Crystal Structure of *Tritrichomonas foetus* Inosine Monophosphate Dehydrogenase in Complex with the Inhibitor Ribavirin Monophosphate Reveals a Catalysis-dependent Ion-binding Site*

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Inosine monophosphate dehydrogenase (IMPDH) catalyzes the rate-limiting step in GMP biosynthesis. The resulting intracellular pool of guanine nucleotides is of great importance to all cells for use in DNA and RNA synthesis, metabolism, and signal transduction. The enzyme binds IMP and the cofactor NAD${}^{+}$ in random order, IMP is converted to XMP, NAD${}^{+}$ is reduced to NADH, and finally, NADH and then XMP are released sequentially. XMP is subsequently converted into GMP by GMP synthase. Drugs that decrease GMP synthesis by inhibiting IMPDH have been shown to have antiproliferative as well as antiviral activity. Several drugs are in use that target the substrate- or cofactor-binding site; however, due to differences between the mammalian and microbial isoforms, most drugs are far less effective against the microbial form of the enzyme than the mammalian form. The high resolution crystal structures of the protozoan parasite *Tritrichomonas foetus* IMPDH complexed with the inactive ribavirin monophosphate, as well as monophosphate together with a second inhibitor, mycophenolic acid, are presented here. These structures reveal an active site cation identified previously only in the Chinese hamster IMPDH structure with covalently bound IMP. This cation was not found previously in apo IMPDH, IMPDH in complex with XMP, or covalently bound inhibitor, indicating that the cation-binding site may be catalysis-dependent. A comparison of *T. foetus* IMPDH with the Chinese hamster and *Streptococcus pyogenes* structures reveals differences in the active site loop architecture, which contributes to differences in cation binding during the catalytic sequence and the kinetic rates between bacterial, protozoan, and mammalian enzymes. Exploitation of these differences may lead to novel inhibitors, which favor the microbial form of the enzyme.

Inosine-5’-monophosphate dehydrogenase (IMPDH) (E.C. 1.1.1.205) is the enzyme that catalyzes the NAD${}^{+}$-dependent oxidation reaction that converts inosine monophosphate (IMP) to xanthosine monophosphate (XMP). This is the rate-limiting step in guanine nucleotide biosynthesis. The kinetic mechanism for both mammalian and microbial IMPDH has been extensively studied (1–3), and the crystal structures have been determined for two bacterial, two mammalian, and one protozoan form of IMPDH (4–9). The enzymatic reaction appears to follow a random-in ordered-out kinetic mechanism where the substrate IMP and cofactor NAD${}^{+}$ bind to the enzyme active site followed by the nucleophilic attack on the C2 position of IMP by the active site cysteine to form E-IMP covalent intermediate. Hydride transfer from the E-IMP intermediate to NAD${}^{+}$ results in the formation of E-XMP intermediate and reduced NADH. A water molecule in the active site helps to hydrolyze the covalent intermediate and to release XMP only after the cofactor NADH has dissociated from the active site. GMP, the next product in purine biosynthesis, supplies an intracellular pool of guanine nucleotide for DNA and RNA synthesis, is used in energy storage, and plays a critical role in signal transduction pathways.

IMPDH has been isolated and characterized from many sources, including human, Chinese hamster, *T. foetus*, *Streptococcus pyogenes*, and *Borella burgdorferi*. The active form of all known IMPDH is a homotetramer with each monomer consisting of an eight-stranded α/β barrel. The active site of the enzyme is located on the C-terminal side of the β sheets. An active site loop between strand 6 and helix 6 (*T. foetus* residues 313–330) contains the catalytic cysteine residue (Cys-319). An active site flap that extends from the C-terminal side of the barrel (residues 408–413) together with the active site loop forms part of the active site. The flap is of great interest for species-specific inhibitor design as it is the least conserved region of the active site. Unfortunately, none of the published IMPDH crystal structures contains a completely ordered flap region. A secondary domain of IMPDH comprised of residues 101–222 (*T. foetus* numbering), which shares homology with cystathionine β-synthase (CBS) (10), has no known function and is not required for catalysis (11). On the human type I enzyme, a D226N (*T. foetus* residue 214) mutation in the CBS domain was reported to be involved in the RP10 form of retinitis pigmentosa (12). The human Asp-226 residue in the CBS domain is conserved in IMPDH from all species, indicating that the CBS domain may have similar functions in other organisms. An active site cation was identified in the Chinese hamster structure; however, this ion is not described in the four other IMPDH structures, and its role in catalysis is not known.

IMPDH is a well established drug target. Drugs that target IMPDH have been approved or are currently evaluated for antiproliferative, antiviral, and anticancer chemotherapies as well as immunosuppressive agents. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a purine nucleoside analog, has been approved for antiviral therapy (Fig. 1). Once
the uncharged ribavirin enters the cell, it is metabolized into its 5'-mono-, di-, and tri-phosphorylated forms. It is the monophosphate form (ribavirin monophosphate or RMP) that is a potent IMPDH inhibitor, and this results in a decrease in the intracellular pool of GTP to suppress viral replication (13). Ribavirin monophosphate is a nucleotide analog that binds competitively with IMP. A second class of inhibitors binds IMPDH uncompetitively in the NAD\(^+\)-binding site. One such inhibitor is mycophenolic acid (MPA). This inhibitor binds to the nicotinamide binding portion of the cofactor-binding site, after NADH but before XMP is released from the enzyme. MPA binding prevents product release, resulting in inhibition.

IMPDH is a highly conserved enzyme; however, the sequence homology between the mammalian enzymes and their microbial counterparts is relatively low (30–35%), which makes it likely that species-selective IMPDH inhibitors can be developed that act as antimicrobial agents. T. foetus is an anaerobic protozoan parasite that infects the urogenital tract of cows, leading to sterility and abortion (14). The major cause of infection is the transfer from asymptomatic bulls to healthy cows—leading to sterility and abortion (14). The major cause of infection is the transfer from asymptomatic bulls to healthy cows.

Kinetic Assays—Steady-state kinetic analysis of T. foetus IMPDH was performed with 4.5 \(\mu\)M enzyme in a reaction buffer containing 100 mM KCl, pH 8.0, 1 mM dithiothreitol, and 1 mM NAD\(^+\). Reactions were initiated with the addition of IMP, and the production of NADH was monitored spectrophotometrically at 340 nm (\(e_{340} = 6.22 \text{ mM}^{-1} \text{cm}^{-1}\)) using a PerkinElmer Lambda 40 (EG\&G, Inc., Wellesley, MA) spectrophotometer at 25 °C. For determination of the apparent value for IMP, the concentration of IMP was varied from 0.2 to 10 \(\mu\)M with the NAD\(^+\) concentration fixed at 1 mM. The initial velocity at various IMP concentrations was measured and was fit to the Michaelis-Menten equation by Sigma Plot (SPSS Inc., Chicago, IL). Since RMP is known to be a competitive inhibitor with IMP, the apparent inhibition constant \(K_i\) of RMP was estimated using a Dixon plot. In the experiments, RMP concentration was varied, whereas IMP concentration remained fixed at 40 \(\mu\)M, and the NAD\(^+\) concentration was 1 mM. The initial velocities at various RMP concentrations were determined by the extraction of the linear portion of the reaction time course. By plotting \(1/v\) versus inhibitor concentration, the apparent inhibition constant \(K_i\) was calculated using Equation 1.

\[
1/v = (K_i/V_{max}) (K_i/IMP) + 1/V_{max}(1 + (K_i/IMP))
\] (Eq. 1)

**Materials and Methods**

Expression, Purification, and Crystallization of T. foetus IMPDH—Recombinant T. foetus IMPDH enzyme was produced from a pBACE plasmid (23) containing the gene for T. foetus IMPDH (24) that was transformed into Escherichia coli strain H712 (E. coli Stock Center, Yale University, New Haven, CT). Expression of T. foetus IMPDH was achieved by modifying previously published protocols (25–27). Briefly, the cells were grown in MOPS medium in a 19-liter fermentor (Wheaton Science Products, Millville, NJ) and were inoculated with 0.5 liters of overnight MOPS culture of H712 containing the IMPDH plasmid. The fermentation was kept at 37 °C and was maintained by aeration, stirring, and glucose addition. The dissolved oxygen was maintained at greater than 40%. The cells were harvested at 8 h when the dissolved oxygen dropped to below 20% at an \(A_{590}\) of roughly 1.5.

The cells were concentrated to 0.5 liter by tangential flow filtration (Millipore, Inc., Bedford, MA) and pelleted by centrifugation at 6,000 \(\times\) g. The pellet was resuspended in a 3-fold volume of buffer A (50 mM Tris, pH 8.0, 50 mM KCl, 10% glycerol, and 1 mM 2-mercaptoethanol) supplemented with protease inhibitors and 1 mM EDTA and was then flash-frozen in liquid nitrogen before storage at –85 °C. This mixture was lysed by French press, and the lysate was clarified by centrifugation at 20,000 \(\times\) g. The supernatant was then run over a cibacron blue column on an AKTA FPLC (Amersham Biosciences). Protein was eluted from the column with 1 mM KCl. This was followed by dialysis into buffer A and concentration to 30 mg/ml.

The protein was crystallized at 20 °C by mixing 15 mg/ml protein in buffer A into 42% of saturated sodium malonate, 100 mM Tris, pH 8.0, 4 mM polyethylene glycol 400, and 1 mM 2-mercaptoethanol in 8 \(\mu\)l of sitting drops in a 1:1 ratio of well solution to protein. The protein was then passed through a monQ column (Amersham Biosciences) using a gradient from 50 to 500 mM KCl over 20 column volumes. The resulting protein was >90% pure and was dialyzed in buffer A and concentrated to 30 mg/ml for storage at –85 °C.

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RESULTS

Steady-state Kinetic Assays—Steady-state kinetic analysis of T. foetus IMPDH using IMP as a substrate was performed using a spectroscopic method. Initial velocity measurements were taken with increasing amounts of IMP and plotted as a Michaelis-Menten graph (Fig. 2). From the plot, an apparent $K_m$ of 3.0 $\mu$M was calculated for IMP. RMP, a competitive inhibitor for the substrate IMP, was then assayed for inhibition of the protozoan enzyme with increasing concentrations of RMP. These data were graphed as a Dixon plot (Fig. 3). The apparent $K_i$ for RMP was determined to be 65 nM.

Data Collection and Refinement—Diffraction quality crystals in the space group P432 grew within 5 days. The cryo-cooled crystals diffracted to 1.90 Å for the RMP co-crystal and to 2.15 Å for the crystal with RMP and MPA using synchrotron radiation. A randomly selected test set of diffraction data (5% of all structure factors) was set aside for $R_{free}$ monitoring. The publication. A randomly selected test set of diffraction data (5% of all structure factors) was set aside for $R_{free}$ monitoring. The cryo-cooled colorless crystals ranging in size from 0.4 to 0.8 mm. The crystals were found in Table I. All data were collected under cryo conditions using colorless crystals ranging in size from 0.4 to 0.8 mm. The crystals were loop-mounted and flash-cooled to $-180 \degree$C in the nitrogen stream. The data were collected from single crystals and integrated and scaled with the Denzo-Scalepack package (28). The published isomorphous T. foetus apo structure (5) was used as an initial model for both structures presented here followed by rigid body and simulated annealing refinement. Several rounds of model building, energy minimization, and B-factor refinement were then performed with O (29) and CNS (30).

Table I

<table>
<thead>
<tr>
<th>Structure</th>
<th>RMP</th>
<th>RMP + MPA</th>
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<tbody>
<tr>
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<td>50–2.15</td>
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<td>Resolution of outer shell (Å)</td>
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<td>23.3 (26.5)</td>
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<td>23.59/2.05</td>
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<tr>
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<td>Number of ordered water molecules</td>
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</tr>
<tr>
<td>Bond length R.M.S.D. (Å)</td>
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<td>0.006</td>
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<tr>
<td>Bond angle R.M.S.D. (°)</td>
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<tr>
<td>Dihedral angle R.M.S.D. (°)</td>
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<tr>
<td>Improper angle R.M.S.D. (°)</td>
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<td>155.1</td>
</tr>
<tr>
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<td>P432</td>
</tr>
<tr>
<td>Mosaicity (°)</td>
<td>0.45</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* R. M. S. D., root mean square deviation.

found in Table I. All data were collected under cryo conditions using colorless crystals ranging in size from 0.4 to 0.8 mm. The crystals were loop-mounted and flash-cooled to $-180 \degree$C in the nitrogen stream. The data were collected from single crystals and integrated and scaled with the Denzo-Scalepack package (28). The published isomorphous T. foetus apo structure (5) was used as an initial model for both structures presented here followed by rigid body and simulated annealing refinement. Several rounds of model building, energy minimization, and B-factor refinement were then performed with O (29) and CNS (30).

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Model Building—IMPDH crystallizes as a homotetramer with monomers related by the 4-fold crystallographic axis (Fig. 4). The active site loop, residues 313–330, was modeled in both the RMP and RMP + MPA structures. The proximal portion of the active site flap (residues 408–416 and 431–433) was modeled, whereas the distal portion (residues 417–430) was disordered. Only very poor density in the region of the CBS domain was observed, and no attempts were made at modeling this domain. The C-terminal amino acids 484–492 were also added to the original T. foetus model, and although these nine residues do not appear to make direct contact with substrate or product, they do lie near the active site of a neighboring monomer in the catalytic tetramer, and the backbone carbonyls of residues 485–487 form part of the active site cation-binding pocket. Although RMP was clearly observable in the electron density of both crystal structures, density for MPA, even with

![Fig. 2. Michaelis-Menten graph.](image-url) The initial velocity increases with increasing concentration of substrate. The concentrations of enzyme and cofactor were held constant.

![Fig. 3. Dixon plot showing IMPDH inhibition with increasing concentration of RMP.](image-url) The concentrations of IMP, NAD$^+$, and enzyme are constant.
saturating concentrations in the crystallization drop, was weak and was therefore modeled at 50% occupancy (Fig. 5). B factors for MPA at this level of occupancy were in agreement with the neighboring RMP atoms.

A cis peptide bond was modeled in both structures between Gly-290 and Asn-291. It is located near the cofactor-binding site but does not appear to be close enough to directly influence cofactor binding. In addition, a strong peak of electron density was found extending from the active site cysteine sulfur in the RMP-bound structure. This appears to be a result of the thiol oxidized to sulfenic acid (Cys-SOH), likely caused by exposure to oxygen during crystal formation prior to freezing. This effect has been described previously (32).

Inhibitor Binding—RMP binding to *T. foetus* IMPDH is very similar to IMP or XMP binding to the protozoan enzyme. As was observed in the IMP-bound structure, the phosphate is coordinated with hydroxyls from Ser-317 and Tyr-405 as well as main chain nitrogens from residues 317, 381, and 382 (Fig. 6). Three solvent waters also form hydrogen bonds with the phosphate. As in all IMPDH structures, the substrate sugar hydroxyls form strong hydrogen bonds with a conserved aspartate carboxylate (Asp-358). Ile-318, which in the substrate and product complexes forms hydrophobic interactions with the purine ring, has moved 1.2 Å away from the RMP inhibitor. The RMP amide oxygen forms hydrogen bonds with Glu-408 and Gly-409. In the RMP+MPA structure, the amide nitrogen is hydrogen bonding to the MPA ring hydroxyl, and the MPA inhibitor has moved 0.6 Å from its position in the XMP+MPA structure in the direction of the RMP purine ring derivative. In this position, the MPA ring O₂ makes a hydrogen bond with the backbone nitrogen of Gly-314, and the C3 oxygen hydrogen bonds with the catalytic sulfhydryl of Cys-319.

Formation of a Catalytic Cation-binding Site—The conformation of the active site loop in the RMP as well as the RMP+MPA structure is different from the loop conformation of the structures with bound substrate or product, resulting in a pocket surrounded by backbone carbonyl oxygens from Gly-314, Gly-316, and the active site Cys-319, as well as carboxyls from Glu-485, Gly-486, and Gly-487 in the neighboring catalytic monomer. A high (7.7 σ) difference density peak was observed at the center of this pocket, indicating the presence of a cation. Both Na⁺ and K⁺ ions were modeled into this site and minimized in CNS. A large peak of negative difference density was observed when K⁺ was modeled as well as high B factors. Because of the high concentration of sodium in the crystallization buffer, the previous observation that a sodium ion binds competitively with K⁺ in microbial IMPDH (33), and B factors that are near those of the neighboring atoms, a Na⁺ ion was placed in the final model (Fig. 7).

**DISCUSSION**

*T. foetus* IMPDH binds RMP in the active site substrate pocket. The RMP+MPA complex was difficult to obtain as the MPA inhibitor was only observed with saturating amounts of MPA present in the crystallization drop. Attempts to obtain a
The ion-binding pocket. Furthermore, the active site loop in the purine ring C2 position to the inhibitor C6 position in order because it was necessary for the active site loop to move from covalently bound inhibitor 6-Cl IMP, no cation was present in the IMP-bound structure. In the human IMPDH structure with the active site loop to occupy this position without forming a covalent intermediate is necessary to recruit the ion to the active site, whereas the inhibitor RMP allows the structures, the covalent intermediate is necessary to recruit the carbonyl oxygen from the active site Cys-319 forms part of the cation-binding site (green) is composed of backbone carbonyls from the active site loop residues, including the active site cysteine and residues from the C terminus of the neighboring catalytic monomer (gray carbons). RMP is to the left.

crystal rather than an MPA soak were unsuccessful as the levels of MPA necessary to form the complex inhibited crystal formation. Although no data have been reported on the additive effects of RMP and MPA, it is unlikely that both inhibitors would occupy the substrate and cofactor-binding pockets of the active site simultaneously, despite their distinct binding sites. The reason might be that MPA appears to rely on stacking its ring against the product XMP purine ring to bind to IMPDH and that the RMP ring is probably too small for effective stacking.

A structure of RMP bound to human IMPDH has not been made available for direct comparison; however, we were able to compare the T. foetus RMP structure with the Chinese hamster structure that contains a covalently bound substrate intermediate (6). These structures show a high degree of similarity in the conformation of the active site loop, recruitment of a catalytic ion, as well as incorporation of the C-terminal residues of the neighboring catalytic monomer to create the ion pocket. The reasons behind this appear to be the lack of the IMP C2 and N3 in the RMP inhibitor, which cause the hydrophobic Ile-318, which normally forms hydrophobic contacts with the purine ring of the substrate or product, to move away from the more polar, less hydrophobic purine derivative. More importantly, with this portion of the purine ring absent, it is now more favorable for the active site cysteine (Cys-319) to move into its catalytic position without the need for the NAT cofactor to bind and for catalysis to occur. This would not be the case with substrate or product present as the Cys-319 sulfhydryl would be within 2.8 Å of the C2 position of IMP or within 1.5 Å of the oxygen bound to the C2 position of XMP.

It appears that in mammalian IMPDH and these T. foetus structures, the covalent intermediate is necessary to recruit the ion to the active site, whereas the inhibitor RMP allows the active site loop to occupy this position without forming a covalent bond with the enzyme. No ion was found in our T. foetus IMP-bound structure. In the human IMPDH structure with the covalently bound inhibitor 6-C1 IMP, no cation was present because it was necessary for the active site loop to move from the purine ring C2 position to the inhibitor C6 position in order for the active site cysteine to form a covalent bond with the inhibitor. This movement in the loop prevented formation of the ion-binding pocket. Furthermore, the active site loop in the apo S. pyogenes structure is in this cation binding conformation without a covalent intermediate, and a possible ion, designated water 179 (Protein Data Bank accession code 1ZF3), appears to occupy the same cation position as in the Chinese hamster (Protein Data Bank accession code 1JR1) and the T. foetus structures presented here. The IMP in the bacterial structure is not covalently bound, and the C2 of the purine ring is rotated slightly away from the active site cysteine. In the T. foetus IMP structure, it is the loop that moves slightly away from the C2 of IMP when compared with the hamster covalently bound structure and the B. burgdorferi apo structure.

The bacterial active site loop appears to be stabilized upon substrate binding by Thr-310, which also makes hydrogen bonds with the active site flap, both directly and through an ordered water. This threonine is conserved in bacteria but is substituted with an isoleucine in eukaryotes, which appears to disrupt the structural coupling of the active site loop to the active site flap. This results in a partial destabilization of the active site loop, allowing the active site cysteine to be displaced instead of the substrate. The bacterial active site loop is in the catalytic position immediately following substrate binding, and this could explain the 10-fold higher Ki, when compared with the mammalian and T. foetus enzymes (24, 2, 1.8, and 4 s−1 for S. pyogenes, T. foetus, and human type I and II, respectively) (1, 9, 21).

The mammalian and T. foetus enzymes, at some point after substrate binding, must first move the active site loop into position and then recruit the active site cation and C-terminal residues of the neighboring monomer before catalysis is possible. These steps may be coupled to cofactor binding. If the positively charged NAD+ binds over the C2 position of IMP, it may expose the substrate C2 to the active site cysteine thiol for subsequent nucleophilic attack. A role for the active site ion may be in stabilization of the active site loop during catalysis. The carbonyl oxygen from the active site Cys-319 forms part of the cation-binding site. When this site is occupied, the cysteine is in an ideal position to form a covalent bond with the C2 carbon of IMP. The cation-binding site appears to be formed before covalent binding in bacterial IMPDH but likely occurs during covalent bond formation in mammalian and T. foetus IMPDH.

In the steady-state kinetic analysis of T. foetus IMPDH, we observe an apparent Km of 3.0 μM for IMP, which is consistent with the previously published Km value of 1.7 μM from a detailed bisubstrate kinetic analysis (1). This value is much smaller than the Km of 14.2 ad 9.2 μM, respectively, for human type I and II IMPDH (21) as well as the Km of 62 μM for S. pyogenes enzyme (9). This result indicates that IMP binds to T. foetus IMPDH about 3–5-fold tighter than to mammalian enzymes. Interestingly, RMP, a nucleotide inhibitor, was shown to have a Ki of 65 nM for T. foetus IMPDH, which is consistent with previous findings (38). This is about 5–10-fold lower than the Ki for human type I and II IMPDH, where the values are 650 nM and 390 nM, respectively (21). In addition, it is considerably lower than the Ki of 6 μM for S. pyogenes IMPDH (9). This result is consistent with steady-state kinetic analysis that shows that T. foetus IMPDH binds IMP (or the IMP analog RMP) more tightly than IMPDH from other species. Furthermore, the inhibition studies with RMP demonstrated that species specificity for an inhibitor does occur in IMPDH. Our in vitro studies showed that RMP is a potent nanomolar inhibitor for T. foetus IMPDH, and it appears that RMP is more effective against the protist form of IMPDH than the human and bacterial forms. A structural explanation for the 100-fold difference in Ki between T. foetus and S. pyogenes IMPDH could not be established from the highly conserved substrate-binding site. The differences in Ki may be related, at
least in part, to the kinetic mechanism. Further in vivo experiments are needed to address whether there are any clinical significance and pharmacological effects of ribavirin on *T. foetus*-infected cows.

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REFERENCES