic fluorescence induced by O-deacylated Kdo_2-LipidA (ODLA) binding. (A) Emission spectra of HepI with ODLA with or without ADP-Hep are blue-shifted when compared with those of apo HepI (neither substrate contributes significantly to the signal). The gray dashed line indicates the 350 nm cut-on filter boundary. (B) Stopped-flow traces show the rapid, biphasic decrease in the fluorescence of HepI when it is mixed with ODLA (1.5 μM) or with ODLA and ADP-Hep (1.5 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.

We performed stopped-flow experiments next, 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 that a 350 nm cut-on filter was used; hence, there is an apparent decrease in fluorescence intensity upon binding of ODLA to HepI). Consistent with the results depicted in Figure 2A, there is no detectable change in signal when HepI is mixed with buffer or ADP-Hep alone (Figure 2B). With ODLA, there is a biphasic decrease in the magnitude of the 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 binding of ODLA to HepI was then investigated over a substrate concentration range of 1–100 μM. The rate of the fast phase increased from 1 to ~5 μM but then became independent of ODLA concentration (Figure 2C); note that at ODLA concentrations of >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 a hyperbolic dependence on ODLA concentration, saturating at a 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). Because 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 binding of ODLA 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 to be <5 s^{-1}).[16] The apparent bimolecular rate constant for ODLA binding (k_{on}) can be estimated to be ~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 to be 0.2 μM ($K_{D1}k_{-2}/k_2$), which is comparable to the K_D of 0.6 ± 0.1 μM obtained from the 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. Because the rate remains nearly constant (~5 s^{-1}) until higher ODLA concentrations are reached (and potentially greater error in the signal), this phase may reflect the 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

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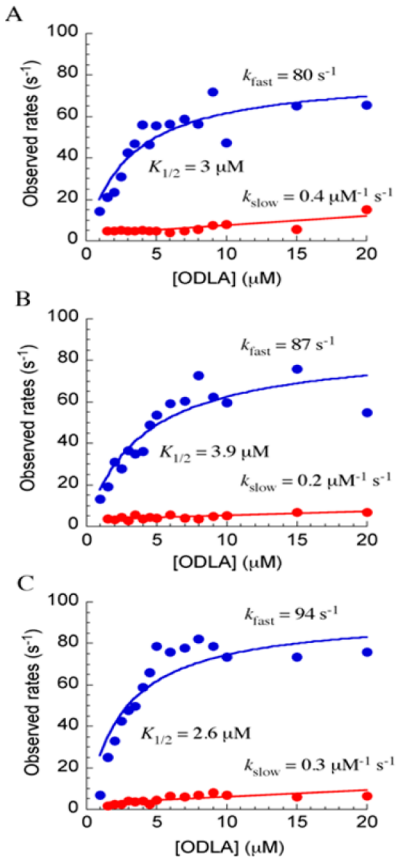

Figure 3. Kinetics of ODLA binding and associated change in HepI conformation (fast phase, blue; slow phase, red). Observed rates of (A) wild-type (WT) HepI titrated with ODLA, (B) WT HepI preincubated with 50 μM ADP-Hep and titrated with ODLA, and (C) HepI D13A preincubated with 50 μM ADP-Hep titrated with ODLA (data for HepI D13A without ADP-Hep are shown in Figure S5).

Scheme 1. Two-Step Binding of ODLA to HepI

$$E + ODLA \underset{K_{D1}}{\rightleftharpoons} E{\bullet}ODLA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} {*}E{\bullet}ODLA$$

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 s^{-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,C, and 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 steps, 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 binding of substrate 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.

■ 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.

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Notes

The authors declare no competing financial interest.

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