ABSTRACT: IMP dehydrogenase (IMPDH) is an essential enzyme that catalyzes the first step unique to GTP synthesis. To provide a basis for the evaluation of IMPDH inhibitors as antimicrobial agents, we have expressed and characterized IMPDH from the pathogenic bacterium *Streptococcus pyogenes*. Our results show that the biochemical and kinetic characteristics of *S. pyogenes* IMPDH are similar to other bacterial IMPDH enzymes. However, the lack of sensitivity to mycophenolic acid and the *K*<sub>m</sub> for NAD (1180 µM) exemplify some of the differences between the bacterial and mammalian IMPDH enzymes, making it an attractive target for antimicrobial agents. To evaluate the basis for these differences, we determined the crystal structure of the bacterial enzyme at 1.9 Å with substrate bound in the catalytic site. The structure was determined using selenomethionine-substituted protein and multiwavelength anomalous (MAD) analysis of data obtained with synchrotron radiation from the undulator beamline (19ID) of the Structural Biology Center at Argonne’s Advanced Photon Source. *S. pyogenes* IMPDH is a tetramer with its four subunits related by a crystallographic 4-fold axis. The protein is composed of two domains: a TIM barrel domain that embodies the catalytic framework and a cystathione β-synthase (CBS) dimer domain of so far unknown function. Using information provided by sequence alignments and the crystal structure, we prepared several site-specific mutants to examine the role of various active site regions in catalysis. These variants implicate the active site flap as an essential catalytic element and indicate there are significant differences in the catalytic environment of bacterial and mammalian IMPDH enzymes. Comparison of the structure of bacterial IMPDH with the known partial structures from eukaryotic organisms will provide an explanation of their distinct properties and contribute to the design of specific bacterial IMPDH inhibitors.

Inosine monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) is a rate-limiting enzyme in the synthesis of guanine ribonucleotides. IMPDH has an essential role in providing critical precursors for DNA and RNA biosynthesis and in signal transduction pathways that mediate cell differentiation (1, 2). This essential nature is illustrated by the utility of IMPDH inhibitors as therapeutic agents. Several potent inhibitors of mammalian IMPDH enzymes are used clinically as antiviral, anticancer, or immunosuppressive agents (3–5). However, the utility of IMPDH inhibitors as antimicrobial agents has not been fully investigated.

Sequence analysis of all known IMPDH enzymes supports a distinction between the bacterial and eukaryotic enzymes.

A deep branching of the bacterial and eukaryotic forms of IMPDH is observed upon phylogenetic analysis of the relationships among the various IMPDH genes (6, 7). The analysis indicates a general functional conservation of amino acid residues and suggests a unique amino acid sequence signature for these kingdoms. The phylogenetic differences between IMPDH enzymes reflect their kinetic differences and differential sensitivity to inhibitors. Enzymes from mammalian sources show distinctly lower values for the *K*<sub>m</sub> for NAD than do those enzymes from bacteria (8–10). In addition, mammalian IMPDH enzymes are several orders of magnitude more sensitive to inhibition by mycophenolic acid (MPA) than are bacterial IMPDH enzymes (8–10). We hypothesize that the biochemical and kinetic differences between bacterial and mammalian enzymes are a consequence of the variance of specific, identifiable amino acid residues. Identification of the critical residues or combination of residues is a prerequisite for the rational identification of agents that specifically target the bacterial enzyme.

IMPDH catalyzes the NAD-dependent oxidation of IMP to XMP, a primary step in the biosynthesis of guanine ribonucleotides. The reaction mechanism has been examined in detail for IMPDH from *Escherichia coli* (11), *Trichomonas foetus* (12), and humans (13). These enzymes use a sequential, ordered, bi-bi kinetic mechanism in which IMP binds before NAD and NADH is released before XMP.
to transform BL21(DE3)lysS bacterial cells. Sequence in-
cloned into a pET23a (Novagen) expression vector and used
residue at the active site (Figure 1). The reaction proceeds through a covalent linkage
between C2 of IMP and the sulfhydryl of an active site
cysteine (Cys310 in S. pyogenes). A hydride transfer to NAD
is thought to occur from this intermediate followed by hydrolysis
of the oxidized enzyme—IMP complex to yield XMP.

Two partial structures of eucaryotic IMPDH enzymes from
Chinese hamster (85% complete, ref 14) and Trichromonas foetus (68% complete, ref 15) have been reported. These structures furnished the initial information about the structure
and reaction mechanism of eukaryotic IMPDH enzymes. The
crystal structure of a bacterial IMPDH enzyme has not yet
been reported.

To examine the differences between bacterial and mammalian IMPDH enzymes and provide a basis for the
evaluation of IMPDH inhibitors as antimicrobial agents, we
have expressed, characterized, and determined the crystal
structure of IMPDH from a pathogenic bacterium, Streptococcus pyogenes. Streptococci are the most common world-
crude cause of pneumonia and a leading cause of pediatric
infections. Our results show that IMPDH from S. pyogenes
has characteristics similar to other bacterial IMPDH enzymes,
suggesting this enzyme will be useful in the identification
of crucial residues that comprise a bacterial IMPDH enzyme
signature. The high-resolution crystal structure of IMPDH
from S. pyogenes, the first structure of a bacterial IMPDH
enzyme, will provide the basis for elucidation of the distinct
structural characteristics of bacterial and eukaryotic IMPDH
enzymes. Knowledge of these characteristics will permit an
understanding of why these enzymes exhibit functionally
distinct behavior and therefore provide a foundation for the
design of specific inhibitors.

MATERIALS AND METHODS

Cloning and Expression of S. pyogenes IMPDH. The
coding region of IMPDH was amplified from S. pyogenes
genomic DNA (kindly provided by Dr. Michael Boyle,
Medical College of Ohio, Toledo, OH) using coding region-
specific primers and a proofreading polymerase (Pfu).
The coding sequence of S. pyogenes IMPDH specifies a protein
of 493 amino acids that contains only a single cysteine
residue at the active site (16). The amplified fragment was
cloned into a pET23a (Novagen) expression vector and used
to transform BL21(DE3)lysS bacterial cells. Sequence int-
tegrity of the expression clones was verified by DNA
sequencing and analysis of the purified protein. The only
notable sequence difference between the expression clones and the published sequence resulted in the substitution of a
Leu in the expression clones for a Val (published sequence)
at amino acid position 419. Expression of Streptococcus IMPDH was induced by the addition of isopropylthiogalac-
topyranoside (IPTG) to a concentration of 0.5 mM.

The Streptococcus IMPDH enzyme was purified using a
modification of the procedure previously described for the
human enzyme (8). The modified procedure replaces the Blue
Sepharose dye column with a Matrix Green resin (Millipore,
Bedford, MA). Since the enzyme elutes as a broad peak from
the dye column, an additional chromatographic procedure
was applied to facilitate enzyme concentration and increase
purity. Peak fractions from the dye column are diluted with
20 mM Tris- HCl, pH 7.4, and applied to a MonoQ HR10/
10 FPLC column (Pharmacia, Piscataway, NJ). The column
was washed with 20 mM Tris- HCl, pH 7.4, 1 mM dithio-
theitol, and the enzyme was eluted with a linear gradient
of 0.2–0.7 M NaCl in wash buffer.

Purified IMPDH from S. pyogenes was characterized by
N-terminal sequencing and analyzed by mass spectroscopy
to validate as much of the internal protein sequence as possible.
An N-terminal sequence was obtained (Yale Biotechnol-
ogy Resource Center) for 19 residues corresponding to
amino acids 2–20 of the predicted sequence and indicated
cleavage of the N-terminal methionine as expected based
on the presence of a serine adjacent to the N-terminal methion-
ine (17). Our characterization of the purified protein also
including matrix-assisted laser desorption ionization mass
spectroscopy (MALDI-MS) analysis of the intact and tryptic-
digested protein provided by The Biotechnology Resource
Laboratory at Yale University. MALDI-MS of the intact
protein indicated a molecular weight (MW) of 52 328, similar
to the predicted MW of 52 657. In addition to N-terminal
sequencing of the intact protein, we analyzed a tryptic
digest of the purified protein by MALDI-MS. This analysis
provided verification of approximately 60% of the internal
protein sequence.

Selenomethionyl (SeMet) IMPDH was obtained by growth
of the native expression bacterium in M9 medium. Prior to
induction of IMPDH expression, de novo methionine syn-
thesis was suppressed by the addition of phenylalanine, valine,
threonine, isoleucine, leucine, and lysine to a final concentra-
tion of 50 µg/mL. Thirty minutes later, SeMet (final
concentration of 50 µg/L) and IPTG (final concentration of
0.25 mM) were added. The induced bacteria were harvested
4–6 h after induction. The purification and crystallization
of selenomethionyl IMPDH was as described for the wild-
type enzyme, and the presence of SeMet was verified by
amino acid analysis of the purified protein.

Kinetic Studies. Kinetic parameters for S. pyogenes IM-
PDH were determined by fitting initial rate data to the
Michaelis–Menten equation:

\[
\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_{m}^{IMP}}{V[IMP]} + \frac{K_{m}^{NAD}}{V[NAD]} + \frac{K_{s}^{IMP}K_{m}^{NAD}}{V[IMP][NAD]}
\]

where v is the initial rate, \(K_i\) is the dissociation constant for
the enzyme/IMP complex, and \(K_m\) is the Michaelis constant.
Initial velocity data were collected at varying concentrations
of IMP (5–1000 µM) and NAD (50–5000 µM). The initial
velocity plots followed Michaelis–Menten kinetics. Double-reciprocal plots provided a set of lines intersecting to the left of the ordinate. For IMP, the values of $K_m$ as well as $V_{max}$ were obtained by reploting the slopes and intercepts of these lines as a function of [NAD]. A similar strategy was employed to determine the Michaelis constant for NAD. The kinetic constants for MPA and ribavirin 5'-monophosphate (ribavirin-P) were determined by modification of Lineweaver–Burk equations for uncompetitive and competitive inhibition, respectively, in multiple substrate reactions as has been previously described (8).

Crystallography and MAD Data Collection. Crystals of IMPDH from *S. pyogenes* were grown by the vapor diffusion method using hanging drops. Crystallization conditions for *S. pyogenes* human IMPDH proteins were evaluated for a variety of conditions using the Crystal Screen II kit (Hampton Research, Laguna Hills, CA). Crystals used for this study were obtained using a reservoir solution of 0.1 M MES (pH 7.2), 1.8 M ammonium sulfate, and 10 mM CoCl$_2$. Crystals were obtained by mixing various ratios of *S. pyogenes* IMPDH protein (20 mg/mL in 10 mM bis-Tris propane, pH 7.4) with reservoir solution containing 1 mM IMP and incubating at 10 °C. Over several days, the crystals grew to a maximum size of about 0.1 × 0.1 × 0.25 mm$^3$. Crystals were transferred into a cryo-protectant solvent prepared by the addition of glycerol to the crystallization solution [final glycerol concentration (v/v), 28%] and flash-cooled in liquid nitrogen. Diffraction data were collected on beamline 19ID of the Structural Biology Center at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL). The approximate X-ray flux on the sample was 4 × 10$^{11}$ photons/s. Diffraction patterns from IMPDH crystals were collected at 100 K using a 3 × 3 mosaic CCD area detector (18), and all data sets were processed using the HKL2000 (19) package.

Crystals for the multiwavelength anomalous diffraction (MAD) study (20) were of SeMet IMPDH from *S. pyogenes* complexed with IMP. We recorded three data sets for a single crystal, each at a unique X-ray wavelength ($\lambda_1 = 1.0781$ Å, $\lambda_2 = 0.9793$ Å, $\lambda_3 = 0.9791$ Å, Table 1). The crystal was not oriented in any special way prior to data collection. The high-resolution data (1.90 Å) were collected from the same crystal at $\lambda = 1.0332$ Å. Details of the experiments and data quality are summarized in Table 1.

---

**Table 1: Crystal and MAD Data Collection Parameters**

<table>
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<tr>
<th>Parameter</th>
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<th>2.5</th>
<th>2.5</th>
<th>2.5</th>
<th>19ID</th>
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<tr>
<td>Wavelength (Å)</td>
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<td>0.9791</td>
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<td>1.0332</td>
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<td>2.5</td>
<td>1.90</td>
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<td></td>
<td></td>
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<tr>
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<td>276365</td>
<td>272576</td>
<td>263355</td>
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<td></td>
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<tr>
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<td>20627</td>
<td>20686</td>
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<td>6.2</td>
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<tr>
<td>Completeness</td>
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<td>99.7</td>
<td>99.6</td>
<td>96.5</td>
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<tr>
<td>$R_{merge}$ (%)</td>
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<td>9.6</td>
<td>5.9</td>
<td>6.8</td>
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**Table 2: Biochemical Comparison of *S. pyogenes* and Human Type II IMPDH**

<table>
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<tr>
<th>Parameter</th>
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<th>Human</th>
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<td>55600</td>
</tr>
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<td>Active form</td>
<td>Tetramer</td>
<td>Tetramer</td>
</tr>
<tr>
<td>pH optimum</td>
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<td>7.2</td>
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<td>Kinetic constants</td>
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<td>$K_m$ for IMP (µM)</td>
<td>62 ± 19</td>
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<tr>
<td>$K_m$ for NAD (µM)</td>
<td>1180 ± 400</td>
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<tr>
<td>$K_{cat}$ (s$^{-1}$)</td>
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<td>MPA</td>
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</tr>
<tr>
<td>Ribavirin 5'-monophosphate</td>
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</tr>
<tr>
<td>Mizzoribine 5'-monophosphate</td>
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</tr>
<tr>
<td>% Activity with cation</td>
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<td></td>
</tr>
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<td>100</td>
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</tr>
<tr>
<td>Na$^+$</td>
<td>&lt;5</td>
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</table>

---

Results

Biochemical Characteristics. *S. pyogenes* IMPDH monomer molecular weight is approximately 52 700 (Table 2) as determined by denaturing gel electrophoresis. Size exclusion chromatography indicates that the native form of the enzyme is a tetramer with a pH optimum of 7.8. As is observed for most IMPDH enzymes, *S. pyogenes* IMPDH has a general requirement for monovalent cations. All IMPDH enzymes thus far characterized have an active site cysteine residue that was proposed to form a covalent intermediate with IMP (13). The coding sequence of *S. pyogenes* IMPDH specifies a protein of 493 amino acids that contains a single cysteine residue at the active site (16). The essential role of this cysteine residue in *S. pyogenes* IMPDH was established by the abolition of activity observed upon treatment with 6-chloropurine, an irreversible inhibitor, or by incubation with low concentrations of iodoacetamide, a sulfhydryl reactive agent. Characterization of the kinetic parameters for this enzyme (Table 2) indicates a concurrence with other bacterial IMPDH enzymes (9, 10). The bacterial enzymes effectively bind IMP but have a low affinity for NAD. Although there is considerable amino acid sequence conservation of IMPDH enzymes in the active site region (6), the bacterial enzymes differ from the mammalian enzymes in their affinity for NAD and in their relative sensitivity to various inhibitors (8–10). The enzyme from *S. pyogenes* has a high $K_m$ value for NAD and is relatively insensitive to inhibition by MPA, an effective inhibitor of the mammalian enzymes. In contrast to bacterial IMPDH enzymes, enzymes from mammalian sources show distinctly lower $K_m$ values for NAD and are effectively inhibited by MPA. These characteristics of *S. pyogenes* IMPDH suggest this enzyme is an appropriate representative of bacterial IMPDH and can be used to examine the differences between the bacterial and eukaryotic forms of IMPDH.

Structure Determination. To investigate the basis for the different characteristics of bacterial and mammalian IMPDH, we determined the crystal structure of *S. pyogenes* IMPDH at 1.9 Å resolution by replacing all methionine residues in the enzyme with selenomethionine and applying MAD phasing methods (20). Phase analysis was initiated by...
molecular replacement using AMORE (21) and the T. foetus atomic coordinates (15) from the PDB as a search model. IMPDH from T. foetus, a eucaryotic organism, shows poor homology to S. pyogenes IMPDH (35% identity). As a consequence of the poor homology and the incompleteness of the T. foetus IMPDH structure, the initial molecular replacement solution produced phases that were not sufficiently close to the correct values for the structure to be interpreted further. However, this phase set was sufficiently good to identify 6 of the 13 selenium sites in the structure. Refinement using MLPHARE (22) permitted identification of all remaining selenium atoms. Successive refinement cycles produced a map from which we could complete the interpretation of the model (Figure 2). Phases were improved during subsequent refinement with CNS (23) (see below), permitting modeling of 97% of the structure.

Model Building and Refinement. All model building was carried out with FRODO (24) on an Evans and Sutherland ESV10 graphics workstation. Refinement of the initial model against the MAD data was carried out using torsion-angle molecular dynamics (25) and the phase-restrained MLHL target (26) implemented in CNS (23). Diffraction data from 6 to 1.90 Å were used throughout the refinement except for target (Rpositional refinement, restrained experimental phases. Alternate cycles of model rebuilding, 

\[ \text{average B-factor (Å}^2) \]

\[ \text{protein atoms 37.5} \]

\[ \text{catalytic domain 34.4} \]

\[ \text{CBS dimer domain 43.4} \]

\[ \text{solvent atoms 50.1} \]

\[ \text{residues in core phi-psi regions} \]

\[ \text{91.2} \]

\[ \text{residues in disallowed regions} \]

\[ \text{0.0} \]

\[ \text{Figure of merit is a measure of the relative reliability of a phase based on the consistency of the MIR analysis from one derivative to the next. The maximum value is 1.0.} \]

\[ \text{α MAD phasing power is defined:} \]

\[ \text{R-value obtained for a test set of 4% randomly selected test set required for cross-validation of the } \]

\[ \text{the } \]

\[ \text{main chain torsion angles within the ”allowed regions” of the Ramachandran plot and 8.8% within the ”additional allowed regions”.} \]

\[ \text{There are no residues in ”disallowed regions”.} \]

\[ \text{Tetramer Organization.} \]

S. pyogenes IMPDH is a tetramer with its four subunits related by a crystallographic 4-fold axis. Each monomer has a two-domain structure (Figure 3A): a catalytic domain (40 × 40 × 50 Å, amino acid residues 2–92 and 224–492) forming the interior core of the active tetrameric enzyme and a CBS dimer domain (20...
The CBS designation arises from the original identification of this folding motif in the enzyme cystathionine β-synthase. The catalytic domain contains the active site positioned near the tetramer 4-fold at the subunit interface (Figure 3B).
A feature observed in the tetrameric structure is an extended region projecting from the C-terminal face of each monomeric subunit (Figure 3B). These finger structures are composed of two antiparallel $\beta$-strand structures ($\beta_\ell$, $\beta_k$; Figure 4A) stabilized by hydrogen bonding and interactions with the $\beta_\ell$ region. The finger region is not involved in catalysis but is found in all IMPDH enzymes.

**Catalytic Domain.** The core of the catalytic domain (Figure 4B) is a TIM barrel providing a scaffold for the attachment of additional structural and catalytic moieties. This core contains eight parallel $\alpha/\beta$ motifs with the active site near the C-terminus of the $\beta$-strands. Sequence comparisons suggest limited sequence conservation within the TIM barrel core relative to the high level of conservation observed for residues forming the catalytic site pocket (6). The sequence conservation of the TIM barrel is restricted to residues adjacent to the active site pocket and to a region representing the junction between the catalytic and CBS dimer domains.

![Figure 4: Secondary structure of the IMPDH monomer.](image-url)
There are several large structural and catalytic protrusions from the TIM barrel surface. The N-terminus protrudes from strand β₀ on the distal face of the TIM barrel (furthest from the IMP binding pocket). The CBS dimer domain is located between helix α₂ and strand β₁. Connections between the remaining α/β motifs are short (2–5 amino acid residues) and contain many proline, glycine, and hydrophobic residues. The C-terminal region exits from helix α₈, on the opposite face of the tetramer from the N-terminus.

The protrusions on the proximal face of the TIM barrel scaffold range in size from 3 to 67 residues and define the character of the active site. Three of the barrel connections (β₁/α₁, β₂/α₂, and β₃/α₃) show greater than a 50% amino acid sequence conservation for all IMPDH proteins. The β₃/α₃ protrusion is the largest (67 residues) of the proximal face motifs and contains the “finger” structure (Figure 3B), short helices α₄ and α₁₀, strands β₁₋₁, and regions that have a role in catalysis and that interact with other IMPDH monomers in the tetramer. This protrusion sequence is also highly conserved, with regional sequence conservation of 60–80% in 3 distinct 10-amino-acid-residue segments. A distinct feature of this region is a “flap” (residues 396–419) on one edge of the active site that shows very poor electron density and apparently projects into the solvent (Figure 4B). In the S. pyogenes IMPDH 1.9 Å structure, 14 residues in this flap are disordered in the presence of substrate in the active site. In crystals of eucaryotic IMPDH containing product and transition-state analogue and complexed with MPA (14, 15), this region is also disordered. It has been suggested (15) that this flap functions by folding over the catalytic pocket, controlling access to and ordering the active site, perhaps in a manner similar to the active site flap of lactate dehydrogenase (30).

**CBS Dimer Domain.** The CBS dimer domain, found in IMPDH proteins from all three kingdoms, is composed of two CBS motifs (Figure 4A,B) related by approximate 2-fold symmetry (rms deviations between related Cα atoms: 2.7 Å). Each CBS motif has the characteristic sheet/helix/sheet/Sheet/helix topology. This is the first reported complete structure of a CBS dimer domain: a folding motif originally identified in cystathionine β-synthase and proposed to act as a regulatory element since mutations lead to the human disease homocystinuria (31). The CBS dimer domain in IMPDH does not interact with the other subunits in the active tetrameric enzyme and may not be required for activity (9, 14). In S. pyogenes IMPDH, two CBS domains form a minicore barrel structure that has a distinct hydrophobic core. Among bacteria, the degree of amino acid conservation is highest in the EF/H/I β-strands (Figure 4A,B) that span the interior of the CBS dimer domain and provide a resource of hydrophobic residues. The α-helices on the exterior maintain the character of this domain with hydrophilic residues on the exterior surfaces and hydrophobic residues positioned on the interior. There is a well-defined cleft between CBS motifs (approximately 15 Å in length) that may function as a potential binding site for regulatory molecules. There is no known role for CBS motifs in bacteria, but in eukaryotic organisms they appear to mediate cytoplasmic targeting, protein–protein interactions, or protein regulation (31).

**Catalytic Site and Implication for the Mechanism of Bacterial IMPDH.** A unique aspect of the S. pyogenes IMPDH structure is that it allows examination of the initial stage of the catalytic cycle. IMP is bound at one end of the TIM barrel, with the other end blocked by the β₉β₁C sheet (Figure 4A,B). Short helices H, J, and L are structural motifs containing many of the active site residues. During the reaction, a hydride is transferred from the C2 carbon of the hypoxanthine ring to NAD, and an oxygen atom is substituted in the C2 position, resulting in the formation of xanthine.

The high-resolution (1.9 Å) crystal structure of S. pyogenes IMP dehydrogenase allows examination of the catalytic site in greater detail than was previously possible. The are a number of protein interactions with the IMP ribose and phosphate moieties (Figure 5A). The ribose is in the C2′-endo-conformation. Its 2′- and 3′-hydroxyls are hydrogen-bonded with Asp343 and a water molecule that, through a water relay system, connects with N3 of the hypoxanthine ring. The phosphate group is anchored in its site by a number of amino acid side chains (Ser308, Ser367, and Tyr 390) and three main chain nitrogen atoms (Gly345, Gly366, and Ser367). Ile309 stacks on top of the hypoxanthine ring, and four hydrogen bonds further stabilize the ring (Figure 5A).

Cysteine 310 has been identified previously as a key residue in catalysis (12, 13). The ability of the thiol residue to ionize appears to be critical for the reaction involving nucleophilic attack. The hydroxyl of Thr312 is in position (3.3 Å) to activate Cys310 (Figure 5B). Covalent bond formation requires reorientation of the hypoxanthine ring (rotation around the glycosidic bond) and nucleophilic attack on C2 by Cys310. This mechanism is consistent with the proposed formation of a tetrahedral intermediate (32). However, our data show that IMPDH does not form a covalent bond with the substrate in the absence of the NAD cofactor. This suggests that NAD may have multiple roles as hydride acceptor and substrate activator, as well as in contributing to the structure of the active site pocket.

The hamster IMPDH structure (14) contains the hypoxanthine ring covalently bound to the active site cysteine and the inhibitor MPA bound in the active site. It appears that the hamster IMPDH structure represents the covalent thioimidate intermediate of the reaction in which MPA, an uncompetitive inhibitor, prevents the hydrolysis of the thioimidate covalent intermediate, as suggested by Link and Straub (33). It has been suggested that MPA restricts the access of the solvent molecules and blocks subsequent steps of the reaction (14). This observation also suggests that the hydrolysis of the thioimidate intermediate is mediated by an activated water molecule originating from the NAD site. In the S. pyogenes IMPDH structure, we have located two water residues that are potential candidates for nucleophilic attack on the thioimidate (Figure 5B), and several residues (Glu421, Thr312, and Tyr450') can act as activators of a water molecule. However, since the enzyme does not contain NAD, a required cofactor, elucidation of the mechanistic details of the hydrolysis reaction will require additional studies. Because MPA can stabilize the thioimidate intermediate in the human enzyme (14), hydrolysis of thioimidate must be several orders of magnitude slower than the dissociation of NADH, a result consistent with the mechanism proposed by Wang et al. (11).

**Site-Specific Mutants.** To validate the role of specific residues in catalysis and to provide a basis for comparing the bacterial and mammalian enzymes, we constructed several point mutants. The sites for mutation were selected on the
basis of previous studies suggesting a catalytic role for the region and supported by information derived from the *S. pyogenes* IMPDH crystal structure. One region targeted for site-specific mutagenesis was the active site flap. This flap is present in all IMPDH enzymes and is disordered in the *S. pyogenes* IMPDH structures and in the IMPDH structures from hamster and *T. foetus*. Although this region has not been previously implicated in the catalysis mechanism of IMPDH enzymes, the presence of a conserved RY(FY) motif and the similarities to the flap region in lactate dehydrogenase (30) suggest a potential role in catalysis. Mutation of Arg406 to alanine in this flap region results in a complete loss of enzyme activity (Table 4) as might be expected for a residue conserved in all IMPDH enzymes. This loss of activity confirms the importance of the active site flap in catalysis. Since there is little sequence conservation of this region, this structure is an attractive target for specific inhibitors.

The catalytic mechanism of *S. pyogenes* IMPDH involves the hydrolysis of a thioimidate intermediate that we believe is mediated by an activated water molecule originating from

Table 4: Site-Specific Mutants of *S. pyogenes* Impdh

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<thead>
<tr>
<th>mutant</th>
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<th>region</th>
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</tbody>
</table>

**Figure 5:** IMPDH active site. (A) Cartoon of bound IMP showing side chain interactions and active site residues. Water molecules are colored green. (B) Stereoview ball-and-stick diagram of bound IMP illustrating the alignment of the hypoxanthine ring relative to the catalytic Cys310 residue. The dashed ring cartoon indicates the proposed realignment of the hypoxanthine ring initiated by NAD binding. Residues targeted for mutagenesis (E421 and Tyr450') are underlined. The "′" symbol on T450 indicates a symmetry-related molecule.
the NAD site. In the *S. pyogenes* IMPDH structure, we have located two water residues that are potential candidates for nucleophilic attack on the thioimidate. Tyr450', originating from an adjacent subunit, is a residue in the active site pocket that can act as an activator of one of these water molecules. This residue is located at the noncatalytic end of a conserved helix (helix 8) that forms the TIM barrel core. Replacement of Tyr450 with aspartic acid or alanine (Table 4) results in substantial loss of enzyme activity. Approximately 25% activity is retained for an alanine replacement, but substitution of aspartic acid results in a loss of enzyme activity. This region is conserved in the IMPDH enzymes, but the sequence pattern is different in bacteria and eukaryotes, suggesting this region may contribute to the differential signature of the bacterial and mammalian enzymes.

The NAD binding region (between the α/β loop) was also selected as a target for site-specific mutagenesis. The selection of Glu421 for mutation was based on an analysis of sequence differences at residues corresponding to or near amino acids identified as MPA binding sites in human IMPDH. The conserved glutamate in bacteria is replaced with a conserved glutamine in eukaryotes. This substitution does not alter the apparent activity of *S. pyogenes* IMPDH (Table 4). This result was unexpected since replacement of the corresponding residue in the hamster enzyme (Gln441) with alanine results in a significant decrease in activity (14).

**DISCUSSION**

Because of its central role in purine metabolism, IMPDH is an attractive therapeutic target. Several recent reviews have outlined the utility of mammalian IMPDH inhibitors as anticancer (34) or antiviral (35) agents or as immunosuppressive drugs (36). Although there are no selective inhibitors of bacterial IMPDH enzymes, such compounds could have potential application as antimicrobial agents. This proposal is supported by the observation that bacterial and mammalian IMPDH enzymes provide the same catalytic function but have a set of unique structural and biochemical characteristics. The kinetic and biochemical characteristics of *S. pyogenes* IMP dehydrogenase are similar to other bacterial IMPDH enzymes but different from mammalian IMPDH enzymes. Relative to the mammalian enzymes, the bacterial enzymes bind NAD poorly and are inhibited by MPA only at high concentrations (Table 2). Elucidation of the basis of these distinct characteristics will aid the design of specific IMPDH inhibitors.

The structure of *S. pyogenes* IMPDH provides a new resource to define the distinct characteristics of bacterial and mammalian IMPDH enzymes. Features such as the catalytic pocket loops, active site flap region, and CBS dimer domain are structurally conserved but show a different pattern of sequence conservation in bacteria and eukaryotes, suggesting that they could contribute to the differential signature of the bacterial and mammalian enzymes. Analysis of sequence alignments for this region indicates a pattern of catalytic residues conserved in all enzymes and a secondary pattern of amino acid conservation associated with either bacterial or eukaryotic IMPDH enzymes. This observation is supported by our site-specific mutants at positions Glu421 and Tyr450 that appear to differentially alter the activity of the mammalian and bacterial IMPDH enzymes. Residue Tyr450 in *S. pyogenes* IMPDH is located at the noncatalytic end of the TIM barrel. However, this region has contacts with another molecule in the tetramer and contributes to the catalytic environment of the adjacent monomer (Figure 5B). Our site-specific mutagenesis results show partial retention of activity with an alanine substitution but no activity with an aspartic acid substitution for this residue. Aspartic acid was selected as a replacement on the basis of sequence alignments that show 12 of 13 eucaryotic enzymes contain aspartic acid at the corresponding position (the exception being asparagine in *T. foetus*). The partial activity observed with the Ala replacement suggests Tyr450 does not have an essential role in catalysis but does contribute to the environment of the catalytic pocket. Further analysis of this region will provide insight into the differences in the environment of the catalytic pocket in bacterial and eucaryotic enzymes and also the role of the tetrameric form of the active enzyme.

The Glu421 in *S. pyogenes* IMPDH is conserved in bacteria while eucaryotic IMPDH enzymes contain glutamine in the corresponding position. In hamster IMPDH, the corresponding residue, Gln441, is implicated in the binding of MPA. Comparison of the residues involved in MPA binding in the hamster enzyme (Asp274, Ser276, Asn303, Arg322, Gly326, Thr333, Gln441) with the equivalent residues in *S. pyogenes* IMPDH indicates that these residues are largely conserved. The aspartic acid, asparagine, glycine, and threonine residues are identical, but threonine replaces Ser276 (although serine is present in other bacterial enzymes), and Lys301 replaces the hamster Arg322 residue. The most significant change appears to be replacement of Glu421 with Gln441 (interestingly, this residue is part of the active site flap). Although this suggests that the NAD binding pockets of hamster and bacterial IMPDH differ, we did not observe a change in activity upon substitution of glutamine for glutamic acid at position 421. It is possible that this substitution does not affect the observed activity but may alter the sensitivity to MPA. Further studies are in progress to define the contribution of the sequence signature in this region to the bacterial kinetic profile.

The active site flap represents another region that could account for the kinetic and biochemical differences between IMPDH enzymes. This flap is present in all IMPDH enzymes and is disordered in the IMPDH structures from hamster, *T. foetus*, and *S. pyogenes*. This persistent disorder suggests that NAD binding is essential for structuring the flap, a suggestion supported by the resistance of this region to proteolysis acquired by NAD binding (37). This also implies that MPA binding does not involve an interaction with this flap and does not entirely mimic NAD binding. This flap may therefore be important in mediating NAD binding specificity in the active site and may be responsible for some of the kinetic differences observed between IMPDH enzymes from bacteria and eukaryotes. Sequence comparisons indicate that loop size is conserved but sequence conservation is limited. A conserved feature of this region is the presence of arginine adjacent to one or two aromatic residues. Since IMP and NAD bind sequentially to the active site, these residues may bind to the NAD moiety, thereby ordering the active site. Our preliminary site-specific mutagenesis results implicate this region as essential for enzyme activity, but further studies will be necessary to define the specific role of the flap region. The sequence heterogeneity observed in this flap region may
also account for the discriminatory features of bacterial and mammalian IMPDH enzymes.

The finger region and the CBS dimer domain are not involved in catalysis but are found in all IMPDH enzymes. These regions show little sequence conservation but have been structurally conserved. The finger structure is composed of two antiparallel β-strand structures stabilized by hydrogen bonding and interactions with the βL region. The CBS dimer domain contains two CBS motifs arranged on a pseudodyad axis. In other proteins (e.g., cystathionine β-synthase and chloride channel proteins), mutations in these domains are associated with pathologic consequences. It has also been suggested (31) that these domains may be involved in cytoplasmic targeting or other regulatory functions. In either case, the metabolic expenditure required for conservation of these structures suggests an underlying functional role.

A unique aspect of the S. pyogenes IMPDH structure is that it allows examination of the initial stage of the catalytic cycle. Our data show IMP does not form a covalent bond in the absence of NAD. Covalent bond formation requires reorientation of the hypoxanthine ring and nucleophilic attack on C2 by Cys310. We propose that NAD binding initiates realignment of the hypoxanthine ring and facilitates the electron shift with the ring required for formation of the thioimidate intermediate. This suggests that NAD may have multiple roles as hydride acceptor, substrate activator, and also in contributing to the structure of the active site pocket.

The structure of S. pyogenes IMPDH allows for a detailed comparison of the eukaryotic and bacterial enzymes. It will also provide the basis for an explanation for the unique properties of the bacterial enzymes. In conjunction with additional site specific mutants and kinetic analyses, the characteristics and structure of S. pyogenes IMPDH will be useful in the delineation of specific characteristics of bacterial and mammalian IMPDH enzymes. This knowledge will contribute to the design of inhibitors that specifically target bacterial IMPDH enzymes.

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REFERENCES


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