Structures of \textit{Mycobacterium tuberculosis} 1-Deoxy-\textit{d}-xylulose-5-phosphate Reductoisomerase Provide New Insights into Catalysis*

Received for publication, March 6, 2007, and in revised form, April 5, 2007 Published, JBC Papers in Press, May 9, 2007, DOI 10.1074/jbc.M701935200

Lena M. Henriksson‡, Torsten Unge†, Jens Carlsson‡, Johan Åqvist‡, Sherry L. Mowbray‡, and T. Alwyn Jones‡ 1

From the †Department of Cell and Molecular Biology, Uppsala University, Biomedical Center, Box 596, SE-751 24 Uppsala, Sweden and the ‡Department of Molecular Biology, Swedish University of Agricultural Sciences, Biomedical Center, Box 590, SE-751 24 Uppsala, Sweden

Isopentenyl diphosphate is the precursor of various isoprenoids that are essential to all living organisms. It is produced by the mevalonate pathway in humans but by an alternate route in plants, protozoa, and many bacteria. 1-Deoxy-\textit{d}-xylulose-5-phosphate reductoisomerase catalyzes the second step of this non-mevalonate pathway, which involves an NADPH-dependent rearrangement and reduction of 1-deoxy-\textit{d}-xylulose 5-phosphate to form 2-C-methyl-\textit{d}-erythritol 4-phosphate. The use of different pathways, combined with the reported essentiality of the enzyme makes the reductoisomerase a highly promising target for drug design. Here we present several resolution structures of the \textit{Mycobacterium tuberculosis} 1-deoxy-\textit{d}-xylulose-5-phosphate reductoisomerase, representing both wild type and mutant enzyme in various complexes with Mn2+, NADPH, and the known inhibitor fosmidomycin. The asymmetric unit corresponds to the biological homodimer. Although crystal contacts stabilize an open active site in the B molecule, the A molecule displays a closed conformation, with some differences depending on the ligands bound. An inhibition study with fosmidomycin resulted in an estimated IC50 value of 80 nM. The double mutant enzyme (D151N/E222Q) has lost its ability to bind the metal and, thereby, also its activity. Our structural information complemented with molecular dynamics simulations and free energy calculations provides the framework for the design of new inhibitors and gives new insights into the reaction mechanism. The conformation of fosmidomycin bound to the metal ion is different from that reported in a previously published structure and indicates that a rearrangement of the intermediate is not required during catalysis.

Within the non-mevalonate pathway (1, 2), present in plants, protozoa, green algae, and many bacteria (1–3), pyruvate and \textit{d}-glyceraldehyde 3-phosphate are used for the production of isopentenyl diphosphate. Isopentenyl diphosphate in turn is the precursor of vital isoprenoids, such as steroid hormones, carotenoids, cholesterol, and ubiquinone (4). In eukaryotes and Archaea (5) isopentenyl diphosphate is instead formed through the mevalonate pathway (6) starting from acetyl-CoA. The fact that humans use a different route from plants, protozoa, and many bacteria for the formation of isopentenyl diphosphate combined with the essentiality of isoprenoids makes all the enzymes within the non-mevalonate pathway interesting as potential targets for drugs and herbicides.

Tuberculosis is one of the most serious diseases of our times. According to the World Health Organization, the causative bacterium, \textit{Mycobacterium tuberculosis}, currently infects one-third of the world population, resulting in two million deaths annually. The increased prevalence of drug-resistant and multidrug-resistant strains together with the lethal combination that tuberculosis and AIDS represents, makes the need for new and better drugs urgent. In the search for new potential drug targets a number of aspects have to be considered. For instance, the target enzyme should perform a reaction vital for the bacteria, and if possible it should not have any human homologues. All enzymes within the non-mevalonate pathway fulfill these and other requirements and are, therefore, potential drug targets in \textit{M. tuberculosis}.

1-Deoxy-\textit{d}-xylulose-5-phosphate reductoisomerase (DXR, also referred to as IspC; EC 1.1.1.267) catalyzes the NADPH-dependent rearrangement and reduction of 1-deoxy-\textit{d}-xylulose 5-phosphate (DXP) to form 2-C-methyl-\textit{d}-erythritol 4-phosphate (MEP) as the second step in the non-mevalonate pathway. The reaction requires the presence of a divalent cation such as Mg2+, Co2+, or Mn2+ (7). All the enzymes within the non-mevalonate pathway in \textit{Bacillus subtilis} have been shown to be essential (8), and knockouts of the DXR enzyme in \textit{Escherichia coli} (7, 9) are lethal. The essentiality of DXR in \textit{M. tuberculosis} has also recently been demonstrated.

Fosmidomycin is a known inhibitor of the non-mevalonate pathway in plants and bacteria (10) and has been shown to

---

* This work was supported by funding from the Foundation for Strategic Research, the Swedish Research Council, the European Union Sixth Framework Program NM4TB CT:018923, and by Uppsala University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The atomic coordinates and structure factors (code 2JCV, 2JCL, 2JCH, 2JCA, 2JD0, and 2JD1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

‡ To whom correspondence should be addressed. Tel.: 46-18-4714982; Fax: 46-18-536971; E-mail: Alwynxray.bmc.uu.se.

---

2 The abbreviations used are: DXR, 1-deoxy-\textit{d}-xylulose-5-phosphate reductoisomerase; DXP, 1-deoxy-\textit{d}-xylulose 5-phosphate; EcDXR, DXR from \textit{E. coli}; F, fosmidomycin; M, Mn2+; MEP, 2-C-methyl-\textit{d}-erythritol 4-phosphate; MD, molecular dynamics; MdDXR, DXR from \textit{M. tuberculosis}; N, NADPH; S, sulfate; ESRF, European Synchrotron Radiation Facility; Bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; PDB, Protein Data Bank.

3 A. Brown and T. Parish, personal communication.
FIGURE 1. Inhibition of MtDXR by fosmidomycin. The inhibitory capacity of fosmidomycin was evaluated in a spectrophotometric assay, in which the MtDXR-catalyzed NADPH-dependent rearrangement and reduction of DXP to form MEP is monitored at 340 nm. The inset shows the relationship between the change in absorbance per second, $\Delta A_{340}$ s$^{-1}$, and the fosmidomycin concentration, for those reactions containing the inhibitor. The reaction rate in the absence of fosmidomycin was 0.0036 $\Delta A_{340}$ s$^{-1}$.

EXPERIMENTAL PROCEDURES

Cloning, Protein Expression, and Purification—The sequence corresponding to MtDXR (Rv2870c, originating from M. tuberculosis strain H37Rv (24)) amino acids 1–389 (of 413 in the full-length protein, molecular mass 41.7 kDa) was amplified by PCR from a previous construct (16). The primers 5'-CACCATGCACTCATCATCATC-ATCATGTAAGCAACTGCTACCGAC-3' (forward) and 5'-CTCATACTAGATACGGGCAGTGTAAGCTGG-3' (reverse), used together with Pfu Ultra DNA polymerase (Stratagene), simultaneously introduced an N-terminal His$_6$ tag. The Champion™ pET101 Directional TOPO® expression kit (Invitrogen) was used for ligation of the PCR product into the pET101D-TOPO vector. The primers 5'-GTCAGATCG-TGGCGGTCAACTCCGAACACCTTCAGGTCATCGAAACCCACCTG-3' and 5'-CTGGTCAACAGGGAAGCTCGG-3' were used together with the QuikChange mutagenesis kit (Stratagene) to introduce the replacements of the MtDXR double mutant, MtDXRNQ, using the new MtDXR clone as template.

Cloning for both MtDXR and MtDXRNQ was performed in E. coli TOP10F’ cells (Invitrogen). Positive clones were selected for by growth on Luria agar plates containing 50 µg/ml ampicillin, and plasmids were isolated after the QIAprep Spin Miniprep kit protocol (Qiagen). For MtDXR, an analytical PCR was performed using the T7 forward primer from the Champion™ pET101 Directional TOPO® expression kit (Invitrogen) and the reverse primer. For MtDXRNQ the analytical PCR was performed using the T7 reverse primer, and the analytical primers 5'-GTCACTGTCATGCTGGTCAAAACCCTCTC-3' and 5'-CTGGTCAACAAGGGACTTC-3' were used with the pBluescript vector (Stratagene) as template. The clones were verified by DNA sequence analysis (Uppsala Genome Center, Rudbeck Laboratory).

Expression was carried out in BL21-STARTM (DE3) cells (Invitrogen) at 37 °C. The cultures were induced with 100 mg/l isopropyl-β-D-thiogalactopyranoside (Sigma) at an A$_{595}$ of 0.6, and growth was continued for 3 h. The cells were harvested and washed with 1× SSPE buffer (150 mM NaCl, 10 mM NaH$_2$PO$_4$, pH 7.5, 1 mM EDTA), then stored at −20 °C.

Purification followed the previous protocol (16) with the following modifications. After purification on a nickel-NTA column (Qiagen), the buffer was replaced on a PD10 column (GE Healthcare) with one containing 150 mM NaCl, 10% glycerol, and 20 mM Tris-HCl, final pH 7.5. The protein was further purified by size exclusion chromatography on a HiLoad™ 16/60 Superdex™ 200 column (Amersham Biosciences) developed with the same buffer.

The pooled fractions were concentrated to 4.3 mg/ml, and the glycerol concentration was at the same time lowered to 2%.

specifically inhibit DXR (11, 12). The compound is active against the protozoan parasite Plasmodium falciparum in humans (13, 14) and Plasmodium vinckei in mice (15).

We have recently solved the structure of the M. tuberculosis DXR with a sulfate ion bound at the site of the phosphate moiety of the substrate DXP (16), which is available in the Protein Data Bank (17) under accession code 2C82. In the PDB, 12 other DXR structures are currently available representing enzymes from E. coli (18–22) and Zymomonas mobilis (23). Among the E. coli DXR (EcDXR) entries are two apo structures (PDB codes 1K5H and 1ONN) (18, 19), one structure in complex with the inhibitor fosmidomycin and a Mn$^{2+}$ ion (PDB code 1K5H) (19), and one in complex with NADPH and the substrate DXP (PDB code 1QOO) (21). The two Z. mobilis entries represent structures in complex with an acetate ion and NADPH, respectively. In this paper only the EcDXR structures will be discussed because they provided by far the most complete view of DXR structure and function.

Here we report a new cloning, expression, and purification procedure for the M. tuberculosis DXR (MtDXR), Rv2870c (24) that makes this target more amenable to structural analysis. Kinetic studies of this new construct explore the inhibition of the enzyme with fosmidomycin together with the properties of a double mutant containing the mutations D151N and E222Q (MtDXR$^\text{NQ}$). We also report the x-ray structures of MtDXR in four different complexes: fosmidomycin, Mn$^{2+}$, and NADPH; fosmidomycin and NADPH; Mn$^{2+}$, NADPH, and SO$_4^{2-}$; Mn$^{2+}$ and SO$_4^{2-}$. Structures of MtDXR$^\text{NQ}$ are also reported in three complexes: SO$_4^{2-}$; NADPH and SO$_4^{2-}$; fosmidomycin and NADPH. These structures and the information they provide contribute to an improved understanding of the reaction mechanism catalyzed by MtDXR and also to the development of new inhibitors.
## TABLE 1

Data collection and refinement statistics

Values in parenthesis are for the highest resolution shell.

<table>
<thead>
<tr>
<th></th>
<th>MtDXR-MS</th>
<th>MtDXR-MNS</th>
<th>MtDXR-FN</th>
<th>MtDXR-FMN</th>
<th>MtDXR-HPLC</th>
<th>MtDXR-NS</th>
<th>MtDXR-FMN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection statistics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beam line</td>
<td>1911-2, MAX-lab</td>
<td>ID14-2, ESRF</td>
<td>ID14-4, ESRF</td>
<td>ID14-3, ESRF</td>
<td>I911-2, MAX-lab</td>
<td>ID14-2, ESRF</td>
<td>ID14-4, ESRF</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.061</td>
<td>0.933</td>
<td>0.940</td>
<td>0.931</td>
<td>1.042</td>
<td>0.933</td>
<td>0.940</td>
</tr>
<tr>
<td>Cell axial lengths (Å)</td>
<td>67.59, 64.81, 85.96</td>
<td>67.33, 64.89, 86.06</td>
<td>67.35, 65.48, 86.09</td>
<td>67.28, 65.26, 86.04</td>
<td>68.11, 64.82, 86.33</td>
<td>67.61, 65.61, 86.02</td>
<td>67.61, 65.32, 86.18</td>
</tr>
<tr>
<td>Cell angles (°)</td>
<td>90.0, 101.9, 90.0</td>
<td>90.0, 101.7, 90.0</td>
<td>90.0, 101.6, 90.0</td>
<td>90.0, 101.7, 90.0</td>
<td>90.0, 101.7, 90.0</td>
<td>90.0, 101.7, 90.0</td>
<td>90.0, 101.9, 90.0</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>35.00-2.15 (2.27-2.15)</td>
<td>35.00-2.00 (2.11-2.00)</td>
<td>30.00-2.20 (2.32-2.20)</td>
<td>30.00-2.05 (2.16-2.05)</td>
<td>40.00-2.35 (2.48-2.35)</td>
<td>35.00-2.30 (2.42-2.30)</td>
<td>30.00-2.10 (2.21-2.10)</td>
</tr>
<tr>
<td>Number of reflections measured</td>
<td>152,495</td>
<td>226,619</td>
<td>135,226</td>
<td>141,985</td>
<td>126,833</td>
<td>148,670</td>
<td>171,156</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>39,797</td>
<td>49,295</td>
<td>36,913</td>
<td>45,559</td>
<td>30,603</td>
<td>32,964</td>
<td>42,772</td>
</tr>
<tr>
<td>Average multiplicity</td>
<td>3.8 (3.8)</td>
<td>4.6 (4.6)</td>
<td>3.7 (3.4)</td>
<td>3.1 (3.1)</td>
<td>4.1 (3.6)</td>
<td>4.5 (4.5)</td>
<td>4.0 (3.5)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>100.0 (100.0)</td>
<td>100.0 (100.0)</td>
<td>98.5 (91.5)</td>
<td>99.2 (99.9)</td>
<td>99.0 (93.1)</td>
<td>100.0 (100.0)</td>
<td>99.3 (96.2)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>0.077 (0.236)</td>
<td>0.116 (0.475)</td>
<td>0.090 (0.266)</td>
<td>0.134 (0.514)</td>
<td>0.074 (0.191)</td>
<td>0.102 (0.431)</td>
<td>0.068 (0.195)</td>
</tr>
<tr>
<td>(I/σ(I))norm</td>
<td>16.2 (6.3)</td>
<td>12.4 (3.3)</td>
<td>13.8 (6.0)</td>
<td>9.8 (3.0)</td>
<td>17.5 (6.9)</td>
<td>13.4 (3.5)</td>
<td>16.7 (7.0)</td>
</tr>
</tbody>
</table>

**Refinement statistics**

<table>
<thead>
<tr>
<th></th>
<th>MtDXR-MS</th>
<th>MtDXR-MNS</th>
<th>MtDXR-FN</th>
<th>MtDXR-FMN</th>
<th>MtDXR-HPLC</th>
<th>MtDXR-NS</th>
<th>MtDXR-FMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>35.00-2.15 (2.21-2.15)</td>
<td>35.00-2.00 (2.05-2.00)</td>
<td>30.00-2.20 (2.26-2.20)</td>
<td>30.00-2.05 (2.10-2.05)</td>
<td>40.00-2.35 (2.41-2.35)</td>
<td>35.00-2.30 (2.46-2.30)</td>
<td>30.00-2.10 (2.15-2.10)</td>
</tr>
<tr>
<td>Number of reflections used in working set</td>
<td>37,758 (2,767)</td>
<td>46,754 (3,407)</td>
<td>35,057 (2,149)</td>
<td>43,246 (3,200)</td>
<td>29,050 (1,911)</td>
<td>31,255 (2,285)</td>
<td>40,602 (2,774)</td>
</tr>
<tr>
<td>Number of reflections for Rfree calculation</td>
<td>1,996 (140)</td>
<td>2,493 (181)</td>
<td>1,848 (121)</td>
<td>2,297 (175)</td>
<td>1,540 (86)</td>
<td>1,672 (117)</td>
<td>2,156 (117)</td>
</tr>
<tr>
<td>R (%)</td>
<td>17.9 (19.3)</td>
<td>17.7 (20.7)</td>
<td>16.3 (17.2)</td>
<td>17.7 (21.2)</td>
<td>18.3 (19.3)</td>
<td>20.2 (23.4)</td>
<td>18.0 (19.6)</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>22.5 (27.4)</td>
<td>22.1 (26.0)</td>
<td>20.9 (26.8)</td>
<td>22.4 (28.0)</td>
<td>23.6 (31.6)</td>
<td>25.5 (32.8)</td>
<td>21.5 (25.7)</td>
</tr>
<tr>
<td>Number of non-hydrogen atoms</td>
<td>5,986</td>
<td>6,178</td>
<td>9,85 (91.5)</td>
<td>99.2 (99.9)</td>
<td>100.0 (100.0)</td>
<td>99.3 (96.2)</td>
<td></td>
</tr>
<tr>
<td>Number of solvent waters</td>
<td>388</td>
<td>507</td>
<td>521</td>
<td>502</td>
<td>327</td>
<td>263</td>
<td>440</td>
</tr>
<tr>
<td>Mean B-factor, protein atoms (Å²)</td>
<td>23.2, 17.5</td>
<td>17.9, 15.0</td>
<td>15.8, 16.9</td>
<td>16.7, 17.1</td>
<td>22.6, 17.4</td>
<td>31.7, 26.0</td>
<td>23.2, 20.2</td>
</tr>
<tr>
<td>Mean B-factor, solvent atoms (Å²)</td>
<td>24.4</td>
<td>23.7</td>
<td>24.1</td>
<td>24.7</td>
<td>21.7</td>
<td>26.8</td>
<td>28.2</td>
</tr>
<tr>
<td>Mean B-factor, fosmidomycin (Å²)</td>
<td>22.5</td>
<td>14.8</td>
<td>15.5</td>
<td>15.5</td>
<td>15.5</td>
<td>33.8</td>
<td></td>
</tr>
<tr>
<td>Mean B-factor, NADPH (A, B) (Å²)</td>
<td>25.5</td>
<td>31.5</td>
<td>18.8, 26.6</td>
<td>16.3, 40.1</td>
<td>12.7, 11.5</td>
<td>56.1 (b)</td>
<td>32.1 (c)</td>
</tr>
<tr>
<td>Mean B-factor, Mn²⁺ (Å²)</td>
<td>29.3, 15.3</td>
<td>16.0, 9.4</td>
<td>-23.1, 28.6</td>
<td>-23.1, 28.6</td>
<td>-23.1, 28.6</td>
<td>33.0, 32.7, 37.8</td>
<td>-30.8, 25.8</td>
</tr>
<tr>
<td>Mean B-factor, sulfate ligands (A, B, AB) (Å²)</td>
<td>24.2, 15.6, 23.2</td>
<td>16.5, 11.3, 22.9</td>
<td>-23.1, 28.6</td>
<td>-23.1, 28.6</td>
<td>22.4, 27.8, 29.8</td>
<td>33.0, 32.7, 37.8</td>
<td>-30.8, 25.8</td>
</tr>
<tr>
<td>Ramachandran plot outliers (%)</td>
<td>1.3</td>
<td>0.9</td>
<td>0.6</td>
<td>1.2</td>
<td>1.5</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Root mean square deviation from ideal bond length (Å)</td>
<td>1.16</td>
<td>1.17</td>
<td>1.23</td>
<td>1.20</td>
<td>1.10</td>
<td>1.11</td>
<td>1.27</td>
</tr>
<tr>
<td>Root mean square deviation from ideal bond angle (°)</td>
<td>0.010</td>
<td>0.010</td>
<td>0.012</td>
<td>0.010</td>
<td>0.008</td>
<td>0.008</td>
<td>0.011</td>
</tr>
</tbody>
</table>

---

* Multiplicity-corrected Rmerge (57).
* Calculated on the part of NADPH with full occupancy.
* Calculated using a strict-boundary Ramachandran plot (58).
* Calculated using small molecule-based parameters (59).
Structures of M. tuberculosis DXR

A

B

C

D
The material was more than 98% pure as deduced from SDS/PAGE analysis (PhastSystemTM, Amersham Biosciences). The yield of pure protein was 1.4 mg/liter culture for MtxDXR and 1.7 mg/liter culture for DXP-R. —DXP and fosmidomycin were generously provided by AstraZeneca India Pvt. Ltd. The inhibitory potential of fosmidomycin was evaluated in a spectrophotometric assay (7, 25) in which the NADPH-dependent rearrangement and reduction of DXP to form MEP, catalyzed by MtxDXR, was monitored at 340 nm using the absorption of NADPH (ε₃₄₀ nm = 6220 M⁻¹ cm⁻¹). Each assay contained 350 μl of reaction buffer (129 mM NaCl, 2.9 mM β-mercaptoethanol, 5.7% Me₃SO, 2.1 mM MnCl₂, and 64 mM HEPES-NaOH, pH 7.5), 50 μl of 3.85 μM MtxDXR (in 100 mM NaCl, 0.01% Brij-35, and 50 mM HEPES-NaOH pH 7.5), 50 μl of 1.1 mM NADPH (in distilled H₂O), 50 μl of 2.2 mM DXP (in distilled H₂O), and 50 μl of fosmidomycin (in distilled H₂O).

One measurement was made in the absence of fosmidomycin together with six measurements with final fosmidomycin concentrations ranging between 25 and 200 nm. The decrease in absorbance at 340 nm was followed at 22 °C (DU® 640 spectrophotometer, Beckman) with time points every 5 s during a 3-min period. The slope of the linear phase (25–125 s) of each reaction was used in calculating the initial velocity (Fig. 1). The velocities were plotted against the corresponding fosmidomycin concentrations. A line was fitted to the points in the approximately linear region of the curve (inset in Fig. 1), and the equation so generated was used to estimate the IC₅₀ value of fosmidomycin.

Crystallization—MtxDXR was co-crystallized with fosmidomycin using vapor diffusion. The sitting drop contained 0.9 μl of protein solution (3.5 mg/ml MtxDXR in the buffer used during concentration, plus 10 mM fosmidomycin, 3 mM NADPH, 0.1 mM EDTA, and 10 mM dithiothreitol) and 0.9 μl of reservoir solution (0.2 M AmSO₄, 25% polyethylene glycol 3350, and 0.1 M Bis-Tris, final pH 5.7) at 22 °C. Needle-like crystals appeared within a few days. Crystallization of both MtxDXR and MtxDXR<sup>NQ</sup> was then optimized with the aid of seeding in a batch experiment. The 4-μl sitting drops consisted of 2 μl of protein solution (3.3 mg/ml MtxDXR or MtxDXR<sup>NQ</sup> in the buffer used in the concentration with the addition of 0.1 mM EDTA and 10 mM dithiothreitol) and 2 μl of crystallization buffer (0.2 M AmSO₄, 25% polyethylene glycol 3350, and 0.1 M Bis-Tris, final pH 5.7). The reservoir solution consisted of 0.1 M AmSO₄, 12.5% polyethylene glycol 3350, 0.05 M Bis-Tris, 75 mM NaCl, and 10 mM Tris-HCl, final pH 5.9.

MtxDXR was co-crystallized with ligands in the combinations: DXP and MnSO₄; DXP and NADPH; DXP, MnSO₄, and NADPH; fosmidomycin and NADPH; fosmidomycin, MnSO₄, and NADPH. The mutant MtxDXR<sup>NQ</sup> was crystallized without ligands as well as in the following combinations: DXP, MnSO₄, and NADPH; fosmidomycin and NADPH. The following concentrations of the ligands were added to the protein solution in co-crystallization experiments: 20 mM DXP, 10 mM fosmidomycin, 12 mM MnSO₄, and 3 mM NADPH. Needle-like crystals appeared within a few days and grew to average dimensions of 0.2 × 0.05 × 0.02 mm in 2 weeks. Before the crystals were flash-cooled in liquid nitrogen, they were transferred to a drop of cryo solution containing 0.1 mM AmSO₄, 13% polyethylene glycol 3350, 0.06 M Bis-Tris, 75 mM NaCl, 25% glycerol, final pH 5.7. Depending on which ligands were used for co-crystallization, the following concentrations of the ligands were also present in the cryo solution: 10 mM DXP, 10 mM fosmidomycin, 12 mM MnSO₄, and 3 mM NADPH.

Inhibition Assay—DXP and fosmidomycin were generously provided by AstraZeneca India Pvt. Ltd. The inhibitory potential of fosmidomycin was evaluated in a spectrophotometric assay (7, 25) in which the NADPH-dependent rearrangement and reduction of DXP to form MEP, catalyzed by MtxDXR, was monitored at 340 nm using the absorption of NADPH (ε₃₄₀ nm = 6220 M⁻¹ cm⁻¹). Each assay contained 350 μl of reaction buffer (129 mM NaCl, 2.9 mM β-mercaptoethanol, 5.7% Me₃SO, 2.1 mM MnCl₂, and 64 mM HEPES-NaOH, pH 7.5), 50 μl of 3.85 μM MtxDXR (in 100 mM NaCl, 0.01% Brij-35, and 50 mM HEPES-NaOH pH 7.5), 50 μl of 1.1 mM NADPH (in distilled H₂O), 50 μl of 2.2 mM DXP (in distilled H₂O), and 50 μl of fosmidomycin (in distilled H₂O).

One measurement was made in the absence of fosmidomycin together with six measurements with final fosmidomycin concentrations ranging between 25 and 200 nm. The decrease in absorbance at 340 nm was followed at 22 °C (DU® 640 spectrophotometer, Beckman) with time points every 5 s during a 3-min period. The slope of the linear phase (25–125 s) of each reaction was used in calculating the initial velocity (Fig. 1). The velocities were plotted against the corresponding fosmidomycin concentrations. A line was fitted to the points in the approximately linear region of the curve (inset in Fig. 1), and the equation so generated was used to estimate the IC₅₀ value of fosmidomycin.

Crystallization—MtxDXR was co-crystallized with fosmidomycin using vapor diffusion. The sitting drop contained 0.9 μl of protein solution (3.5 mg/ml MtxDXR in the buffer used during concentration, plus 10 mM fosmidomycin, 3 mM NADPH, 0.1 mM EDTA, and 10 mM dithiothreitol) and 0.9 μl of reservoir solution (0.2 M AmSO₄, 25% polyethylene glycol 3350, and 0.1 M Bis-Tris, final pH 5.7) at 22 °C. Needle-like crystals appeared within a few days. Crystallization of both MtxDXR and MtxDXR<sup>NQ</sup> was then optimized with the aid of seeding in a batch experiment. The 4-μl sitting drops consisted of 2 μl of protein solution (3.3 mg/ml MtxDXR or MtxDXR<sup>NQ</sup> in the buffer used in the concentration with the addition of 0.1 mM EDTA and 10 mM dithiothreitol) and 2 μl of crystallization buffer (0.2 M AmSO₄, 25% polyethylene glycol 3350, and 0.1 M Bis-Tris, final pH 5.7). The reservoir solution consisted of 0.1 M AmSO₄, 12.5% polyethylene glycol 3350, 0.05 M Bis-Tris, 75 mM NaCl, and 10 mM Tris-HCl, final pH 5.9.

MtxDXR was co-crystallized with ligands in the combinations: DXP and MnSO₄; DXP and NADPH; DXP, MnSO₄, and NADPH; fosmidomycin and NADPH; fosmidomycin, MnSO₄, and NADPH. The mutant MtxDXR<sup>NQ</sup> was crystallized without ligands as well as in the following combinations: DXP, MnSO₄, and NADPH; fosmidomycin and NADPH. The following concentrations of the ligands were added to the protein solution in co-crystallization experiments: 20 mM DXP, 10 mM fosmidomycin, 12 mM MnSO₄, and 3 mM NADPH. Needle-like crystals appeared within a few days and grew to average dimensions of 0.2 × 0.05 × 0.02 mm in 2 weeks. Before the crystals were flash-cooled in liquid nitrogen, they were transferred to a drop of cryo solution containing 0.1 mM AmSO₄, 13% polyethylene glycol 3350, 0.06 M Bis-Tris, 75 mM NaCl, 25% glycerol, final pH 5.7. Depending on which ligands were used for co-crystallization, the following concentrations of the ligands were also present in the cryo solution: 10 mM DXP, 10 mM fosmidomycin, 12 mM MnSO₄, and 3 mM NADPH.

Data Collection, Structure Determination, Refinement, and Analysis—X-ray data were collected under cryo conditions at various beamlines at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France, and at MAX-lab in Lund, Sweden (Table 1). Diffraction data were indexed and integrated with MOSFLM (26) and processed with SCALA (27) as implemented in the CCP4 program suite (28). Statistics are shown in Table 1. The crystals possessed the symmetry of space group P₂₁. The Matthews coefficient (29) suggested that there were two molecules in the asymmetric unit; the value was predicted to be 2.2 Å³/Da with 44.5% solvent. The structures were solved by molecular replacement using AMoRe (30) with the apo M. tuberculosis DXR structure (PDBe code 2C82) (16) as a search model.
Structures of M. tuberculosis DXR

A

FIGURE 3. Fosmidomycin, DXP, and MEP. A, carbon atom numbering of fosmidomycin, DXP, and MEP. B, the conformation of fosmidomycin around the metal ion is illustrated as well as the modeled conformations of DXP and MEP. The H+ hydrogen atom at C1 of MEP is derived from the NADPH cofactor. In our proposal the O3 and O2 atoms of the substrate remain coordinated to the active site ion, becoming the O1 and O2 hydroxyl groups of the product without substantial rearrangement.

Docking, Molecular Dynamics, and Empirical Valence Bond Calculations—The free energy profile for proton abstraction from the O4 hydroxyl of DXP by the carboxylate group of Glu-153 (see “Discussion”) was investigated using the empirical valence bond method (35, 36). Molecular dynamics (MD) simulations combined with the free energy perturbation technique were used to describe the corresponding process in the solvated protein-substrate complex (35, 36). The valence bond states used here correspond to the negatively charged carboxylate of Glu-153 and the O4 hydroxyl of DXP (φ1) and to the protonated carboxylate of Glu-153 and the charged O4 alkoxy anion of DXP (φ2). For DXP, restrained electrostatic potential (37) charges were derived for all atoms except for the diatomic phosphate group, for which partial charges were extracted from the CHARMM22 force field (38). From the pKₐ difference between the O4 hydroxyl of DXP and glutamic acid, the reaction free energy for the proton abstraction in water, ΔG°wat, was estimated to be 14.3 kcal/mol. The corresponding activation free energy, ΔG*wat, was predicted from linear free energy relationships to be 15.4 kcal/mol after correction for bringing the donor and acceptor to contact distance (39, 40).

The docking calculations and MD simulations were carried out based on the M. tuberculosis DXR structure in complex with fosmidomycin, Mn²⁺, and NADPH. The binding mode of DXP was modeled with the program GOLD Version 3.0 using default settings and 50 docking runs (41). The top-ranked solution was essentially identical to the interactively docked DXP. In the MD simulation of the reaction, we used the MtDXR structure in complex with NADPH, the docked DXP, and an active site Mg²⁺ ion, which was placed in the same position as the Mn²⁺ ion in the original structure. The complex was solvated by using TIP3P water molecules (42) in a sphere with a 24-Å radius centered on DXP.

RESULTS

Enzyme Properties—The shortened construct used was designed based on observations in the previous apo structure (16), where the last four residues of the construct (390-393) could not be observed in the electron density. The protein with an N-terminal His₆ tag was overexpressed in E. coli. It behaved as a homogenous dimer during purification, and the activity showed no activity. To test the inhibitory capacity of fosmidomycin, the NADPH-dependent rearrangement and reduction of DXP to form MEP was evaluated using a spectrophotometric assay; see Fig. 1. With MtDXR at a concentration of 0.35 μM and substrate concentrations of 0.2 mM for DXP and 0.1 mM for NADPH, the IC₅₀ value for fosmidomycin was estimated to be 80 nM.
MtDXR and MtDXR<sub>NQ</sub> Structures—The MtDXR and MtDXR<sub>NQ</sub> complex structures were solved in space group P2<sub>1</sub> by molecular replacement using the apo MtDXR structure as a search model. The various structures were refined to resolutions ranging between 2.00 and 2.35 Å; data collection and refinement statistics are summarized in Table 1. The asymmetric unit in each case contains one homodimer. Unless otherwise indicated, the electron density for the main chain was of very good quality except for the N-terminal His<sub>4</sub> tag and the first 10 residues. The subunit is, as previously described, composed of three domains, an N-terminal NADPH binding domain, a central catalytic domain, and a C-terminal α-helical domain. The domains are arranged in a V shape, where the N-terminal and C-terminal domains form the two arms, and the catalytic domain lies at the vertex (Fig. 2A). The dimer interface is created by interactions between the catalytic domains and the connecting regions that lie between the catalytic and the C-terminal domain of each subunit. A sulfate ion is also placed close to the dimer interface interacting with the side chain of Arg-162 (NE and NH<sub>2</sub>) from the A molecule.

During refinement of the structures crystallized in the presence of DXP, it became clear that the substrate could not be modeled in any of the structures. Instead, a sulfate ion was found in the active site at the expected position of the DXP phosphate. Therefore, the structures presented in this paper are designated as MtDXR-FMN, MtDXR-FN, MtDXR-MNS, MtDXR-MS, MtDXR<sub>NQ</sub>-S, MtDXR<sub>NQ</sub>-NS, and MtDXR<sub>NQ</sub>-FN (where F stands for fosmidomycin, M for Mn<sup>2+</sup>, N for NADPH, and S for SO<sub>4</sub><sup>2-</sup>).

The root mean square difference between Cα positions in the two subunits when they are superimposed ranges between 1.6 and 2.0 Å in the different structures. The rather large values reflect significant conformational differences between the two subunits, as was also observed for the first published DXR structure (EcDXR, PDB code 1K5H). The differences are best described as rigid body domain rotations combined with flexibility of the active site flap (residues 198–209). In the B molecule the flap is locked into an open conformation by crystal packing interactions, which results in poor or no electron density for inhibitor or cofactor. By contrast, the conformations of the active site flap in the A molecules depend on the interactions with substrates, cofactors, and inhibitors and are linked to well defined ligand electron density; thus, most of the discussion that follows will focus on the active site of the A molecule.

When aligning the dimers (Cα positions) of the seven M. tuberculosis structures presented here using a restrictive pair cutoff of 0.5 Å, one can see that the complete B chains and dimer-forming central catalytic domain of the A chains are well aligned. This indicates that the crystal contacts keep the B chains fixed while allowing the A chains to flex depending on the ligand state. Using the distance across the active site cleft (between the Cα atoms of residues Gly-47 and Ala-339) as a simple yardstick, the relative motions of the N- and C-terminal domains can be assessed (Table 2). The S.D. for this distance is 0.7 Å for the A chains but only 0.2 Å for the B chains. Furthermore, the A chains that bind NADPH show similar, more closed conformations, whereas the other A chains from the mutant and from the structure lacking NADPH show a larger variation and also a larger separation across the active site cleft.

MtDXR-MS displays octahedral coordination of the Mn<sup>2+</sup> ions, each of which interacts with three water molecules and the σ<sub>syn</sub> orbitals of residues Asp-151 (OD1 and OD2, respectively, in A and B), Glu-153 (OE1), and Glu222 (OE1). The sulfate ion interacts with three water molecules and with residues Ser-177 (OG and N), Ser-213 (OG), Asn-218 (ND2), and Lys-219 (NZ), which are conserved residues in DXR sequences (47) and make up a phosphate/phosphonate binding pocket that has been described in EcDXR-N,DXP and EcDXR-FN. Poor electron density is observed for residues A199–A206 and B204–B206 in the active site flap regions.

The MtDXR-MNS structure shows that the binding of NADPH has little effect on the Mn<sup>2+</sup> coordination. However, the interactions with the sulfate ion are affected by NADPH binding, albeit indirectly. The flap of the A molecule is better defined than for the MtDXR-MS structure, with improved density for the imidazole ring of His-200, which interacts with the sulfate via NE2. However, the temperature factors of key flap side chains are still somewhat elevated (in the range of 30–40 Å<sup>2</sup>). The density for the nicotinamide ring and also for a portion of the sugar in the NADPH is also quite weak, with correspondingly high B-factors. The nicotinamide phosphate and AMP portion of the cofactor have better density and lower B-factors. In the B molecule, the metal ion and the sulfate are present, but the NADPH could not be modeled from the electron density (although some density is seen at the 2′-phosphate). Poor electron density is also observed for residues B204–B205.

MtDXR-FN shows clearly the position of fosmidomycin and NADPH in the absence of the Mn<sup>2+</sup> ion. The electron density for the sugar and nicotinamide ring in the NADPH is better than that observed in MtDXR-MNS. Here only a small portion of the nicotinamide ring is mobile, indicating the importance of inhibitor and most probably also substrate binding for optimal binding of NADPH. The residues interacting with fosmidomycin are strictly conserved in M. tuberculosis, whereas only 70% of the residues interacting with NADPH in the M. tuberculosis structure are found in the E. coli enzyme. In the B molecule, a sulfate is bound at the phosphonate position of the inhibitor. Given the high concentration of AmSO<sub>4</sub> used in the crystallization, this is unlikely to be part of a disordered fosmidomycin molecule.

MtDXR-FMN provides the most complete picture of interactions in the active site of MtDXR (Fig. 2). NADPH is bound in the N-terminal domain and comes in close contact with the inhibitor fosmidomycin (closest contact is 3.1 Å from the C5 to O2 of the inhibitor; the naming convention is shown in Fig. 3A) and the Mn<sup>2+</sup> ion that are positioned underneath the active site flap; see Fig. 2A. Residues Thr-21 (OG1), Gly-22 (N), Ser-23 (N), Ile-24 (N), Gly-47 (N), Gly-48 (N), Ala-49 (N), and Glu-129 (OE1) of the N-terminal domain interact with NADPH as well as residues Gly-206 (N) and Asn-209 (ND2) from the active site flap in the catalytic domain (Fig. 2B). The amine group of the Asn-209 side chain points directly at the center of the nicotinamide ring in a classic amide aromatic ring interaction (48). The Mn<sup>2+</sup> ion is coordinated by interactions with residues Asp-151 (OD2), Glu-153 (OE1), and Glu-222 (OE2) as well as to the
inhibitor fosmidomycin (O1 and O2). The inhibitor also interacts with residues Ser-152 (OG and N), Ser-177 (OG and N), His-200 (NE2), Asn-218 (ND2), and Lys-219 (NZ), all located in the catalytic domain. The key side chains from the flap are well defined and cause the inhibitor to be completely shielded from the bulk solution. A number of solvent molecules, however, are trapped in an enclosed pocket (Fig. 2C), and one of these interacts with the phosphonate group. The electron density for the active site residues as well as for the Mn$^{2+}$ ion, fosmidomycin, and NADPH is well defined (see Fig. 2D). When comparing MtDXR-FMN with the most analogous E. coli DXR structure, that with PDB code 1ONP (19), it is evident that the coordination of the inhibitors around the Mn$^{2+}$ ion is different. In MtDXR-FMN one can see that when fosmidomycin binds, the octahedral coordination of the metal, as seen in MtDXR-MNS and MtDXR-MS, is slightly disturbed. Three water molecules are displaced. The two oxygen atoms of the (N-formyl-N-hydroxy) amino group of the inhibitor replace only two of these three water molecules, and so the sixth coordination site is empty. This may be a steric effect due to the lack of space between the C3 atom of the inhibitor and the OE2 of Glu-153. In the coordination seen in MtDXR-FMN, the N-hydroxyl oxygen of the inhibitor, corresponding to the hydroxyl group at C3 in the DXP substrate (Fig. 3A), is positioned opposite Glu-153, in close proximity to C4 and C5 of the nicotinamide ring of NADPH (3.5 and 3.1 Å, respectively). The N-formyl oxygen, corresponding to O2 of the substrate, is positioned opposite residue Glu-222. Thus, the free coordination position is opposite Asp-151. By contrast, in 1ONP, the N-hydroxyl oxygen of fosmidomycin is positioned opposite the residue equivalent to Asp-151 in MtDXR-FMN, whereas the N-formyl oxygen lies opposite the residue equivalent to Glu-222. A water
molecule positioned opposite the residue corresponding to Glu-153 completes the full octahedral coordination of the Mn\(^{2+}\) ion in the \(E.\ coli\) structure.

The \(Mt\)DXR-FMN active site contains two enclosed pockets; see Fig. 2C. The larger one, referred to earlier, is hydrated, whereas the other is smaller and bounded by the inhibitor, cofactor, and side chains of Trp-203, Met-205, and Met-267. A comparison of \(Mt\)DXR-FMN with \(Mt\)DXR-FN and \(Mt\)DXR-MNS shows that they are highly similar overall. The side chains of Asp-151 and Ser-152 together with fosmidomycin and side chains of Trp-203, Met-205, and Met-267, whereas the other is smaller and bounded by the inhibitor, respectively. The larger one, referred to earlier, is hydrated, see Fig. 2.

The occupancy has been excluded from the refinement. Whereas the complete nicotinamide portion in the B molecule for residues A199–A205. The density for the nicotinamide portion of NADPH is poor, although not as closed as those observed in the \(Ec\) structures, based on their N-terminal NADPH-binding sites are occupied, but in an open conformation. Structure factors have not been deposited for this entry, and so the electron density cannot be inspected, although it is apparent that the NADPH molecules have elevated B-factors compared with the protein. This may indicate that crystal packing forces have trapped this protein conformation.

When grouping the \(Mt\)DXR structures by the same criteria, one can see that all B molecules as well as both A and B molecules from \(Mt\)DXR-FN show an open conformation. The A molecules generally display a more closed conformation, although not as closed as those observed in the \(Ec\) structures \(Ec\)DXR-FN and \(Ec\)DXR-N,DXP. \(Ec\)DXR-NS needs special mention since both sites are occupied but in an open conformation. Structure factors have not been deposited for this entry, and so the electron density cannot be inspected, although it is apparent that the NADPH molecules have elevated B-factors compared with the protein. This may indicate that crystal packing forces have trapped this protein conformation.

Comparison to Other DXR Structures—Our previous comparison (16) of 13 DXR structures from \(M.\ tuberculosis\), \(E.\ coli\), and \(Z.\ mobilis\) showed that the overall three-dimensional structural arrangement is conserved. However, the \(Mt\)DXR structure exhibits some differences from the others. These include a shorter \(a3/b4\) unit (residues 70–84 and shorter \(a7/b7\) and \(a8/b8\) loops (residues 142–145 and 164–169, respectively). Although the overall sequence identity is \(\sim 40\%\), the central catalytic domain shows a higher degree of conservation (45–50% amino acid sequence identity) than the N- and C-terminal domains (40 and 35% amino acid sequence identity, respectively). A comparison of the various DXR structures also showed that there is variation in the way that the domains move relative to each other, triggered by the binding of cofactors, substrates and inhibitors, and differences in crystal forms (21). The \(E.\ coli\) structures can be grouped into closed, open and super-open forms, as reflected in the distances across the active site cleft (Table 2). The A and B chains of \(Ec\)DXR (PDB code 1K5H) are the only representatives of the super-open conformation. Among the structures showing the open conformation are those binding a single substrate or inhibitor with or without a metal ion present. Structures showing closed conformations include those where both substrate and cofactor binding sites are occupied, i.e. \(Ec\)DXR-FN (with and without citric acid) and \(Ec\)DXR-N,DXP. \(Ec\)DXR-NS needs special mention since both sites are occupied but in an open conformation. Structure factors have not been deposited for this entry, and so the electron density cannot be inspected, although it is apparent that the NADPH molecules have elevated B-factors compared with the protein. This may indicate that crystal packing forces have trapped this protein conformation.

When grouping the \(Mt\)DXR structures by the same criteria, one can see that all B molecules as well as both A and B molecules from \(Mt\)DXR-FN show an open conformation. The A molecules generally display a more closed conformation, although not as closed as those observed in the \(Ec\) DXR-N,DXP. This difference could be a consequence of the species difference. The larger variations of the distance across the active site among the \(E.\ coli\) structures may be associated with the different crystallization conditions and space groups involved. A superposition of \(Mt\)DXR and \(Ec\)DXR structures, based on their N-terminal NADPH-binding domains, is shown in Fig. 4C to illustrate the variation.

MD Simulations and Empirical Valence Bond Calculations—The free energy profiles for the proton abstraction from the O4 hydroxyl of DXP by a glutamic acid residue in solution and by Glu-153 in \(Mt\)DXR are shown in Fig. 5. The activation and reaction free energy in the protein are significantly lower than for the reaction in solvent; evidently the enzyme exerts a large catalytic effect on this reaction step. It is particularly notewor-

FIGURE 4. Changes in the structure on ligand binding. A, stereo view of the super-positioning of the active sites of \(Mt\)DXR-FN (pink) and \(Mt\)DXR-FMN (gold), showing the movement of foremost Asp-151 and fosmidomycin in the presence or absence of Mn\(^{2+}\). B, stereo view of the active site of \(Mt\)DXR-MNS showing the octahedral coordination of the Mn\(^{2+}\) ion by Asp-151, Glu-153, Glu-222, and three water molecules. C, stereo view of the structural comparisons. The \(M.\ tuberculosis\) structures \(Mt\)DXR-S (red), \(Mt\)DXR-MS (magenta), \(Mt\)DXR-MNS (brown), \(Mt\)DXR-FN (black), and \(Mt\)DXR-FMN (cyan) and the \(E.\ coli\) structures \(Ec\)DXR-N,DXP (green), \(Ec\)DXR-blue (blue, PDB code 1ION), and \(Ec\)DXR-FM (yellow) have been superimposed based on their N-terminal domains (Ca atoms) with the \(lsq\)-\(explicit\) command in O (32). The \(M.\ tuberculosis\) enzyme displays a smaller movement of the domains than the \(E.\ coli\) enzyme upon the binding of different substrates and cofactors. D, stereo view of the active site of \(Mt\)DXR-FMN with models of DXP (magenta) and MEP (cyan) super-positioned onto our experimentally observed fosmidomycin structure. Golden bubbles show the observed interactions with the Mn\(^{2+}\) ion. Magenta and cyan bubbles indicate the close contacts between the ion and the O4 and O3 hydroxyl groups of DXP and MEP, respectively.
move as rigid bodies with respect to the catalytic domain under the influence of ligand/substrate binding and crystal contacts. Our structural studies of \textit{MtDXR} show similar effects. In the structures presented here, crystal contacts stabilize an open active site in the B molecule. The active site in the A molecule is more closed, although some variation is possible depending on the binding of ligands and substrates. An active site flap is present in both \textit{E. coli} and \textit{M. tuberculosis} enzymes. Well defined density for the \textit{M. tuberculosis} flap requires well defined density for the complete NADPH cofactor in the closed active site cleft. It may be of particular relevance that in the best-defined structures, the main-chain nitrogen of Gly-206 interacts with the nicotinamide phosphate, and the side chain of Asn-209 interacts with the nicotinamide sugar and ring. The side chains of two flap residues, Trp-203 and Met-205, help to shield the substrate, whereas a third, His-200, contributes to the binding of the substrate phosphate group. All five residues are highly conserved among DXR enzymes (47).

The structures reveal that the mutant can still bind NADPH, sulfate, and fosmidomycin but that it has lost its ability to bind the metal ion, which in turn causes loss of activity. In the \textit{MtDXR} structure with a sulfate in the active site (PDB code 2C82) (16) the short distance of 2.45 Å between Asp-151 (OD1) and Glu-222 (OE2) suggests that one of these residues has to be protonated, which is unsurprising given the pH of 5.1 used to produce crystals. In \textit{MtDXR}\textsubscript{NO\textsubscript{2}-S} the interaction is lost, which seems to affect not only the metal binding but also NADPH binding. By comparing the mutant structures with the corresponding wild type structures, for example \textit{MtDXR}\textsubscript{NO\textsubscript{2}-FN} and \textit{MtDXR}-FN, one can see that the position of NADPH is less defined in the mutant. The collection of structures also shows that the adenosine part of NADPH and especially the 2' phosphophate bound to it is better defined than the nicotinamide part, confirming the importance of this group for binding, as previously proposed (49).

By comparing the structures of \textit{MtDXR-FN} and \textit{MtDXR-FMN}, it can be seen that the metal binding residues and the (N-formyl-N-hydroxy) amino group of the inhibitor must move to coordinate the metal. By contrast, the positions of the phosphonate part of the inhibitor and of NADPH remain fixed. Comparison of \textit{MtDXR-FN} with \textit{EcDXR-FN} shows that the position of the inhibitors in these structures is equivalent. However, when comparing \textit{MtDXR-FMN} and the \textit{E. coli} structure in complex with fosmidomycin and Mn\textsuperscript{2+} (PDB code 1ONP) (19) it becomes clear that the conformation of the inhibitor is different. This could be due to the lack of NADPH in the 1ONP structure, \textit{i.e.} a real effect, or to a misinterpretation of the crystallographic results. The 1ONP study was carried out at 2.5 Å resolution and clearly showed that the inhibitor was bound in the active site. However, since structure factor data are not available, we are unable to evaluate which mode of ligand (and possibly water) binding is in best agreement with their experimental data. The coordination around the metal ion has important mechanistic consequences. The production of MEP from DXP is carried out in two steps, an isomerization followed by an NADPH-dependent reduction. The intramolecular rearrangement of DXP to form the aldehyde intermediate, 2-C-methylerythrose 4-phosphate, is thought to occur via a retroaldoliza-

**FIGURE 5. Reaction free energy profiles.** Calculated free energy profiles for the proton abstraction from the O4 hydroxyl by a glutamic acid in solution \textit{(open diamonds)} and by Glu-153 in \textit{MtDXR} \textit{filled diamonds).} The reaction coordinate, \(\Delta f_{1,2}\), is the energy difference between the two valence bond states \(\phi_1\) and \(\phi_2\). The reactants \(R\) and products \(P\) corresponding to \(\phi_1\) and \(\phi_2\), respectively, are shown below the free energy profiles. In the enzyme the transition state \(TS\) is stabilized by more than 7 kcal/mol compared with the corresponding uncatalyzed reaction in solution.

**DISCUSSION**

The sequence corresponding to \textit{M. tuberculosis} Rv2870c codes for a fully functional DXR (16, 49, 50). Our previous construct, which contained a C-terminal truncation of 20 amino acids, showed similar kinetic parameters to the full-length and a proteolytically cleaved enzyme (49) truncated by 18 amino acids. The even shorter construct used here showed similar activity, indicating that a C-terminal truncation of 24 amino acids still results in an active enzyme. We have now also shown that the inhibitor fosmidomycin, at an enzyme concentration of 0.35 \textmu M and substrate concentrations of 0.2 mM DXP and 0.1 mM NADPH, inhibits the enzyme with an estimated IC\textsubscript{50} value of 80 nM. This is similar to values of 310 and 30 nM that have been published for the \textit{M. tuberculosis} enzyme with an estimated IC\textsubscript{50} value of 80 nM. This is similar to values of 310 and 30 nM that have been published for the \textit{M. tuberculosis} enzyme with an estimated IC\textsubscript{50} value of 80 nM.

### The reaction coordinate

The reaction coordinate, \(\Delta f_{1,2}\), is the energy difference between the two valence bond states \(\phi_1\) and \(\phi_2\). The reactants \(R\) and products \(P\) corresponding to \(\phi_1\) and \(\phi_2\), respectively, are shown below the free energy profiles. In the enzyme the transition state \(TS\) is stabilized by more than 7 kcal/mol compared with the corresponding uncatalyzed reaction in solution.

### Structures of \textit{M. tuberculosis} DXR

Crystallography is usually considered to provide a static view of an enzyme structure. However, by solving structures of different crystal forms with and without different substrates, inhibitors, ligands, and cofactors, one can build up insights into the dynamic properties of an enzyme. Extensive studies on the EcDXR (listed in Table 2) indicate well defined catalytic domains that provide much of the rather rigid dimer interface. The N-terminal NADPH binding and C-terminal domains...
tion or an α-ketol rearrangement (52). Three different studies of DXRs from the cyanobacterium Synechocystis (53), E. coli (54), and M. tuberculosis (49) agree that the pro-R hydrogen of C1 of MEP is derived from the 4S-H of NADPH in the reduction step. However, the mode of fosmidomycin binding reported in the 1ONP structure is not suitable for this stereochemistry. To explain the stereochemistry of the hydride transfer from this model, a rearrangement of the intermediate 2-C-methylerythrose 4-phosphate around the Mn²⁺ ion was, therefore, proposed (19). This new coordination would in fact be directly equivalent to a fosmidomycin binding mode that we observe in our structure. In Figs. 3B and 4D we have made a simple modeling experiment of the substrate and product binding based on the position of fosmidomycin observed in our MtDXR-FMN structure. In this model, hydride transfer from C4 of NADPH can take place to provide the stereochemically correct product and without major rearrangement of the metal coordination. Furthermore, in MtDXR-FMN, the hydrogen atom of NADPH is transferred from the position below C4 in the nicotinamide ring, which would be appropriate if DXR is a class B dehydrogenase, as proposed earlier (21) and as suggested by the stereochemical course of the reaction (49, 53, 54). Our model for substrate binding may provide yet another mechanistic insight. The O4 hydroxyl of our DXP model comes close to the unoccupied Mn²⁺ coordination site and to the carboxylate group of Glu-153 (Fig. 4D). Glu-153 is, therefore, ideally positioned for the proton abstraction step, which leads to the known enzyme intermediate via a proposed retroaldolization mechanism (52).

We have evaluated the likelihood of this proton transfer step using MD simulations and free energy calculations. The free energy profiles, which showed that the enzyme exerts a large catalytic effect on the reaction, therefore provide support for the first step in a possible retroaldolization mechanism. Both of the isomerization mechanisms (52) also require at some stage deprotonation of the DXP O3 hydroxyl group, and Glu-222 is suitably positioned for such a task.

Our main interest in studying MtDXR is to contribute toward the development of new drugs against M. tuberculosis. Fosmidomycin is not active against M. tuberculosis (50), although it is active against Plasmodium species that cause malaria (13–15). It has been suggested that the only possibilities to build upon the fosmidomycin scaffold would require either a displacement of the nicotinamide ring of NADPH or targeting the open conformation of DXR (21). Our studies suggest otherwise. Fosmidomycin-like compounds have already been produced that are more active than fosmidomycin against P. falciparum (51). We believe that the most active of these new compounds (with a 3,4-dichlorophenyl substitution at the C4 atom of fosmidomycin) does not extend into the NADPH site but that instead it extends into the hydrated cavity, lined by highly conserved amino acids (Thr-175, Ser-245, and His-248) (47), that is located close to the substrate binding site. As far as we know this compound has not been tested on mycobacteria, although, as for fosmidomycin itself, the phosphate group makes it unlikely that the compound will be transported into M. tuberculosis. Although efforts should be made to replace the phosphate group with one that is more compatible with bacterial uptake, this does not preclude the use of the NADPH binding site as a target for the design of new inhibitors. Initial attempts at targeting the NADPH binding site have already produced mimics that bind with ~200 nm affinity (55), which provides incentive for further efforts. No compounds have yet been produced, however, that make full use of the binding potential available in the active site of DXR. Our structural work, therefore, provides a strong framework for the design of DXR inhibitors, with the desired bacteriocidal activities.

Acknowledgments—We are grateful to AstraZeneca India Pvt. Ltd for providing the template plasmid, the inhibitor fosmidomycin, and the substrate 1-deoxy-D-xylulose 5-phosphate. We are also thankful to Evalena Andersson, Annette Roos, Johan Linge, Alina Castell, Patrik Johansson, Anna Jansson, and Emma Jakobsson for assistance in data collection.

REFERENCES
Structures of *Mycobacterium tuberculosis* 1-Deoxy-D-xylulose-5-phosphate Reductoisomerase Provide New Insights into Catalysis

Lena M. Henriksson, Torsten Unge, Jens Carlsson, Johan Aqvist, Sherry L. Mowbray and T. Alwyn Jones

doi: 10.1074/jbc.M701935200 originally published online May 9, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M701935200

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

This article cites 55 references, 10 of which can be accessed free at http://www.jbc.org/content/282/27/19905.full.html#ref-list-1