Crystal Structure of *Brucella abortus* Deoxyxylulose-5-phosphate Reductoisomerase-like (DRL) Enzyme Involved in Isoprenoid Biosynthesis*

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Background: The current antibiotic resistance epidemic demands new drugs specifically targeting infective agents.

Results: The crystal structure of the *Brucella* DRL enzyme shows major differences compared with DXR, which catalyzes the same reaction in most other bacteria.

Conclusion: Structural information will allow development of inhibitors targeting only DRL.

Significance: Drugs against DRL could function as highly specific, narrow-range antibiotics.

Most bacteria use the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway for the synthesis of their essential isoprenoid precursors. The absence of the MEP pathway in humans makes it a promising new target for the development of much-needed new and safe antimicrobial drugs. However, bacteria show a remarkable metabolic plasticity for isoprenoid production. For example, the NADPH-dependent production of MEP from 1-deoxy-d-xylulose 5-phosphate in the first committed step of the MEP pathway is catalyzed by 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXR) in most bacteria, whereas an unrelated DXR-like (DRL) protein was recently found to catalyze the same reaction in some organisms, including the emerging human and animal pathogens *Bartonella* and *Brucella*. Here, we report the x-ray crystal structures of the *Brucella abortus* DRL enzyme in its apo form and in complex with the broad-spectrum antibiotic fosmidomycin solved to 1.5 and 1.8 Å resolution, respectively. DRL is a dimer, with each polypeptide folding into three distinct domains starting with the NADPH-binding domain, in resemblance to the structure of bacterial DXR enzymes. Other than that, DRL and DXR show a low structural relationship, with a different disposition of the domains and a topologically unrelated C-terminal domain. In particular, the active site of DRL presents a unique arrangement, suggesting that the design of drugs that would selectively inhibit DRL-harboring pathogens without affecting beneficial or innocuous bacteria harboring DXR should be feasible. As a proof of concept, we identified two strong DXR inhibitors that have virtually no effect on DRL activity.

The increased prevalence of antibiotic resistance, mainly caused by the use of broad-spectrum antibiotics at a huge scale in medicine and industry, is an ecological disaster that threatens to end the golden age of antibiotic therapy. One of the most promising novel antibiotic targets is the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway for isoprenoid biosynthesis (1, 2). Isoprenoids are a large group of compounds synthesized by all free-living organisms from the universal precursors isopentenyl diphosphate and its isomer dimethylallyl diphosphate (3–5). Two pathways have been described for the synthesis of these precursors. Archaea (archaeabacteria), fungi, and animals synthesize isopentenyl diphosphate from mevalonate (MVA) and then convert it into dimethylallyl diphosphate by an isopentenyl diphosphatidemethylallyl diphosphate isomerase enzyme. By contrast, most bacteria (eubacteria) and Apicomplexa protozoa (including the malaria parasites) use the MEP pathway, a completely different route that simultaneously produces both isopentenyl diphosphate and dimethylallyl diphosphate (3, 6). Specific inhibitors of the MEP pathway (which is absent in mammals) are expected to develop into much-needed antibiotics and novel antimalaria agents (1, 2). Among them, fosmidomycin (FSM) and derivatives such as FR900098 (Fig. 1) have already been shown to act as broad-spectrum agents against multidrug-resistant bacteria and malaria parasites.

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The atomic coordinates and structure factors (codes 3UPL and 3UPY) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: MEP, 2-C-methyl-d-erythritol 4-phosphate; MVA, mevalonate; FSM, fosmidomycin; DXP, 1-deoxy-d-xylulose 5-phosphate; DXR, DXP reductoisomerase; DRL, DXR-like; BaDRL, *B. abortus* DRL; EcDXR, *E. coli* DXR.
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A

\[
\begin{align*}
\text{DXP} & \xrightarrow{\text{NADPH}} \text{MEP} \\
\text{OH} & \xrightarrow{\text{OH}} \text{PO}_4^2- \\
\text{HO} & \xrightarrow{\text{HO}} \text{O} \\
\end{align*}
\]

B

\[
\begin{align*}
1: R=\text{H} & \text{(fosmidomycin, FSM)} \\
2: R=\text{CH}_3 & \text{(FR900098)} \\
3: R=\text{H} \\
4: R=\text{CH}_3 \\
\end{align*}
\]

FIGURE 1. Schemes of chemical structures. A, reaction catalyzed by DXR and DRL enzymes. B, structure of DXR inhibitors used in this work.

(7–9). FSM is an inhibitor of 1-deoxy-d-xylulose-5-phosphate (DXP) reductoisomerase (DXR), the enzyme catalyzing the NADP-dependent production of MEP from DXP (Fig. 1) (10, 11). DXR crystal structures from different bacteria (including *Escherichia coli* and the human malaria parasite *Plasmodium falciparum*) are currently available (12–19).

Selection acts to maintain isoprenoid production but not necessarily at the level of individual enzymes (20, 21). Thus, two types of isopentenyl diphosphate:dimethylallyl diphosphate isomerase enzymes with no sequence, structural, or catalytic similarity have been found in bacteria (22–24). In the case of DXR, we recently identified a DXR-like (DRL) enzyme that shows some sequence similarity to DXR only at the level of the NADPH-binding domain but catalyzes the same biochemical reaction (25). DRL is found instead of DXR in emerging animal and human pathogens such as *Bartonella* (which is responsible for a variety of HIV-associated infections) and *Brucella* (which usually infects livestock but can also spread to humans) (26–28). Although DRL can be inhibited with FSM (25), it would be most interesting to develop specific antibiotics against DRL-harboring pathogens that would not affect beneficial bacteria that use DXR (like those present in the intestine). However, the identification and development of such narrow-spectrum drugs require structural information for both enzymes.

Here, we report the crystal structure of the *Brucella abortus* DRL (*BdDRL*) enzyme in its apo form and in complex with FSM solved to 1.5 and 1.8 Å resolution, respectively. DRL catalytic residues were found to be structurally nonequivalent to DXR catalytic residues. Furthermore, comparison of the active sites of both enzymes led to the identification of FSM derivatives that inhibited DXR but had virtually no effect on DRL activity in vitro, opening the door to the design of new, safe, and highly specific antibiotics against only one of these two types of enzymes.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Protein expression and purification were carried out as described (25). Protein-containing fractions were pooled, concentrated, and loaded onto a HiLoad Superdex 200 16/60 gel filtration column (GE Health-care) that was equilibrated in buffer solution containing 0.02 M Tris-HCl (pH 7.5), 0.1 M NaCl, 2 mM MgCl₂, 2 mM DTT, and 5% (v/v) glycerol. The correct size of the recombinant *BdDRL* protein was verified by SDS-PAGE. Size exclusion chromatography and dynamic light scattering experiments confirmed *BdDRL* to be a monomer in solution by showing a monodisperse peak of the dimer size. Purified *BdDRL* was concentrated to 10 mg/ml and flash-frozen in liquid nitrogen for storage at −80 °C.

**Data Collection and Reduction**—Crystals were briefly soaked in a drop of cryoprotectant solution composed of 100 mM HEPES (pH 7.0), 20% (v/v) PEG 4000, 200 mM MgCl₂, and 5% (v/v) glycerol, and 0.5 mM FSM when complexation with the inhibitor was intended) before being flash-cooled in liquid nitrogen and exposed to x-rays. Diffraction data were collected in several beam lines of the European Synchrotron Radiation Facility (Grenoble, France) (supplemental Table S1). Data were processed and scaled using DENZO and SCALEPACK (30). Processing statistics are summarized in supplemental Table S1.

**Structure Determination and Refinement**—The program package HKL2MAP (31) was used both to locate the osmium sites in the primitive orthorhombic P2₁2₁2₁ crystal form, which had been soaked with OsCl₄, and to calculate initial phases to 2.8 Å resolution. To improve the electron density, the osmium derivative was then combined with data from two other crystal forms: a SeMet-labeled *BdDRL* crystal belonging to space group C2 and a native data set from a primitive triclinic (P1) crystal. The crystal density modification protocol was performed with DMM (32), including averaging, solvent flattening, and histogram matching. A model containing *BdDRL* residues 2–437 was derived from progressive improvement of the electron density map using iterative rounds of restrained refinement with Refmac5 (33) and manual building with Coot (32). Refinement statistics are summarized in supplemental Table.
S1. TLS refinement in Refmac5 was applied in the last stage using eight TLS groups. Atomic isotropic B-factors were refined only in the final cycles. The stereochemical quality of the final models, assessed with MolProbity (34), showed no residues in the disallowed region of the Ramachandran plot. Atomic coordinates have been deposited in the Protein Data Bank (apo-BaDRL, code 3UPL; and BaDRL-FSM complex, code 3UPY).

Activity Assays—In vivo activity assays were carried out by complementation of the MVA auxotrophy of DXR-defective E. coli EcAB4-10 cells as described (35). Positive transformants carrying plasmids with either wild-type or mutant versions of BaDRL were selected on MVA-supplemented LB plates. Three independent colonies were then streaked on LB plates with no MVA or used to inoculate 5 ml of LB liquid medium with no MVA. After incubation at 37 °C, complementation was assessed by visually monitoring the development of colonies on MVA. After incubation at 37 °C, complementation was assessed by visually monitoring the development of colonies on plates and by quantifying the growth of liquid cultures by light scattering studies. The same dimer was also found in the other two BaDRL crystal forms available, where the molecular binary axis coincides with a crystallographic 2-fold axis, leaving only one BaDRL subunit in the asymmetric unit of these crystals.

Results and Discussion

Overall Architecture—The crystal structure of the full-length BaDRL enzyme fused to a C-terminal hexahistidine tag was solved by the single-wavelength anomalous dispersion method of an osmium derivative together with density averaging between the three crystal forms available (see “Experimental Procedures”). The structures of the apoprotein and a binary complex, obtained by soaking with the inhibitor FSM (Fig. 3), were refined to 1.5 and 1.8 Å resolution, respectively (supplemental Table S1). The refined models comprise Thr-2 to Gly-437, with most of the side chains defined unambiguously in the electron density maps for the two subunits found in the asymmetric unit of the triclinic crystals (Fig. 2). The subunits are related by an accurate molecular binary axis with a buried surface of ~2000 Å². A dimeric form of BaDRL had been previously detected by gel filtration (25) and confirmed by dynamic light scattering studies. The same dimer was also found in the central domain in the other two BaDRL crystal forms available, where the molecular binary axis coincides with a crystallographic 2-fold axis, leaving only one BaDRL subunit in the asymmetric unit of these crystals.

Each BaDRL subunit is organized in three structural domains: N-terminal, central, (catalytic), and C-terminal (Fig. 2A). The N-terminal domain, formed by residues 1–166 and 328–339, corresponds to the NADPH-binding domain, as anticipated from the sequence similarity between DRL and DXR in this region (25). This N-terminal domain starts with a helix (α1) followed by a Rossmann-like motif composed of seven parallel β-strands surrounded by seven α-helices (supplemental Fig. S1). The central or catalytic domain, stretching from residues 167 to 327, has an α/β-fold with a central four-stranded mixed β-sheet (Fig. 2A). Two long α-helices (α8 and α11) are situated on the sheet face facing the N-terminal domain, whereas helix α12 is located on the opposite side of the sheet. A protrusion in the central domain, formed by two antiparallel helices (α9 and α10) connected by a short loop, forms the active site lid (see below). The central domains of BaDRL and DXR proteins present a distant structural relationship, with a root mean square difference of 2.4 Å for 90 equivalent residues (55%) for the E. coli DXR (EcDXR) enzyme (supplemental Fig. S1). Finally, the small C-terminal domain of BaDRL, starting at residue 340, adopts a distorted β-sandwich topology that includes two three-stranded antiparallel β-sheets (Fig. 2A). The C-terminal domain is completed by helices α15 and α16, the later located at the C-terminal end of the domain.
and oriented perpendicularly to helices α6 and α7. A search with Dali (36) found that the C-terminal domain of BaDRL has a clear structural similarity only to the ice-binding domain of type III antifreeze proteins, with a root mean square difference of 2.2 Å for 57 equivalent residues (87%) for the ocean pout (Macrozoarces americanus) protein (supplemental Fig. S2) (37). The C-terminal domain of DXR presents a four-bundle helix topology unrelated to the one found for DRL. However, the two domains have similar overall volumes and occupy approximately the same position relatively to the N-terminal and central domains.

The interactions between the two subunits of the BaDRL dimer involve the N-terminal and central domains (Fig. 2B), with only one interaction being formed exclusively by residues of the central domains (Phe-178 and Tyr-322). A cavity at the dimer interface contains bulky blobs of electron density that were modeled as glycerol molecules forming hydrogen bonds with side chains from Glu-174, Arg-320, and Tyr-322, which are highly conserved in DRL enzymes (supplemental Fig. S3).

**Active Site and FSM Binding**—The active site, identified at the cleft formed between the N-terminal and central domains, contains a Mg^{2+} ion bound with an octahedral coordination to Asp-167, Glu-168, and two water molecules (equatorial ligands) and to Glu-232 and a third water (axial ligands) (Fig. 2A). These three residues are fully conserved in all of the bona fide DRL enzymes, i.e. those belonging to the DRL phylogenetic clade (supplemental Fig. S3) (25). The presence of the catalytic Mg^{2+} ion and its coordination geometry in BaDRL resemble those in DXR structures. Asp-167, Glu-168, and Glu-232 in BaDRL are structurally close to the Mg^{2+}-coordinating residues Asp-150, Glu-152, and Glu-231 in EcDXR. In the vicinity of the Mg^{2+} coordination sphere of DXR enzymes there is a highly conserved triad of charged residues (Lys-125, Glu-126, and Asp-221 in EcDXR), which are assumed to play an important role in catalysis. Although not structurally equivalent, a similar triad of charged residues (Glu-141, Glu-222, and Lys-228) is also found in BaDRL, with side chains of Glu-141 and Glu-222 interacting with each other in the SeMet crystal. Other catalytic residues identified in EcDXR such as Met-214, His-257, and Met-276 (12) appear to be maintained in BaDRL but by the structurally nonequivalent residues Met-215, His-323, and Met-34, respectively (supplemental Fig. S1).

In DXR enzymes, the active site requires the closing of a lid formed by residues from a flexible loop that is generally not well defined in the available structures. A similar role might be performed in BaDRL by the protrusion formed by the central domain helices α9 and α10 (Fig. 2 and supplemental Fig. S1). The electron density in the BaDRL-FSM complex shows a low
The EcDXR residues involved in the binding of FSM are Trp-212, which participates in stacking with the hydroxamic acid moiety, and Ser-186, Asn-227, and Lys-228, which interact with the phosphonate moiety of the inhibitor (Fig. 3B). It is remarkable that, despite the overall similarity of the binding of FSM to DRL and DXR, none of the residues participating directly in the binding are structurally equivalent, and even the identities of many of the residues differ between both families of enzymes (Fig. 3).

Inhibition Studies—The described data indicate that, despite the fact that DRL and DXR enzymes catalyze the same biochemical reaction (25), the arrangement of their active sites shows major differences, particularly between the lids. Interestingly, such substantial differences create the possibility of identifying new drugs selectively inhibiting either DRL or DXR. As a first step in this direction, several FSM derivatives known to inhibit the activity of DXR enzymes in vitro (compounds 2–4 in Fig. 1) were positioned into the BaDRL-FSM structure such that the phosphonate group and the hydroxamic acid skeleton common to all of these compounds coincided with the experimental position of FSM (supplemental Fig. S4). In FR900098 (compound 2 in Fig. 1), the formyl hydrogen of FSM is replaced by a methyl group that was shown to favorably interact with the lid residue Trp-296 in the P. falciparum DXR enzyme (Trp-212 in EcDXR), resulting in an increased affinity (supplemental Fig. S4A) (8, 11, 19). In BaDRL, the extra methyl group of FR900098 is predicted to establish new hydrophobic interactions, without steric clashes, with the side chains of Thr-325 and Leu-308 (supplemental Fig. S4B), suggesting that FR900098 might also be more efficient than FSM in inhibiting DRL activity. On the other hand, FSM derivatives with an α-phenyl substitution and a reverse orientation of the hydroxamic acid group (such as compounds 3 and 4 in Fig. 1) are excellent DXR inhibitors that consistently fit well into the active site of EcDXR (supplemental Fig. S4C) (39, 40). However, positioning of compounds 3 and 4 into the BaDRL structure revealed that, for the two possible stereoisomers of both compounds, the α-phenyl group would either clash against the side chain of Phe-223 (supplemental Fig. S4D) or remain exposed. This is expected to either prevent or destabilize binding, suggesting that these two compounds might not be good inhibitors of BaDRL.

To substantiate these predictions experimentally, purified recombinant BaDRL and EcDXR enzymes were used for in vitro activity assays in the presence of different concentrations of FSM and compounds 2 (FR900098), 3, and 4 (Fig. 5). FR900098 inhibited BaDRL activity more efficiently than FSM (FR900098 IC₅₀ = 1.09 ± 0.19 µM versus FSM IC₅₀ = 2.39 ± 0.62 µM), suggesting that the interactions predicted for the methyl group of FR900098 with BaDRL residues (supplemental Fig. S4B) might indeed improve the affinity for this enzyme. Interestingly, both FSM and FR900098 inhibited BaDRL with less efficiency than EcDXR (Fig. 5A), resulting in IC₅₀ values for BaDRL that were ∼10-fold higher than those reported in the literature for DXR (39, 41). This might be due to the structural differences between the two types of enzymes since the large size of the pocket around the hydroxamic acid tail of both FSM and FR900098 in BaDRL is predicted to result in looser binding compared with DXR enzymes (Fig. 3 and supplemental Fig. S4).

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In vivo and in vitro activity assays of wild-type and mutant BaDRL proteins. A, complementation of E. coli EcAB4-10 cells with plasmids carrying WT and mutant E209S/R213A (209/213) versions of the B. abortus DRL gene. DRL activity was deduced from the ability of the cloned gene to rescue growth of the DXR-deficient strain in the absence of MVA. Positive transformants selected on MVA-supplemented plates were used for inoculation in solid (left) or liquid (right) LB medium lacking MVA. After incubation at 37 °C for 18 h, growth was observed only with the WT construct. B, enzymatic activity of purified WT (black circles) and mutant E209S/R213A (white squares) BaDRL proteins in vitro. DRL activity was estimated from the decrease in NADPH levels monitored by absorbance at 340 nm. Means ± S.D. of three experiments (n = 3) are represented.

In vivo and in vitro activity assays of wild-type and mutant BaDRL proteins. A, complementation of E. coli EcAB4-10 cells with plasmids carrying WT and mutant E209S/R213A (209/213) versions of the B. abortus DRL gene. DRL activity was deduced from the ability of the cloned gene to rescue growth of the DXR-deficient strain in the absence of MVA. Positive transformants selected on MVA-supplemented plates were used for inoculation in solid (left) or liquid (right) LB medium lacking MVA. After incubation at 37 °C for 18 h, growth was observed only with the WT construct. B, enzymatic activity of purified WT (black circles) and mutant E209S/R213A (white squares) BaDRL proteins in vitro. DRL activity was estimated from the decrease in NADPH levels monitored by absorbance at 340 nm. Means ± S.D. of three experiments (n = 3) are represented.
Most strikingly, compounds 3 and 4 inhibited EcDXR activity at similar concentrations compared with FSM and FR900098 (Fig. 5A), but they were unable to significantly inhibit BaDRL activity at concentrations up to 1 mM (Fig. 5B). These results confirm the structural analysis and represent a proof of concept for the development of highly specific antibiotics targeting only one of the two enzyme families.

**Perspectives**—The large-scale, indiscriminate use of broad-spectrum antibiotics has resulted in a worldwide epidemic of antibiotic resistance. A solution to this problem is to find new antibiotics with novel targets that are present only in the specific pathogenic bacteria causing the infection. Here, we show that DRL can be one such target. The determination of the crystal structures of the BaDRL enzyme in its apo form and in complex with FSM showed a low structural relationship to DXR proteins and revealed important differences in the active sites of these enzymes, opening the door to the development of highly specific inhibitors against only one of the two types of enzymes. Because DRL (but not DXR) is the enzyme catalyzing the essential production of MEP in important human and livestock pathogens such as *Brucella*, which can be potentially used as a biological weapon (42), inhibitors exclusively targeting DRL should be useful as highly specific, narrow-range antibiotics that would not cause undesired side effects by affecting beneficial or innocuous bacteria harboring only DXR. Although finding and developing such inhibitors are not straightforward tasks, we have shown how the structural information can contribute to their identification.

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