Characterization of a Carbon-Carbon Hydrolase from *Mycobacterium tuberculosis* Involved in Cholesterol Metabolism*§

Nathan A. Lack‡,†, Katherine C. Yam§,†, Edward D. Lowe§, Geoff P. Horisman§, Robin L. Owen†, Edith Sim§, and Lindsay D. Eltis†,‡

From the Departments of †Pharmacology and §Molecular Biophysics, University of Oxford, Oxford OX1 3QT, United Kingdom, the ¶Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, and the ‡Swiss Light Source, Paul Scherrer Institut, 5232 Villigen, Switzerland

In the recently identified cholesterol catabolic pathway of *Mycobacterium tuberculosis*, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (HsaD) is proposed to catalyze the hydrolysis of a carbon-carbon bond in 4,5,9,10-diseco-3-hydroxy-5,17-tri-oxoandrosta-1(10),2-diene-4-oic acid (DSHA), the cholesterol meta-cleavage product (MCP) and has been implicated in the intracellular survival of the pathogen. Herein, purified HsaD demonstrated 4–33 times higher specificity for DSHA (K_{cat}/K_m) than for the biphenyl MCP 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) and the synthetic analogue 8-(2-chlorophenyl)-2-hydroxy-5-methyl-6-oxoocta-2,4-dienoic acid (HOPODA), respectively. The S114A variant of HsaD, in which the active site serine was substituted with alanine, was catalytically impaired and bound DSHA with a K_d of 51 ± 2 μM. The S114A:DSHA species absorbed maximally at 456 nm, 60 nm red-shifted versus the DSHA enolate. Crystal structures of the variant in complex with HOPDA, HOPODA, or DSHA to 1.8–1.9 Å indicate that this shift is due to the enzyme-induced strain of the enolate. These data indicate that the catalytic serine catalyzes tautomerization. A second role for this residue is suggested by a solvent molecule whose position in all structures is consistent with its activation by the serine for the nucleophilic attack of the substrate. Finally, the α-helical lid covering the active site displayed a ligand-dependent conformational change involving differences in side chain carbon positions of up to 6.7 Å, supporting a two-conformation enzymatic mechanism. Overall, these results provide novel insights into the determinants of specificity in a mycobacterial cholesterol-degrading enzyme as well as into the mechanism of MCP hydrolases.

*Mycobacterium tuberculosis* is the leading cause of bacterial mortality, causing an estimated 2 million deaths/year (1). The mechanisms underlying the remarkable ability of this pathogen to survive for long periods of time within the host are poorly understood (2). Although it was well known that saprophytic mycobacteria could metabolize cholesterol (3), it was only recently demonstrated that pathogenic strains can also utilize this nutrient as a growth substrate (4, 5). Interestingly, cholesterol has been found in high concentrations within caseating granulomas in both humans and mice (6, 7), and bacteria have been observed congregating around cholesterol foci (7). Highlighting the importance of cholesterol in bacterial pathogenesis, the deletion of genes involved in cholesterol metabolism reduces the virulence of *M. tuberculosis* (5, 8). Therefore, further knowledge of cholesterol metabolism in *M. tuberculosis* is crucial to our understanding of bacterial virulence.

*M. tuberculosis* catabolizes cholesterol using a metabolic pathway similar to that identified in *Rhodococcus jostii* RHA1 (4, 9). In this pathway, the aerobic degradation of the four-ribbed steroid nucleus occurs through the opening of ring B, aromatization of ring A, and hydroxylation of the phenolic metabolite. The resulting catechol undergoes oxygenolytic cleavage, yielding 4,5,9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (DSHA), (see Fig. 1 for IUPAC numbering) a meta-cleavage product (MCP; Fig. 1). The latter is then hydrolyzed by the MCP hydrolase, HsaD, to yield 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (DOHNAA) and 2-hydroxy-hexa-2,4-dienoic acid (HHD). HHD is then metabolized to tricarboxylic acid cycle intermediates, whereas the metabolic fate of DOHNAA is unknown. In this pathway,

---

*This work was supported by grants from the Wellcome Trust (to E. S.) and the Canadian Institutes of Health Research (to L. D. E.).

†The on-line version of this article (available at [http://www.jbc.org](http://www.jbc.org)) contains supplemental Figs. S1 and S2.

‡The atomic coordinates and structure factors (codes 2wud, 2wue, 2wuf, and 2yvg) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/)

†Recipient of a studentship from the Natural Sciences and Engineering Research Council of Canada.

‡Recipient of a studentship from the Natural Sciences and Engineering Research Council and the Michael Smith Foundation for Health Research.

§To whom correspondence should be addressed: 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada. Tel.: 604-822-0042; Fax: 604-822-6041; E-mail: leltis@interchange.ubc.ca.

The abbreviations used are: DSHA, 4,5,9,10-diseco-3-hydroxy-5,9,17-tri-oxoandrosta-1(10),2-diene-4-oic acid; HOPDA, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid; HOPODA, 8-(2-chlorophenyl)-2-hydroxy-5-methyl-6-oxoocta-2,4-dienoic acid; DSHA, 3,4-dihydroxy-6-methyl-7,8-dihydrostilbene; MCP, meta-cleavage product; HsaD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase; DOHNAA, 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid; HHD, 2-hydroxy-hexa-2,4-dienoic acid; ht, histidine-tagged; bis-tris, 1,3-bis(tris(hydroxymethyl)methylamino) propane.
HsaD is of particular interest, because transposon mutagenesis has indicated that it is critical for the intracellular survival of M. tuberculosis (10).

MCP hydrolases are involved in the aerobic degradation of aromatic compounds, hydrolyzing vinylogous 1,5-diketones arising from the extradiol ring cleavage of catechols (11). Crystal structures of MCP hydrolases, including those of BphD from Burkholderia xenovorans LB400, MhpC from Escherichia coli, and a ht-HsaD (12–14), reveal that these enzymes belong to the α/β-hydrolase superfamily and contain a conserved Ser-His-Asp catalytic triad as well as an “oxyanion” hole, similar to a number of other hydrolytic enzymes (15). The substrate-binding pocket comprises two subsites: a hydrophilic polar subsite that binds the dienoate moiety of the MCP and a hydrophobic nonpolar subsite that accommodates the remainder of the MCP. In HsaD, the nonpolar subsite is at least twice as large as that of other MCP hydrolases, possibly to fit the larger cholesterol metabolite DSHA (14).

Despite the similar catalytic machinery of MCP hydrolases and serine proteinases, the mechanism of the former appears to differ from the nucleophilic mechanism of the latter in two respects. First, the enolate substrate of MCP hydrolases undergoes tautomerization to a keto intermediate prior to hydrolysis (16). Second, the ketonized substrate (17) is believed to be hydrolyzed through a general base mechanism by a strained enolate (E:Sse) more satisfactorily accounts for the red-shifted absorption spectrum because steric hindrance to planarity about a double bond raises the ground state energy but not the excited state (22). Nevertheless, the refractions did not exclude the 2-enol tautomer, and a strained enolate (E:Sse) more satisfactorily accounts for the red-shifted absorption spectrum because steric hindrance to planarity about a double bond raises the ground state energy but not the excited state (22). Second, the roles of catalytic residues remain unclear. The active site serine has been variably proposed to deprotonate the nucleophilic water (19) and to stabilize the oxyanion (23). Similarly, the active site histidine has been proposed to catalyze tautomerization (23) and to deprotonate the catalytic water (23). Finally, despite evidence supporting a general base mechanism, in the structures of BphD from B. xenovorans LB400 and MhpC from E. coli, no solvent species have been detected near the active site of the enzyme.

FIGURE 1. The successive reactions catalyzed by HsaC and HsaD in the catabolism of cholesterol by M. tuberculosis and alternate substrates of HsaD. DSHA is referred to as 4,5–9,10-diepo-3-hydroxy-5,9,17-trioxandrost-1(10),2-diene-4-oic acid in the steroid literature. However, the carbon atoms are numbered here according to its IUPAC name, 2-hydroxy-5-methyl-8-(7a-methyl-1,5-dioxooctahydro-1H-inden-4-yl)-6-oxooct-2,4-dienoic acid, to be consistent with the numbering in HOPDA and HOPODA. In this study, DSHA, HOPDA, and HOPODA were generated by ring cleavage of DHSA, 2,3-dihydroxybiphenyl, and DHDS, respectively.
Crystal Structure and Kinetics of HsaD

In this study, we determined the specificity of HsaD for various MCPs including its proposed physiological substrate, DSHA. To elucidate the interactions between enzyme and substrates, a S114A variant of HsaD was generated to slow substrate turnover, and the crystal structures of the variantsubstrate complexes were analyzed. These results provide insights into the catalytic mechanism of MCP hydrolases.

EXPERIMENTAL PROCEDURES

Chemicals—HOPDA used for crystallization was synthesized as previously described (24). 2,3-Dihydroxybiphenyl and 2,3-dihydroxy-6-methyl-7,8-dihydrostilbene (DHDS) were synthesized by a combined directed ortho metalation cross-coupling strategy as described elsewhere (8, 25). 3,4-Dihydroxy-9,10-seco-nandrost-1,3,5(10)-triene-9,17-dione (DHSA) was obtained by incubating a culture of the ΔhasC mutant of R. jostii RHA1 with cholesterol as described previously (8). All other chemicals were of analytical grade.

Preparation and Characterization of MCPs—MCPs were produced by dissolving the corresponding catechol in a small volume of ethanol and diluting to the desired volume with 100 mM potassium phosphate, pH 7.5 (ethanol constituted less than 0.1% of the solution). To this solution a sufficient amount of HasC was added to completely transform DHDS and DHSA to HOPODA and DSHA, respectively. The reaction was monitored using a Varian Cary 5000 spectrophotometer equipped with a thermostatted cuvette holder (Varian Canada, Mississauga, Canada) maintained at 25.0 °C. The reaction was initiated by adding 5 mM MCP to a diluted sample, complete cleavage of DHDS and DHSA to HOPODA and DSHA, respectively. MCPs used for steady-state kinetic studies were prepared enzymatically using 100 mM potassium phosphate, pH 7.5, and used within 2 h.

To determine pKₐ values, HOPODA and DSHA were prepared as described above in unbuffered water. HasC was removed by ultrafiltration using a stirred cell equipped with a YM10 membrane (Amicon). Solutions of ~0.5 mM MCP were acidified to pH 3 using 12N HCl. This solution was titrated with aliquots of 50 mM NaOH, and the pH was determined after the addition of each aliquot. The pKₐ values were determined from plots of pH versus the amount of base added.

The nonenzymatic transformation of MCPs in solution (100 mM potassium phosphate, pH 7.5, at 25 °C) was determined by monitoring the absorption spectra. The experiments were performed using two concentrations of each MCP (0.03, 0.3 mM). The half-lives were determined by fitting equations to the decay at 396 nm using Excel.

Protein Production and Purification—Ser¹¹⁴ of HsaD was substituted with alanine to generate the S114A variant using the QuikChange II-E site-directed mutagenesis kit (Stratagene) as per the manufacturer’s instructions. In this reaction, the template was pT7HD1, a previously constructed pT7–7 derivative carrying hsaD under control of the T7 promoter (4). The primers were 5′-CTGCTTTGCGGAACCGCTTGGCGCGGG-3′ and 5′-CCCCGCCCACCCGTGGCACCACG-3′. The nucleotide sequence of the mutated gene was confirmed using an ABI 373 Stretch DNA sequencer (Applied Biosystems, Foster City, CA) and a Big-Dye Terminator v3.1 kit. The mutated hasD was subcloned into pEMBL18 downstream of the P₈₋₈ promoter as an XbaI fragment, yielding pEMHSA.

HsaD was produced and purified from E. coli strain GJ1158 containing pT7HD1. The cells were grown at 30 °C in salt-depleted Luria-Bertani broth (10 g of tryptone extract, 5 g of yeast extract/liter) supplemented with 100 µg/ml ampicillin. The culture was grown to an optical density at 600 nm of ~0.5, at which point NaCl was added to a final concentration of 300 mM. The cultures were incubated for an additional 20 h, and then the cells were harvested by centrifugation. The S114A variant was produced using E. coli strain DH5α containing pEMHSA. The cells were grown at 30 °C in LB broth supplemented with 100 µg/ml ampicillin. The culture was grown to an optical density at 600 nm of ~0.5, and isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM. The culture was incubated for an additional 20 h before the cells were harvested.

The cell pellet obtained from 4 liters of culture was resuspended in 40 ml of 20 mM HEPES, pH 7.5, and disrupted using three passages through a Emulsiflex C-5 cell disrupter (ATA Scientific, Sutherland, Australia) operating at a pressure of 15,000 p.s.i. The cell debris was removed by ultracentrifugation at 120,000 × g for 60 min. The supernatant was removed and filtered through a 0.45-µm cellulose filter to yield ~45 ml of raw extract. The raw extract was loaded onto 28 ml of Source 15Q anion exchange resin (GE Healthcare) packed in an AP-2 column (Waters Corp., Milford, MA) equilibrated with 20 mM HEPES, pH 7.5. The proteins were eluted using a linear gradient of 70–220 mM NaCl in 280 ml. HsaD eluted at ~140 mM NaCl. Fractions of 10 ml containing activity (in the case of the wild-type enzyme) or the protein of the expected size (in the case of the variant) were pooled and concentrated to 10 ml using an Amicon stirred cell concentrator equipped with an YM10 ultrafiltration membrane (Millipore, Billerica, MA). The HsaD-containing solution was brought to 1 M (NH₄)₂SO₄, and the sample was briefly centrifuged to harvest the pellet. The cloudy white precipitate was washed twice with 1 M (NH₄)₂SO₄ solution; exchanged into 20 mM HEPES, pH 8.5, to resolubilize the enzyme; concentrated to >20 mg/ml; flash frozen as beads in liquid nitrogen; and stored at ~80 °C. Coomassie Blue-stained denaturing gels revealed that preparations of the wild-type and variant proteins were >97% HsaD.

Kinetic Measurements—The enzyme-catalyzed hydrolysis of the MCP was monitored using a Varian Cary 5000 spectrophotometer equipped with a thermostatted cuvette holder (Varian Canada, Mississauga, Canada) maintained at 25.0 ± 0.5 °C. The amount of enzyme used in each assay was adjusted so that the progress curve was linear for at least 2 min. Initial velocities were determined from a least squares analysis of the progress curves using the kinetics module of the Cary WinUV software.

Specificity experiments were performed in a total volume of 1.0 ml of 100 mM ionic strength potassium phosphate, pH 7.5, at 25.0 ± 0.1 °C. The reaction was initiated by adding 5 µl of an appropriately diluted enzyme preparation to the reaction cuvette. The reactions were monitored at the wavelengths corresponding to the enolate. Initial velocities were determined over the following ranges of substrate concentration: 0.5–30 µM HOPDA, 10–300 µM HOPODA, and 2–60 µM DSHA. Steady-state kinetic parameters were evaluated by fitting the
appropriate equations to the data using the least squares and dynamic weighting options of LEONORA (26).

*K*<sub>d</sub> Determination—The dissociation constant of the S114A-DSHA complex was determined by titrating a 200-μl solution of 100 mM ionic strength potassium phosphate, pH 7.5, at 25.0 ± 0.1 °C containing 5 μM DSHA with S114A and monitoring the change in absorbance at 456 nm. The spectra were recorded using a Cary 5000 spectrophotometer. The dissociation constant was evaluated by fitting the binding equation (27) to the data using the curve fitting program and the nonlinear statistical analyses of *R*.<br>

The half-life of S114A-DSHA was determined by monitoring the decay of the complex at 456 nm. The experiment was carried out in a total volume of 150 μl of 100 mM ionic strength potassium phosphate, pH 7.5, at 25.0 ± 0.1 °C containing 14 μM S114A and 6 μM DSHA. A single-exponential equation was fit to the data.<br>

Crystalization of S114A and Preparation of Substrate Complex—Protein crystals were grown by sitting drop vapor diffusion with equal volumes (1 μl) of S114A (8 mg/ml) and precipitant (200 mM KSCN, 24% polyethylene glycol 3.35 K, and 100 mM bis-tris propane, pH 7.0). Disphenoid S114A crystals typically grew in 3–5 days at 19 °C. Complexes between S114A and HOPDA, HOPODA, and DSHA were obtained by soaking S114A crystals in 10 μl of precipitant supplemented with 15 mM of each ligand for 30–60 min.<br>

Diffraction Data Measurement and Processing—The crystals were briefly transferred to a cryoprotectant solution (1:4 (v/v) mixture of glycerol and precipitant) and then flash-frozen in liquid nitrogen. For enzyme-ligand complexes, the cryoprotectant solution was supplemented with the ligand (15 mM).<br>

S114A crystal x-ray data were collected in-house using a Bruker Microstar x-ray generator with a Smart6000 CCD detector and integrated with the commercial software SAINT (Bruker-AXS). X-ray data from S114A-HOPDA crystals were collected at the Swiss Light Source on Beamline X06SA with a Pilatus 6M detector and integrated with MOSFLM (28). X-ray data from HOPODA and S114A-DSHA crystals were collected at the Diamond Light Source on Beamline I03 with a Quantum ADSC CCD detector and integrated with MOSFLM (28).<br>

Structure Determination and Refinement—The initial phases of S114A were determined by molecular replacement using hT-HsD (Protein Data Bank code 2vf2), refined to 2.3 Å, as a search template (14) with the program MOLREP (29). For the enzyme-ligand complexes the phases were determined as described above, using the refined S114A structure as a search template. Simulated annealing refinement was followed by iterative cycles of manual density fitting with COOT (30) and refinement with PHENIX (31). PHENIX eLBOW (31) was used to calculate the geometric restraints for HOPDA, HOPODA, and DSHA. The stereochemical properties and quality of the final model were assessed with the program MOLPROBITY (32). All of the structural figures, alignments, and graphical renderings were done using PYMOL (33).<br>

**RESULTS**<br>

Physical Properties of HOPODA and DSHA—The absorption spectrum of MCPs are strongly pH-dependent because of the

### TABLE 1<br>

| Substrate | λ<sub>max</sub> | ε | Half-life<sup>a</sup> | pK<sub>a</sub><sup>b</sup> |
|-----------|--------------|---|----------------|-----------------|
| HOPDA     | 434          | 25.7 | 58<sup>c</sup> | 7.3<sup>d</sup> |
| HOPODA    | 396          | 6.8  | 25             | 7.5             |
| DSHA      | 396          | 3.8  | 15.4           | 6.8             |

<sup>a</sup> Half-lives were determined in potassium phosphate buffer (I = 0.1 M), pH 7.5, at 25 °C.<br>
<sup>b</sup> The pK<sub>a</sub> values of the respective enolates were determined in unbuffered water.<br>
<sup>c</sup> Data shown are from Ref. 17.<br>

TABLE 2<br>

Steady-state kinetic parameters of HsaD with HOPDA, HOPODA, and DSHA<br>
The experiments were performed using potassium phosphate buffer (I = 0.1 M), pH 7.5, at 25 °C. The values in parentheses indicate standard deviations.<br>

| Substrate | K<sub>m</sub> | k<sub>cat</sub> | k<sub>cat</sub>/K<sub>m</sub> |
|-----------|-------------|--------------|-----------------|
| HOPDA     | 8 (1)       | 0.066 (0.005) | 9 (1)          |
| HOPODA    | 310 (70)    | 0.33 (0.06)  | 1.06 (0.06)    |
| DSHA      | 17 (2)      | 0.55 (0.02)  | 33 (3)         |

<sup>a</sup> Characteristic of S114A in Solution—The catalytic serine of HsD was substituted with alanine to generate a catalytically impaired variant. The addition of a 60-fold excess of S114A to a 5 μM solution of DSHA produced a species with an absorbance maximum of 456 nm (Fig. 2), 60 nm red-shifted with respect to that of the DSHA enolate tautomer. This species had a half-life of 3 h (supplemental Fig. S1), similar to that of the BphD S112A-HOPDA complex (34). Based on this, the upper limit of the k<sub>cat</sub>/K<sub>m</sub> value of S114A for DSHA was 8.5 (± 0.9) × 10<sup>-5</sup> s<sup>-1</sup>, 6500-fold lower than that of the wild-type enzyme. This slow turnover facilitated further study of the S114A-DSHA complex.<br>

Titration of the DSHA with S114A yielded a dissociation constant (K<sub>d</sub>) of 51 ± 2 μM (Fig. 2, inset). In comparison, titration of a 2 μM solution of HOPDA with the variant enzyme resulted in modest spectral shifts; in the presence of a 10-fold excess of S114A, most of the HOPDA (~90%) was present as the free enolate.<br>

Crystallography—To characterize the interactions of HsD with its substrate, crystallization studies were undertaken using the S114A variant. The variant failed to crystallize using condi-
Crystal Structure and Kinetics of HsaD

FIGURE 2. Characterization of the S114A:DSHA complex by UV-visible absorption spectroscopy. A, spectra of S114A:DSHA and DSHA in solution are shown by dotted and solid lines, respectively. The complex absorbs maximally at 456 nm. B, the curve represents the fit of the binding equation to ΔA456 observed upon titrating DSHA with S114A (Kd = 51 ± 2 μM). The experiments were performed using potassium phosphate (I = 0.1 M, pH 7.5) at 25 °C.

TABLE 3
Summary of data collection and refinement statistics

|                     | S114      | S114A-HOPDA | S114A-HOPDOA | S114A-DSHA |
|---------------------|-----------|-------------|--------------|------------|
| X-ray source        | Cu-Ka     | Swiss light source (X06SA) | Diamond (I03) | Diamond (I03) |
| Space group         | F 222     | F 222       | F 222        | F 222      |
| Unit cell parameters| a = 112.3, b = 118.8, c = 182.6, α = β = γ = 90 | a = 111.9, b = 118.0, c = 180.9, α = β = γ = 90 | a = 111.9, b = 118.0, c = 180.9, α = β = γ = 90 | a = 111.9, b = 118.0, c = 180.9, α = β = γ = 90 |
| Data collection statistics | Resolution (Å) | 59.8-2.1 (2.2-2.1) | 29.49-1.80 (1.80-1.90) | 40.66-1.80 (1.80-1.90) | 40.59-1.90 (2.00-1.90) |
| | No. unique reflections | 35,484 (4560) | 55,165 (7928) | 55,043 (8086) | 46,107 (6736) |
| | Multiplicity | 4.2 (3.8) | 4.2 (4.2) | 5.0 (5.0) | 3.5 (3.5) |
| Refinement and model statistics | Resolution (Å) | 49.7789-2.1 (2.11-2.10) | 29.49-1.80 (1.90-1.80) | 36.34-1.80 (1.90-1.80) | 40.59-1.90 (2.00-1.90) |
| | No. reflections used (work + test) | 53,439 | 54,460 | 53,203 | 42,140 |
| | Rwork | 0.192 | 0.080 | 0.069 | 0.094 |
| | Rfree | 0.239 | 0.192 | 0.210 | 0.231 |
| | Total No. of atoms | 4909 | 4871 | 4775 | 4725 |
| | Additional molecules | 2 thiacynate | 1 glycerol, 1 thiacynate | 2 thiacynate | 2 glycerol, 1thiacynate |
| | No. of water molecules | 275 | 280 | 334 | 183 |
| | Root mean square deviation bond lengths (Å) | 0.009 | 0.008 | 0.008 | 0.013 |
| | Root mean square deviation bond angles (°) | 0.746 | 0.783 | 0.716 | 0.933 |
| | Wilson B-factor (Å²) | 16.6 | 16.5 | 21.1 | 28.5 |
| | Mean B-factor (Å²) | 21.0 | 21.6 | 27.3 | 34.4 |
| Ramachandran statistics (%) | Core region | 98.04 | 98.40 | 98.06 | 97.84 |
| | Additional allowed region | 1.96 | 1.60 | 1.94 | 2.16 |
| | Disallowed | 0.00 | 0.00 | 0.00 | 0.00 |

*a Rwork and Rfree = Σ||Fo(h)|| - |Fc(h)||/Σ||Fo(h)|| for the working set and test set (%). of reflections, where the Fo(h) and Fc(h) are the observed and calculated structure factor amplitudes for reflection h.

Strains previously developed for h-t-HsαD (14). After screening and optimization, diffracting disphenoid crystals of S114A could be reproducibly grown. Using these conditions, the structures of S114A were determined alone or in complex with HOPDA, HOPDOA, or DSHA at resolutions between 1.8 and 2.1 Å. The crystals have space group F222 (a = 112 Å, b = 118 Å, c = 181 Å) with two protomers/asymmetric unit. There was clear electron density in all structures for at least residues 7–288. For all of the models, >98% of the residues were in the favored region, and none were in the disallowed region of Ramachandran plots as defined by MolProbity (32). The statistics of the data collection and refinement for these structures are summarized in Table 3.

Structure of S114A—The structure of S114A is essentially identical to that of h-t-HsαD (14) (root mean square deviation, 0.26 Å, Cα) except for the substituted active site residue. Briefly, this structure comprises a core α/β hydrolase domain with a four-helix lid domain, inserted between residues 137 and 233 in HsαD, which “caps” the active site (supplemental Fig. S2). As with other MCP hydrolases (12, 13), the residues of the lid structure (19.7 ± 0.2 Å S.E.), with the highest root mean square deviation bond angles (°) (19.7 ± 0.2 Å S.E.), with the highest R-factor found in helix αL4 (residues 199–212; 36.2 ± 1.2 Å S.E.) (Fig. 3). The substituted active site residue is found within a tightly turning “nucleophilic elbow” (Gly112, Asp113, Ala114, Leu115, and Gly116) similar to other α/β hydrolases. This strand-turn-helix motif introduces unfavorable main chain torsional angles but contributes to the “oxyanion hole” formed by the backbone nitrogen atoms from Leu115 and Gly116. His260 of the catalytic
triad forms a hydrogen bond via N/H9254 with O/H9254 of A sp241 (2.7 Å) and likely also interacts with O/H9254 of this residue (3.2 Å).

Structures of S114A-MCP Complexes—In the structure of S114A crystals soaked with HOPDA, there was clear electron density in the active site, supporting the presence of HOPDA in both protomers A and B (Fig. 4A). However, in crystals soaked with either DSHA or HOPODA, there was well-defined electron density only in the active site of protomer B (Fig. 4, B and C). In protomer A of DSHA-soaked crystals, there was density supporting the presence of a ligand, but it was not sufficiently well resolved for accurate placement. Similar to previous published work, attempts to fit the 3E,5Z,2E,4E and 2Z,4Z 2-enol or the monoanionic (Z) 2-keto tautomers of the ligand were unsuccessful (34). The ligands were therefore initially refined as both the 2Z,4E enol and monoanionic (E) 2-keto tautomers. Protomer B of S114A-HOPDA was refined first, because this structure had the clearest ligand electron density. Although both tautomers fit the electron density of HOPDA equally well, the refinement of the ligand introduced considerable deviations of the torsion angles for the enol tautomer. Specifically, the refinement of the enol tautomer introduced a 67° deviation from planarity of the torsion angle about the C-4–C-5 bond (Table 4). By contrast, the keto tautomer refined without significant deviations in the expected torsional angle of the C-3=C-4 double bond. A similar trend was seen in protomer A of the S114A-HOPDA, protomer B of S114A-HOPDA, and S114A-DSHA structures, in which the C-4=C-5 bond refined with 51, 77, and 67° deviations from planarity, respectively (Table 4). Therefore, all of the ligands were refined as the monoanionic (E) 2-keto tautomers.

In each of the three S114A-MCP structures, the ligands oriented similarly in the active sites; the dienoate was bound to the polar subsite, and the rings (bicycloal- kanone in the case of DSHA and phenyl in the case of HOPODA and HOPDA) were found in the nonpolar subsite (Fig. 5). More specifically, the C-6-oxo group of each ligand forms hydrogen bonds with the backbone amides of Gly45 (2.7–2.9 Å) and Leu115 (2.8–3.1 Å) (Fig. 6A). This interaction keeps the ligands in close proximity with active site residue 114. The C-5-methyl group of both DSHA and HOPODA was located within a hydrophobic pocket lined by the side chains of Phe173, Met177, and Leu115. When in the presence of a ligand, Arg192 was found to adopt two conformations:
one similar to that observed in the absence of ligand and a second conformation involving a rotation of ~90° about χ^3 such that the guanidium group is in closer contact with ligand. This movement allowed the formation of a salt bridge (2.7–3.4 Å) between Nε of Arg^{192} and the carboxylic acid group of the substrate. In addition, the substrate carboxylic acid interacts with Asn^{54} (3.1–3.4 Å). Although likely too distant to form hydrogen bonds with the C-2-oxo group of HOPDA, HOPODA, and DSHA, the NH_{2} of Trp^{270} (3.6–4.1 Å), Nδ of Asn^{113} (3.7–4.0 Å), and Ne2 of His^{269} (3.5–4.0 Å) may contribute to a polar environment important for substrate binding and positioning (Fig. 6A). In all structures, a solvent species was present 3.2–3.4 Å from the C-6-carbonyl (Fig. 6B) and in two of them, S114A-DSHA and S114A-HOPODA, formed an angle (water O–C–6–O-6) that was within 10°–20° of the Bürgi-Dunitz angle of 107°. Stabilized by hydrogen bonds with Nδ of Asn^{244} (3.0–3.1 Å), this water molecule has a relatively low B-factor (30.8 ± 12.3 Å^2), suggesting essentially 100% occupancy. A similarly positioned solvent species was also present in the ligand-free S114A structure. The bicycloalkanone and phenylchloro moieties of DSHA and HOPODA, respectively, were located within the nonpolar subsite in a hydrophobic region formed by Phe^{212}, Met^{208}, Leu^{115}, and Val^{155}. However, the ring moieties of all MCPs were found to have elevated B-factors compared with the remainder of the ligand, suggesting that these groups are mobile. As such, there is weak electron density for these groups (Fig. 4, B and C).

Comparison of the five ligand-containing protomers with the three ligand-free protomers of S114A revealed two conformations of the L4 helix within the lid domain. In the presence of the ligand, the lid domain is observed in a “closed” orientation, whereas the lid domain of the ligand-free protomers is in an “open” orientation. As displayed in Fig. 7, for the DSHA-containing protomer B, these two conformations differ in the position of Cα (Gly^{209}) by up to 2.8–3.3 Å, and the position of individual side chain carbons (Phe^{212}) differs by up to 4.2–6.7 Å (Fig. 7). The net effect of the implied movement places Met^{208} and Phe^{212} closer in the nonpolar subsite of the binding pocket. Although neither residue is likely to directly influence the binding of HOPDA, both could form hydrophobic interactions with the ring moieties of DSHA and HOPODA, respectively. The conformation of the α-helix in the ligand-free protomers is

### TABLE 4
Torsional angles of the refined 2-enol and 2-keto tautomers of HOPDA, HOPODA, and DSHA in S114A

| Bonding | C2-C3 | C3-C4 | C4-C5 | C2-C3 | C3-C4 | C4-C5 |
|---------|-------|-------|-------|-------|-------|-------|
| Double  | 174   | 175   | 113   | 177   | 177   | 116   |
| Single  | 164   | 168   | 129   | 149   | 173   | 139   |
| Double  | 177   | 157   | 103   | 180   | 165   | 98    |
| DSHA    | 159   | 167   | 113   | 158   | 175   | 97    |

**FIGURE 5.** Overlay of the MCP substrates HOPDA, HOPODA, and DSHA in complex with S114A. The protein (white) is shown in with the side chains involved in ligand binding shown as sticks. The ligands are represented as sticks and are colored pink (HOPDA), cyan (HOPODA), and purple (DSHA). In all structures the nitrogen, oxygen, and sulfur atoms colored blue, red, and yellow, respectively.

**FIGURE 6.** Interaction of DSHA with S114A. A, the structure of S114A (white) in complex with DSHA (purple) is shown in cartoon representations with the amino acid side chains involved in binding and DSHA displayed in stick representation. The interactions between S114A and the C-2-oxo and C-1 carboxylic acid are shown by yellow dashes, whereas the interactions between C-6-oxo and S114A are shown by red dashes. B, the structure of S114A (white) in complex with DSHA (purple). The water molecule (HOH89) is shown by a red star. All of the distances are in angstroms.
Crystal Structure and Kinetics of HsaD

The relatively high specificity of HsaD from M. tuberculosis for DSHA ($k_{\text{cat}}/K_m = 33,000 \text{ M}^{-1} \text{s}^{-1}$) indicates that the enzyme is involved in cholesterol catabolism. Similarly, the structural data indicate that the substrate-binding pocket is best adapted to the steroid substrate. Although the specificity of HsaD is ~2–3 orders of magnitude lower than other MCP hydrolases for their physiological substrates (13, 23, 35), this is consistent with what has been reported for other M. tuberculosis enzymes involved in cholesterol metabolism, including KshAB (36) and HscC (8).

It is unclear why HOPODA is a poorer substrate for HsaD than HOPDA; the chloro-phenyl moiety of HOPODA was designed to mimic the bulk of the bicycloalkanone portion of DSHA, and the structures of the enzyme-substrate complexes indicate that the dienolate moiety of the three MCPs were identically positioned in the active site of S114A (Fig. 5). Nevertheless, the $K_m$ of HOPODA was 30-fold higher than that of HOPDA or DSHA. The increased $K_m$ and $k_{\text{cat}}/K_m$ is in contrast to the high specificity of BphDs for HOPDA variants bearing chloro substituents on the phenyl ring, particularly in the meta- and para-positions (17). Although chlorophenyl HOPDAs are thought to stabilize the negatively charged tetrahedral intermediate at C-6, the chlorophenyl of HOPODA is unlikely to contribute to stabilization because of the saturated two carbon bridge between the chlorophenyl and the dienolate moieties. Redesigning the chlorophenyl moiety of the substrate analogue may improve HsaD-analogue interactions and provide clues to the binding mechanism.

This study provides the clearest evidence to date that the active sites of MCP hydrolase exist in two conformations: open in the absence of ligand and closed in its presence. Two observations in previous structures of MCP hydrolases had suggested this possibility. First, the $\alpha$-helix lid domains had higher B-factors than the remainder of the protein (12, 37) as observed here for HsaD. Second, structures of BphD obtained in the different states revealed similar but smaller differences in lid positions (34, 38). The presence of malonate in the active site cleft of substrate-free BphD may have reduced the observed differences in this enzyme. The conformational differences seen in the lid domain of HsaD are lesser in magnitude than those observed in another $\alpha$/$\beta$ hydrolase, the lipase from Rhizomucor miehei (39, 40), however, they are remarkably similar in location and in net effect.

The observation of two active site conformations in HsaD is consistent with the half-site mechanism proposed on the basis of the MphC structure (37) as well as from kinetic studies of BphD and the ordered release of the two reaction products (13). According to the half-site mechanism, the hydrolase cycles between two interconverting conformations: one that is enzymatically inactive and one that is enzymatically active. More particularly, benzoate dissociation followed that of the dienolate, was nondiffusive, and occurred at a rate that was roughly equivalent to the $k_{\text{cat}}$ (13). Consistent with this model, the electron density of the bound substrate in the S114A-MCP complexes was always better defined in one protomer (B) than the other. Moreover, in the structure with the weakest ligand density, S114A-HOPODA, the $\alpha$4 helix was in a mixed conformation, with one protomer in an open conformation and another in a closed conformation. Although speculative, the movement of the $\alpha$4 helix could assist in the nondiffusive release of the second product: benzoate and DOHNAA in the cases of HOPDA and DSHA, respectively. Because the release of the second product is rate-limiting, the increased distance between the $\alpha$4 helix and the non-dienolate portion of HOPDA compared with that of DSHA and HOPODA (Fig. 5) could explain the 6-fold reduction of the $k_{\text{cat}}$ of HOPDA. However, further data are required to determine the effect of the C-5 methyl substituent on the $k_{\text{cat}}$ of HsaD.

The spectroscopic (Fig. 2) and structural data (Fig. 4 and Table 4) of the S114A-MCP complexes are very similar to those reported for the equivalent S112A-MCP complexes of BphD. Specifically, the orientation and the torsional angles of the MCPs in the BphD structure suggest that the substrate had undergone ketonization, yielding an E:Sk species. However, the spectroscopic data indicated that the bound MCPs were conjugated because the disrupted $\pi$-conjugation of E:S is predicted to result in a blue shift. Interestingly, the 60-nm red shift in S114A-DSHA is similar in magnitude to those observed in S112A-HOPDA (72 nm) (12, 13) and in the L photointermediate of the retinal chromophore of bacteriorhodopsin (60 nm) (41). The latter has been explained on the basis of twist-about double bonds, a distortion of the chromophore that has recently been observed crystallographically (42). The dihedral angles of the twisted double bond in the L photointermediate of bacteriorhodopsin were up to 40° from the resting state molecule, similar to the values observed with the 2-enol tautomers of the S114A-MCP structures (Table 4). Although it is possible that different species of S114A-DSHA occur in solution and in crystallo, a strained enolate, E:S$^{\text{en}}$, is most consistent with the structure and red-shifted absorption spectrum of S114A-DSHA. Moreover, the similar electronic
absorption spectra of S114A/DSHA and E:S\textsuperscript{red} indicate the occurrence of a strained enolate species as a catalytic intermediate and imply that the serine residue plays a significant role in tautomerizing the bound MCP.

In MCP hydrolases, the enzymatic cleavage of the ligand has been proposed to occur through a general base mechanism (19). The structures of the S114A-MCP complexes are most consistent with the catalytic serine acting as a general base to activate a water molecule for nucleophilic attack of the C-6 carbonyl of the substrate. Notably, a solvent species is located 3.2–3.6 Å from the carbonyl and 3.7–4.1 Å from the C\textsubscript{\textbeta} of Ala\textsubscript{114} (Fig. 6B). Although the location of the water molecule may differ in the native enzyme, the overlaid structure of h-HsaD with S114A/MCP places the O\textgamma from Ser\textsuperscript{114} within ~3 Å from the water and 2.4 Å from the C-6 carbonyl in the overlaid structures. However, the O\textgamma–C-6–O-6 angle of 46° deviates significantly from the Bürgi-Dunitz angle. Finally, His\textsuperscript{269} is over 5 Å away from the closest water molecule (Fig. 6B).

In conclusion, the results presented herein suggest a dual role for the catalytic serine of MCP hydrolases and enable further refinement of the proposed catalytic mechanism for these enzymes. As summarized in Fig. 8, hydrolysis of the bound substrate is initiated by deprotonation of the C-2 hydroxyl by His\textsuperscript{269}. The bulk of the latter helps to generate a strained enolate intermediate, E:S\textsuperscript{se}. Protonation of C-5 by Ser\textsuperscript{114} drives tautomerization of the substrate to generate a keto intermediate, E:S\textsuperscript{k}. C-5 is then protonated by Ser\textsuperscript{114}, inducing tautomerization of the substrate and further rotation around the C-4–C-5 to yield a ketonized intermediate, E:S\textsuperscript{k}. Subsequently, Ser\textsuperscript{114} deprotonates a nearby water molecule, which attacks the C-6 carbonyl to yield a gem-diol intermediate. The tetrahedral intermediate collapses, releasing HHD and triggering a conformational change of the lid domain, which releases DOHNAA. This proposal provides a basis for further study on the mechanism of MCP hydrolases.

Acknowledgments—We thank the beamline scientists at the Diamond Light Source (Oxfordshire, UK) and Swiss Light Source (Villigen, Switzerland) for valuable technical support, Jie Liu for help constructing the S114A variant, Jeffrey T. Bolin for critically reading the manuscript, and Isaac Westwood for expert crystallography advice.

REFERENCES
1. Dye, C. (2006) Lancet \textbf{367}, 938–940
2. Schnappinger, D., Ehr, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efren, B., Butcher, P. D., Nathan, C., and Schoolnik,
Crystal Structure and Kinetics of HsaD

G. K. (2003) J. Exp. Med. 198, 693–704
3. Sobel, H., and Plaut, A. (1949) J. Bacteriol. 57, 377–382
4. Van der Geize, R., Van, K., Heuser, T., Wilbrink, M. H., Harra, H., Ander-

ton, M. C., Sim, E., Dijkhuizen, L., Davies, J. E., Mohnt, W. W., and Eltis,
L. D. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 1947–1952
5. Pandey, A. K., and Sassetti, C. M. (2008) Proc. Natl. Acad. Sci. U.S.A. 105,
4376–4380
6. Pagel, W., and Pagel, M. (1925) Virchows Arch. Pathol. Anat. 256,
629–640
7. Hunter, R. L., Olsen, M., Jagannath, C., and Actor, J. K. (2006) Am. J.
Pathol. 168, 1249–1261
8. Yam, K. C., D’Angelo, I., Kalscheuer, R., Zhu, H., Wang, J. X., Snieckus, V.,
Ly, L. H., Converse, P. J., Jacobs, W. R., Jr., Strynadka, N., and Eltis, L. D.
(2009) PLoS Pathog. 5, e1000344
9. Horinouchi, M., Hayashi, T., Yamamoto, T., and Kudo, T. (2003)
J. Biol. Chem. 278, 43761–43769
10. Rengarajan, I., Bloom, B. R., and Rubin, E. J. (2005) Proc. Natl. Acad.
Sci. U.S.A. 102, 8327–8332
11. Li, C., Hassler, M., and Bugg, T. D. (2008) ChemBioChem. 9, 71–76
12. Nanddagopal, N., Yamada, A., Hatta, T., Massi, E., Fukuda, M., Mitsui, Y.,
and Senda, T. (2001) J. Mol. Biol. 309, 1139–1151
13. Horsman, G. P., Ke, J., Dai, S., Leah, S. Y., Bolin, J. T., and Eltis, L. D. (2006)
Biochemistry 45, 11071–11086
14. Lack, N., Lowe, E. D., Liu, J., Eltis, L. D., Noble, M. E., Sim, E., and West-
wood, I. M. (2008) Acta Crystallogr. Sect. F Struct. Biol. Crystal Commun.
64, 2–7
15. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M.,
Harel, M., Remington, S. J., Silman, I., and Schrag, J. (1992) Protein Eng. 5,
197–211
16. Lam, W. W., and Bugg, T. D. (1997) Biochemistry 36, 12242–12251
17. Seah, S. Y., Labbé, G., Nerdinger, S., Johnson, M. R., Snieckus, V., and Eltis,
L. D. (2000) J. Biol. Chem. 275, 15701–15708
18. Li, J., and Bugg, T. D. (2007) Org. Biomol. Chem. 5, 507–513
19. Fleming, S. M., Robertson, T. A., Langley, G. J., and Bugg, T. D. (2000)
Biochemistry 39, 1522–1531
20. Li, J. J., Li, C., Blidenauer, C. A., and Bugg, T. D. (2006) Biochemistry 45,
12461–12469
21. Gruber, K., Gartler, G., Krammer, B., Schwab, H., and Kratky, C. (2004)
J. Biol. Chem. 279, 20501–20510
22. Williams, D. H., and Fleming, I. (1995) Spectroscopic Methods in Organic
Chemistry, 5th Ed., McGraw-Hill, London
23. Li, C., Montgomery, M. G., Mohammed, F., Li, J. J., Wood, S. P., and Bugg,
T. D. (2005) J. Mol. Biol. 346, 241–251
24. Lack, N. A., Kawamura, A., Fullam, L., Laurieri, N., Bead, S., Russell, A. I.,
Evangelopoulos, D., Westwood, I., and Sim, E. (2009) Biochem. J. 418,
369–378
25. Nerdinger, S., Kendall, C., Cai, X., Marchart, R., Riebel, P., Johnson, M. R.,
Yin, C. F., Hénaff, N., Eltis, L. D., and Snieckus, V. (2007) J. Org. Chem. 72,
5960–5967
26. Cornish-Bowden, A. (1995) Analysis of Enzyme Kinetic Data, Oxford Uni-
versity Press, Oxford
27. Erman, J. E., and Vitello, L. B. (1980) J. Biol. Chem. 255, 6224–6227
28. Leslie, A. G. W. (1992) Joint CCP4 + ESF-EAMCB Newsletter on Protein
Crystallography 26
29. Vagin, A., and Teplyakov, A. (1994) Acta Crystallogr. D Biol. Crystallogr.
50, 760–763
30. Emsley, P., and Cottan, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60,
2126–2132
31. Adams, P. D., Grosse-Kunstleve, R. W., Hung, L. W., Joerger, T. R., Mc-
Coy, A. J., Moriarity, N. W., Read, R. J., Sacchettini, J. C., Sauter, N. K., and
Terwilliger, T. C. (2002) Acta Crystallogr. D Biol. Crystallogr. 58,
1948–1954
32. Davis, I. W., Leaver-Fay, A., Chen, V. B., Block, J. N., Kapral, G. J., Wang,
X., Murray, L. W., Arendall, W. B., 3rd, Snoeyink, J., Richardson, J. S., and
Richardson, D. C. (2007) Nucleic Acids Res. 35, W375–383
33. DeLano, W. L. (2002) The PyMOL Molecular Graphics System, 0.99 Ed.,
DeLano Scientific, LLC, Palo Alto, CA
34. Horsman, G. P., Bhownik, S., Seah, S. Y., Kumar, P., Bolin, J. T., and Eltis,
L. D. (2007) J. Biol. Chem. 282, 19894–19904
35. Seah, S. Y., Ke, J., Denis, G., Horsman, G. P., Fortin, P. D., Whiting, C. J.,
and Eltis, L. D. (2007) J. Bacteriol. 189, 4038–4045
36. Capyk, J. K., D’Angelo, I., Strynadka, N. C., and Eltis, L. D. (2009) J. Biol.
Chem. 284, 9937–9946
37. Dunn, G., Montgomery, M. G., Mohammad, F., Coker, A., Cooper, J. B.,
Roberts, T., Garcia, J. L., Bugg, T. D., and Wood, S. P. (2005) J. Mol. Biol.
346, 253–265
38. Bhownik, S., Horsman, G. P., Bolin, J. T., and Eltis, L. D. (2007) J. Biol.
Chem. 282, 36377–36385
39. Derewenda, U., Brzozowski, A. M., Lawson, D. M., and Derewenda, Z. S.
(1992) Biochemistry 31, 1532–1541
40. Brady, L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E., Dodson, G.,
Tolley, S., Turkenburg, J. P., Christiansen, L., Hug-Jensen, B., and Nors-
kov, L. (1990) Nature 343, 767–770
41. Hu, J. G., Sun, B. Q., Petkova, A. T., Griffin, R. G., and Herzfeld, J. (1997)
Biochemistry 36, 9316–9322
42. Lanyi, J. K., and Schobert, B. (2007) J. Mol. Biol. 365, 1379–1392
Characterization of a Carbon-Carbon Hydrolase from Mycobacterium tuberculosis Involved in Cholesterol Metabolism
Nathan A. Lack, Katherine C. Yam, Edward D. Lowe, Geoff P. Horsman, Robin L. Owen, Edith Sim and Lindsay D. Eltis

J. Biol. Chem. 2010, 285:434-443.
doi: 10.1074/jbc.M109.058081 originally published online October 29, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.058081

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2009/10/29/M109.058081.DC1

This article cites 38 references, 14 of which can be accessed free at
http://www.jbc.org/content/285/1/434.full.html#ref-list-1