ESI-MS Assay of *M. tuberculosis* Cell Wall Antigen 85 Enzymes Permits Substrate Profiling and Design of a Mechanism-Based Inhibitor

Conor S. Barry,† Keriann M. Backus,‡ Clifton E. Barry, III,§ and Benjamin G. Davis*†

†Department of Chemistry, University of Oxford, Chemistry Research Laboratory, Mansfield Road, Oxford OX1 3TA, U.K.
‡Tuberculosis Research Section, Laboratory of Clinical Infectious Diseases, U.S. National Institute of Allergy and Infectious Disease, Bethesda, Maryland, United States
§Supporting Information

ABSTRACT: *Mycobacterium tuberculosis* Antigen 85 enzymes are vital to the integrity of the highly impermeable cell envelope and are potential therapeutic targets. Kinetic analysis using a label-free assay revealed both mechanistic details and a substrate profile that allowed the design and construction of a selective in vitro mechanism-based inhibitor.

*Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis, is among the foremost causes of death and morbidity worldwide. It persists within the host with the aid of a complex and highly impermeable cell envelope containing a high content of long-chain fatty acids (mycolic acids) present as mycolate esters of arabinogalactan and of trehalose. High content of long-chain fatty acids (mycolic acids) present as a complex and highly impermeable cell envelope containing a morbidity worldwide. It persists within the host with the aid of plasticity for substrates based upon the trehalose motif. Genetic knockout of natural substrates and products can be monitored by the development of a precise and quantitative MS assay of Ag85 activity based on total ion counts (TICs) of ions corresponding to trehalose, 6,6'-dihexanoyltrehalose 2b (TDH), and 6-hexanoyltrehalose 3b (TMH) (Figure 1). This exploited our previously described calibration approach with a pseudointernal standard (here N-acetyl-D-glucosamine) injected concurrently with the sample aliquot; calibration plots for each monitored mass simultaneously to give an unimpeded view of kinetic processes. Kinetic analysis and substrate/inhibitor profiling were enabled by the development of a precise and quantitative MS assay of Ag85 activity based on total ion counts (TICs) of ions corresponding to trehalose, 6,6'-dihexanoyltrehalose 2b (TDH), and 6-hexanoyltrehalose 3b (TMH) (Figure 1).

The reaction timecourse catalyzed by Ag85.

**Conditions:** 250 μM 1, 250 μM 2b, 2 μM Ag85c, 1 mM TEA buffer (pH 7.2), 37 °C. Full kinetic analysis utilized the full range of conditions [Tre] = 5–125 μM, [2b] = 25–600 μM, [Ag85c] = 10 nM.

**Figure 1.** Reaction timecourse catalyzed by Ag85. Conditions: 250 μM 1, 250 μM 2b, 2 μM Ag85c, 1 mM TEA buffer (pH 7.2), 37 °C. Full kinetic analysis utilized the full range of conditions [Tre] = 5–125 μM, [2b] = 25–600 μM, [Ag85c] = 10 nM.

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Table 1. Bi-substrate Kinetic Analysis of Ag85c

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_1$ (μM/min)</td>
<td>0.70 ± 0.05</td>
</tr>
<tr>
<td>$V_0$ (μM/min)</td>
<td>0.29 ± 0.08</td>
</tr>
<tr>
<td>$K_{M(TDH)}$ (μM)</td>
<td>262.8 ± 42.1</td>
</tr>
<tr>
<td>$K_{M(Tre)}$ (μM)</td>
<td>16.9 ± 5.3</td>
</tr>
<tr>
<td>$K_{TDH}$ (μM)</td>
<td>355.3 ± 105.6</td>
</tr>
</tbody>
</table>

The mechanism of Ag85 involves the hydrolysis (V_0) and acyl transfer (V_1) reactions.

Determination of the full kinetic parameters for Ag85c (Table 1) including $K_{M}$ values for both substrates and separate maximal reaction velocities for acyl transfer ($V_1$) and hydrolysis ($V_0$), resolved this apparent conundrum and revealed considerable acyl hydrolase activity ($k_{cat}$ 0.49 s⁻¹) in contrast with prior reports.6 We reasoned that this previously unreported activity of Ag85 was likely enzyme-mediated.20 Indeed, analysis using a suitably modified ping-pong cycle gave an excellent correlation with the data ($R^2 > 0.99$). The implied mechanism (Figure 2) is therefore one of ping-pong formation of a hydrolytically unstable acyl enzyme intermediate that then releases catalyst through two competitive pathways. Although rare, such mechanisms are not entirely unprecedented.21–23 This insight informed later inhibitor design (vide infra).

This precise dissection of different activities highlighted a strong advantage compared with, for example, single-substrate coupled assays that survey only one part of a given mechanistic cycle.19 The value for $K_{M(Tre)}$ correlated well with the only previous value of 8.3 μM, determined under pseudo-single-substrate conditions.6 A higher $K_{M}$ value determined for TDH may reflect the homogenous, shorter lipid chain of 2b compared with heterogeneous mycolic acid substrates. The relative acyltransferase activities of the three Ag85 isoforms were also determined under pseudo-single-substrate conditions by monitoring the production of both TMH and hexanoate concurrently (Table 2), and confirmed the higher activity of Ag85c compared to those of Ag85a or Ag85b.4,24

Figures 2 and 3. Mechanistic outline for Ag85.

With an accurate automated assay of Ag85 activity in place we then probed substrate selectivity. We have previously shown the utility of a semiquantitative green-amber-red (GAR) moderate-throughput screen to discover novel substrates for other bi-substrate (glycosyltransferase) enzymes.10–13 Relative reaction velocity ($ν_{rel}$) was determined by MS-based GAR assay for a panel of putative substrates (Figure 3, Table S1) to evaluate their suitability as acyl acceptors. Both trehalose (1) itself and related disaccharides 4 and 5 were found to act as substrates, although the measured $ν_{rel}$ for 5 was lower for Ag85a and b, indicating the sensitivity of the enzymes to altering this scaffold configuration away from that found in trehalose (here through inversion at C-4). Interestingly, 6, a known inhibitor of mycobacterial growth and previously described as an $in vitro$ inhibitor of Ag85c activity is also a substrate for all Ag85 isoforms, indicating that it acts as a competitive substrate and suggesting that the observed antibacterial activity may arise from downstream events after Ag85-mediated mycolyltransfer or through inhibition of other pathways.4,25

Table 2. Kinetic Parameters for Ag85 Isoforms Recorded under Pseudo-Single-Substrate Conditions

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Substrate</th>
<th>$K_{M(app)}$ (μM)</th>
<th>$k_{cat(app)}$ (s⁻¹)</th>
<th>$k_{cat(app)}/K_{M(app)}$ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1</td>
<td>175</td>
<td>0.014</td>
<td>82</td>
</tr>
<tr>
<td>b</td>
<td>1</td>
<td>112</td>
<td>0.003</td>
<td>30</td>
</tr>
<tr>
<td>c</td>
<td>1</td>
<td>62</td>
<td>0.182</td>
<td>2952</td>
</tr>
</tbody>
</table>

*Assay conditions: [TDH] = 500 μM, [trehalose] = 10–250 μM, [Ag85a/b] = 500 nM, [Ag85c] = 50 nM.
Despite observed plasticity for variants of the trehalose scaffold, truncated or more dramatically altered sugar variants displayed considerably lower activity. Although trehalose is a 1,1-diglucoside, methyl β-glucosides 9 and 10 were utilized less efficiently. Interestingly, some low-level activity (up to ∼20%) was also observed for the arabinofuranosides 7 and 8; this is consistent with a possible role of Ag85 in mycolate scrambling and mycolation of Mtb arabinogalactan. Notably, for these glucosides and arabinosides differential anomeric selectivity between enzyme isoforms was observed: α-anomers were preferred by Ag85a and b, with Ag85c showing little anomer discrimination. In keeping with this observation of tolerance at the anomeric center, Ag85c also showed the greatest tolerance toward ketosides 4 and 5, which carry an additional methyl substituent at C-1. As for the disaccharides, configurational changes had a more dramatic effect. The very low vrel value determined for β-galactosides 11 and 12 for all three Ag85 isoforms is clear additional evidence that the epimeric content at C-4 is a crucial selectivity determinant. Conversely, α-methyl mannosides 13 was slowly utilized by Ag85c, indicating some degree of flexibility with respect to C-2 configuration. As expected, xyloside 14 was also poorly processed, confirming that the majority of acyl transfer is OH-6. These differences in substrate recognition stand in contrast to the conserved active site of these three isoforms and suggest that the enzymes may well have distinct physiological roles.18

With this understanding of substrate tolerance, we sought to rationally design an effective covalent inhibitor probe. Although nonspecific approaches based simply on serine modification might be considered,16 the true utility of this probe would be vitally dependent on selectivity. Design was therefore essentially informed by both mechanism (Figure 2) as well as by the substrate screen. These dictated an inhibitor structure that would mimic the tetrahedral intermediate (TI) formed in the first unified ‘ping-to-pong’ acylation steps rather than through intervention in the divergent pathways for deacylation (Figure 2). Fluorophosphonate 19 (Figure 4b) was therefore targeted upon (a) the TI of acylation (TI1) through the in situ formation of a tetrahedral mimic (Figure 2);28 (b) by ensuring features that also mimic both lipid and sugar moieties (Figure 2); (c) substrate preferences that highlight trehalose as a superior sugar scaffold (Figure 3). In silico evaluation supported another aspect of design with these features: molecular docking27 was performed to compare binding modes that would correspond to putative Michaelis complexes (Figure 2, MC1 and Figure 4a). Lowest energy conformations of 19 (−7.3 kcal mol−1) overlaid closely with both TMH 2b (−7.0 kcal mol−1) and also with trehalose (observed in the crystal structure of Ag85b, pdb: 1F0P17), placing the acyl carbon (in 2b) and phosphorophosphorus (in 19) in close proximity to the targeted active site serine 124.

19 was readily synthesized from hepta-benzyl-trehalose 18 (Figure 4b). Treatment of 16 with fluoro-N,N,N′,N′-bis(tetramethylene)formamidinium hexafluorophosphate (BTFFH) afforded 18 in good yield; deprotection by catalytic hydrogenolysis gave 19 with no concomitant hydrolysis of the phosphorylfluoride.

The inhibitory potential of 19 against Ag85c was assayed using a saturating concentration of trehalose (>25 × K0.5) to ensure maximal turnover rate. Following incubation of Ag85c with 19 (150 μM) activity was almost entirely ablated (<7%). Phosphonate 17, an unreactive analogue of 19 that cannot effectively mimic the targeted TI resulted in inhibition only to 46%.20 kobs/Ki was determined to be 1420 (±90) min−1 mM−1 (see SI); the reactivity of 19 is such that saturation could not be achieved, as has been reported for other covalent inhibitors.29 This marks 19 as a potent inhibitor in comparison with other fluorophosphonates.31,32 With inhibitory potential confirmed, critical selectivity of 19 for Ag85 over other enzymes that contain similar catalytic triads was investigated. Serine protease subtilisin from Bacillus lentus (SBL), lipase B from Candida antarctica (CalB),
and Ag85c were incubated under identical conditions with 19. Detailed analysis by MS revealed essentially complete protein modification of Ag85c at Ser124 (Figure 4c and SI), while no detectable modification or inhibition of either SBL or CalB was observed.

MS has provided an accurate and automated method to determine the full kinetic parameters of Ag85c, which revealed that the enzyme displays a combination of acyltransferase and acylhydrolase activities. Substrate profiling revealed that, although the Ag85 enzymes show promiscuity for trehalose-based substrates, selectivity for differing monosaccharides and anomic configurations is marked: gluco- and arabinofuranosides are preferred over galacto-, manno-, or xylopyranosides. These data provide additional evidence that Ag85 is responsible for both trehalose—mycolate scrambling and mycolylation of the mycobacterial arabinogalactan. They also suggest that regions distal to the active sites influence activity of the three isoforms. The screen data indicate that disaccharides are better substrates than the constituent monosaccharides, which suggests an extended active site. This in turn suggests that the optimal starting point for rational drug design may be the trehalose scaffold that was used here to design a tailored fluorophosphonate 19. This compound was a potent and highly selective covalent inhibitor probe of Ag85c that shows no reactivity toward other serine acyltransferases. The selectivity of this molecule may allow interrogation of the importance of Ag85 activity in vitro or in infected macrophages. We anticipate that this and related ‘tagged’ compounds may find use as activity-based probes33 of Ag85 function and might serve as a starting point for the design of novel anti-mycobacterial drugs.

ASSOCIATED CONTENT

Supporting Information. Experimental methods and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author
cbarry@niaid.nih.gov; Ben.Davis@chem.ox.ac.uk

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REFERENCES

(14) See SI for derivation.
(15) See SI for details of double reciprocal analysis of Ag85c.
(23) The divergent decylation pathways may also be related to the nature/identity of the fatty acyl chain.
(24) $V_{\text{transfer}} = \frac{V_{\text{PHBH}} \cdot V_{\text{hydrolysis}}}{2}$
(29) See SI for details.
(31) Diisopropylfluorophosphonate and soman have $K_{\text{obs}}/K$ values of 140 and 9200 min $^{-1}$ mM $^{-1}$, respectively, against human acetylcholine esterase.