Key Flap Residues of *Bacillus anthracis* Inosine 5’-Monophosphate Dehydrogenase (IMPDH) Influence Conformational Equilibrium and Interact with Inhibitors

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Abstract:

Antibiotic resistance is becoming an increasing problem in the treatment of bacterial pathogens. A deeper understanding of inosine 5′-monophosphate dehydrogenase (IMPDH) inhibition at a biochemical level may reveal a route to the development of novel antimicrobial drugs. IMPDH catalyzes the oxidation of IMP to XMP, the first committed and rate-limiting step in de novo guanine nucleotide biosynthesis. Several inhibitors of bacterial IMPDH have been developed. Although all bacterial IMPDHs sensitive to these inhibitors have highly conserved inhibitor-binding sites, there is still quite a bit of variation in inhibitor sensitivity between the IMPDHs of various bacterial species. Our goal is to determine which residues within the IMPDH protein affect sensitivity to inhibitors. Specifically, we hypothesized that mutations in the dynamic flap region of Bacillus anthracis IMPDH (BaIMPDH) would alter its sensitivity to inhibitors. Several point mutations were incorporated into BaIMPDH through site-directed mutagenesis (SDM) and mutant proteins were expressed and purified from E. coli with nickel affinity chromatography. The mutants were analyzed with both kinetic and IC₅₀ studies, and compared to wild type BaIMPDH. It was found that the kinetic parameters of the mutants were similar to those of wild type, indicating that the mutants have not greatly disrupted enzymatic activity. IC₅₀ fold changes were constant for some inhibitors and different for others, suggesting that the flap residues both influence conformational equilibrium between the open-flap and closed-flap conformations, as well as interact with some inhibitors. Further studies will correlate inhibitor structures with IC₅₀ fold changes.
Introduction:

I. Introduction

Inosine monophosphate dehydrogenase (IMPDH) is the enzyme that catalyzes the oxidation of IMP to XMP and the coupled reduction of NAD\(^+\) to NADH. This happens in two steps: (1) dehydrogenase reaction in which IMP becomes covalently linked to a catalytic cysteine residue, and (2) hydrolysis reaction in which water releases the XMP product (Figure 1) (Hedstrom 2009). IMPDH controls this rate-limiting step in \textit{de novo} guanine nucleotide biosynthesis (Figure 2) (Makowska-Grzyska \textit{et al.} 2012).

\textbf{Figure 1: Mechanism of IMPDH}

The electron-pushing mechanism for the two very different chemical reactions catalyzed by IMPDH. (A) The dehydrogenase reaction that converts IMP to the covalently linked XMP* and NAD\(^+\) to NADH. (B) The hydrolysis reaction that releases XMP product. Note the conserved arginine residue that acts as a general base catalyst.
Figure 2: Purine Nucleotide Biosynthesis

The *de novo* and salvage pathways involved in purine biosynthesis are shown. The reaction catalyzed by IMPDH is highlighted in the box. Susceptibility to IMPDH inhibitors is dependent on ability to produce sufficient guanine nucleotides through salvage pathways. For example, bacteria that can salvage guanine through only xanthine won’t be a good target of IMPDH inhibitors. Figure adapted from (Hedstrom *et al.* 2011).

We’ve known about IMPDH for decades, yet it was not until recently that IMPDH has been recognized as a potential target for novel antimicrobial drugs (Mandapati *et al.* 2014). This area of research is significant because IMPDH is universal; that is, almost all species have IMPDH (Hedstrom 2009). Additionally, IMPDH is an excellent drug target. As the guanine nucleotide pool controls cell proliferation, IMPDH is highly expressed in rapidly dividing cells (Hedstrom 2009). Thus, it has been used as an anti-cancer, anti-viral, and immunosuppressive therapeutic target, as well (Braun-Sand & Peetz 2010). Furthermore, the NAD\(^+\) binding site is significantly different in human and prokaryotic IMPDHs, which suggests that it would also be an attractive target for the development of novel anti-microbial drugs (Umejiego *et al.* 2004).

Although IMPDH is probably the most studied and most popular target of purine biosynthesis inhibitors, there are still vast gaps in our knowledge that merit additional research.
and have the potential to produce worthwhile discoveries that can be utilized in drug development. In particular, understanding the conformational changes that take place throughout the catalytic cycle of IMPDH, especially those of the dynamic flap, may shed light on the reasons why, despite similar binding sites, different bacterial species have widely varied inhibitor sensitivity.

II. Structure

IMPDH is a tetrameric enzyme that adopts a square planar geometry (Figure 3) (Hedstrom 2009). The four active sites are at the subunit interfaces. Since IMPDH has two different substrates (IMP and the NAD\(^+\) cofactor), each active site has a distinct binding site for each substrate (Hedstrom 2009). There are 400-500 residues in each monomer (depending on whether or not the CBS domain, a subdomain not required for enzymatic activity, is present) and some residues are highly conserved across species (Riera et al. 2008). The crystal structure of IMPDH has been solved for several organisms. While the active site is ordered, the flap, a dynamic component necessary for the hydrolysis step, is often disordered in the crystal structure, due to its mobility (Josephine et al. 2010).
The crystal structure of *Ba*IMPDH and *Ba*IMPDH\(\Delta L\). For *Ba*IMPDH\(\Delta L\), the CBS domain that has been removed is shown in gray and the connector sequence it has been replaced with is shown in red. The mobile flap, which is disordered in the crystal structure, folds into the cofactor site for the hydrolysis step. Figure adapted from (Makowska-Grzyska et al. 2015).

The catalytic domain of IMPDH is the part of the enzyme responsible for the oxidation of IMP to XMP (Hedstrom 2009). As previously described, the cysteine residue involved in the dehydrogenase reaction is highly conserved, as it is functionally important (Josephine et al. 2010). In general, IMPDH is a rather flexible protein in that it is dynamic and can adopt many different conformations (Hedstrom & Gan 2006). Specifically, the cysteine loop is known to be dynamic, which is necessary for enzymatic activity, but normally adopts a more stable conformation when IMP binds (Hedstrom 2009). Of particular relevance to this project is the mobility of the flap containing the conserved hydrolysis dyad, which is disordered in most crystal structures. These important structural features are highlighted in Figure 4.
Figure 4: Model of IMPDH
The blue box indicates the location of the hydrolysis dyad on the mobile flap. The yellow box indicates the conserved cysteine loop that becomes covalently attached in the dehydrogenase step.

The IMP-binding site is stringently conserved across many species, whereas the NAD$^+$-binding site has diverged (Pankiewicz et al. 2004). Drug design takes advantage of this and many IMPDH inhibitors bind to the NAD-binding site, which allows a great deal of selectivity, especially in distinguishing between prokaryotic and eukaryotic IMPDHs (MacPherson et al. 2010). The mobile flap is also considered to be part of the catalytic domain because it interacts with the active site. A highly-conserved arginine and tyrosine dyad are part of the flap (Schlippe et al. 2003). They are catalytic and required for the hydrolysis step. Importantly, the flap may interact directly with IMPDH inhibitors, resulting in a change in inhibitor sensitivity that can be utilized in drug design.

In addition to the catalytic domain, IMPDH also has a CBS subdomain, also known as the Bateman domain. The function of the CBS domain is still being researched and is currently debated. The most current hypotheses suggest that the CBS domain binds RNA polymerase, and is therefore involved in the moonlighting functions of IMPDH, an area of research that necessitates further study. (Kozhevnikov et al. 2012). The CBS domain is not required for enzymatic activity, and is often removed in order to facilitate crystallization (Hedstrom 2009).

Unlike bacteria and other prokaryotes, humans and other mammals have two types of IMPDHs: IMPDH1 and IMPDH2 (Kohler et al. 2005). Mutations in hIMPDH1 are associated
with the hereditary retinal disease retinitis pigmentosa (Hedstrom 2009). Human IMPDHs have also been targets of anti-cancer drug discovery, as rapidly dividing cells require guanine nucleotides and therefore IMPDH (Wei et al. 2016). Additionally, human IMPDHs have been the target of immunosuppressive drugs to treat autoimmune disease and decrease the frequency of rejection during organ transplantation (Shu & Nair 2008).

III. Function

The main function of IMPDH is to convert IMP to XMP. The mechanism is shown in Figure 5. The dehydrogenase step involves covalent catalysis while the hydrolysis step involves an arginine residue acting as a general base (Hedstrom & Gan 2006). While IMP and NAD$^+$ can bind to IMPDH in either order, NADH must be released before the hydrolysis of XMP, as the flap moves into the dinucleotide binding site (Gollapalli et al. 2010). This ordered mechanism is significant because it explains how the inhibitors work. The inhibitors bind to the NADH site after it has left and trap the E-XMP$^*$ complex, thus preventing release of product and regeneration of free enzyme (Umejiego et al. 2008). The conformational changes that the flap region undergoes are especially important to the mechanism, specifically the hydrolysis step (Figure 5).

In terms of kinetics, the dehydrogenase reaction is fast while the hydrolysis reaction is slow, so the E—XMP$^*$ complex builds up, which is why although the inhibitors may also bind to the E•IMP complex, they mostly bind to the E—XMP$^*$ complex, making their mechanism of action more uncompetitive than noncompetitive (Figure 5) (Umejiego et al. 2008).
Figure 5: IMPDH Mechanism
Adapted from (Umejiego et al. 2008).

As briefly described above, the susceptibility of a species to IMPDH inhibitors is the extent to which it relies on IMPDH for guanine nucleotide production (Hedstrom 2009). If flux through the salvage pathways is sufficient, the species will likely not be sensitive to IMPDH inhibitors.

Aside from the main function of IMPDH, which is the conversion of IMP to XMP, it has been proposed that IMPDH also has several moonlighting functions within the cell. For example, the CBS domain of IMPDH regulates adenine and guanine nucleotide pools, as well as associates with poly ribosomes (Mortimer et al. 2008). IMPDH binds nucleotides and is associated with RNA in cell lysates (Hedstrom 2016). Additionally, mutations in human IMPDH type 1 cause autosomal dominant retinisis pigmentosa (Thomas et al. 2012). Furthermore, IMPDH can be phosphorylated and associate with lipid vesicles (Hedstrom & Gan 2006).
IV. Cryptosporidium parvum IMPDH

A good example of how IMPDH inhibitors can be used as an effective therapy is its use against Cryptosporidium parvum. C. parvum is a eukaryotic parasite that is especially devastating in the developing world where malnutrition puts one at risk for death (MacPherson et al. 2010). In the developing world, diarrhea is a bigger cause of mortality than malaria, and C. parvum is the second most common cause of diarrhea (after rotovirus) (Hedstrom 2009). Those especially at risk are the immunosuppressed, like children and those with AIDS. Additionally, C. parvum poses a risk for bio-warfare, as if oocysts get in the water supply, they can’t be removed by simple treatment of the water (Hedstrom 2009). There is no vaccine or effective drug (only drugs that shortens the course of the disease in healthy people) (Hedstrom 2015). C. parvum is difficult to culture and the animal models that we have are poor (Hedstrom 2009).

However, C. parvum IMPDH is a promising drug target. C. parvum has a streamlined purine biosynthesis pathway (Umejiego et al. 2004). At this point, it was already known that IMPDH was a drug target in humans, meaning that the enzyme is “druggable” (it is possible to design drugs that target it) (Hedstrom 2009). Furthermore, the C. parvum IMPDH gene came from bacteria and the eukaryotic one was dropped entirely (Umejiego et al. 2008). Since the enzyme will be different from the human host, it makes a good target for selective inhibitors (Umejiego et al. 2008).

C. parvum IMPDH inhibitors were identified with a high throughput screen that started with about 130,000 compounds (Maurya et al. 2009). The number of compounds was reduced to 60 that did inhibit C. parvum IMPDH, but did not inhibit human IMPDH. Chemical modifications made these original hits more potent, so now there are several hundred selective and potent inhibitors of the parasitic enzyme with several different scaffolds (Gorla et al. 2014).
The binding site that the inhibitors occupy is a pocket that does not exist in the human enzyme, so the inhibitors are sterically excluded from the human form, making them highly selective (Figure 6) (Umejiego et al. 2004). One side of the inhibitors pi-stacks with IMP while the ring on the other side pi-stacks with a conserved tyrosine residue (Mandapati et al. 2014).

![Figure 6: Different Cofactor Binding Sites Between Prokaryotic and Eukaryotic IMPDHs Allows Selective Drug Design](image)

Purple is the prokaryotic *Mycobacterium tuberculosis* IMPDH (PDB 1NFB) and orange is the eukaryotic human type II IMPDH (PDB 4ZQM). IMPDH inhibitors bind to the cofactor (NAD$^+$) site. While the IMP binding site is conserved between prokaryotes and eukaryotes, the NAD$^+$ binding site is not. This disparity allows for selective drug design.

V. Bacterial IMPDH

The inhibitors originally identified in the *C. parvum* program were repurposed to inhibit bacterial IMPDHs (Figure 7). The design of novel antimicrobial drugs is important because
antibiotic resistance is a growing problem, so there is an urgent need to develop novel antibiotics (Hedstrom 2016).

However, there are several problems with re-purposing these compounds to be effective antibiotics. First of all, the transition to an antibiotic introduces the challenge of getting through the cell wall. Additionally, there are inherent problems with targeting IMPDH as a potential treatment for bacterial infections. For instance, some bacteria can survive and still be virulent, even without IMPDH, because of salvage pathways (Hedstrom 2009). Whether or not bacteria can survive off of salvage pathways is dependent upon the type of bacteria and where the infection is, so is difficult to predict from just in vitro studies (Hedstrom 2016).

Nevertheless, despite these difficulties, there is promise in IMPDH as a target for novel antimicrobials, as IMPDH is required for infection for many pathogens. In fact, many of the inhibitors developed for C. parvum IMPDH were able to be successfully re-purposed to be successful against bacterial IMPDHs. As mentioned, the structure of these small molecule inhibitors involves two aromatic regions connected by a linker (Kirubakaran et al. 2012). The aromatic regions pi-stack with IMP on one side and a conserved tyrosine on the other side (Hedstrom 2009). Although not all bacterial IMPDHs are sensitive to the inhibitors, those with an alanine residue (Ala165) and a tyrosine residue (Tyr351) are sensitive (Hedstrom 2009). Thus, we can use presence or absence of these residues to predict sensitivity to the inhibitors. A number of pathogenic bacteria are susceptible to these inhibitors, such as B. anthracis, the bacteria responsible for anthrax, and M. tuberculosis, the bacteria responsible for tuberculosis (Figure 7).
Figure 7: Many Bacterial IMPDHs are Susceptible to \textit{Cp}IMPDH Inhibitors
Figure adapted from (Hedstrom et al. 2011).

However, despite the fact that the inhibitor binding sites are very similar across sensitive orthologs, inhibitor sensitivity varies widely (Mandapati et al. 2014). Importantly, the enzymes that have the most similar sequences do not necessarily have the most similar inhibitor sensitivity (Figure 8). This suggests that certain residues contribute to selectivity in inhibitor sensitivity more than others.
Figure 8: Phylogenetic and Functional Trees for Several Bacterial IMPDHs
(A) Phylogenetic tree of sequence similarity and (B) functional tree of inhibition sensitivity for 9 species of bacteria. Ab = Acinetobacter baumannii IMPDH; Pa = Pseudomonas aeruginosa IMPDH; Ft = Francisella tularensis IMPDH; Bm = Burkholderia mallei IMPDH; Cp = Cryptosporidium parvum IMPDH; Lm = Listeria monocytogenes IMPDH; Sa = Staphylococcus aureus IMPDH; Sp = Streptococcus pyogenes IMPDH; Ba = Bacillus anthracis IMPDH. Note that the trees are not the same: that is, overall sequence similarity cannot explain inhibition sensitivity.

VI. Determining Selectivity in Bacterial IMPDH Inhibitor Sensitivity

As previously mentioned, we know that the dynamic flap of IMPDHs play a role in the selectivity of inhibitor sensitivity. For example, it has been shown that changing the length of the dynamic flap in the IMPDH from Acinetobacter baumannii (AbIMPDH) affects its affinity to some inhibitors (unpublished data). Aside from Arg404 and Tyr405, the highly-conserved hydrolysis dyad that plays an important mechanistic role, the amino acid composition of the flap is variable across many bacterial IMPDH orthologs (Figure 9). We hypothesize that the amino acid composition of this flap is at least partially responsible for the fact that the sensitivity of various IMPDH orthologs to inhibitors varies tremendously, despite their highly conserved catalytic and inhibitor binding sites. Further, we propose that the mechanism responsible for this
phenomenon is described by one of two hypotheses: (1) the flap and inhibitors compete for binding in the cofactor site, but do not interact with each other; or (2) the flap and inhibitors do interact with each other. Each of these models comes with specific, testable predictions, which will be described in more detail.

Figure 9: Multiple Sequence Alignments of IMPDH Flap
Alignment of IMPDH flap in 17 bacterial species generated using the clustal program. Ba = Bacillus anthracis IMPDH; Cld = Clostridium difficile IMPDH; Bb = Borrelia burgdorferi IMPDH; Mtb2 = Mycobacterium tuberculosis IMPDH; Cb = Coxiella burnetti IMPDH; Kp = Klebsiella pneumoniae IMPDH; Ft = Francisella tularensis IMPDH; Bm = Burkholderia mallei IMPDH; Ab = Acinetobacter baumannii IMPDH; Pa = Pseudomonas aeruginosa IMPDH; Clp = Clostridium perfringens IMPDH; Sa = Staphylococcus aureus IMPDH; Sp = Streptococcus pyogenes IMPDH; Lm = Listeria monocytogenes IMPDH; Cp = Cryptosporidium parvum IMPDH; Cj = Campylobacter jejuni IMPDH; Hp = Helicobacter pylori IMPDH. The hydrolysis dyad (R464 and Y465, with this numbering system) is conserved across all 17 bacterial species.

Investigating how the composition of the dynamic flap affects inhibitor sensitivity and direct testing of these hypotheses can be accomplished by creating a series of IMPDH mutants
with mutations in the flap region and comparing their inhibition sensitivity profiles to those of
the wild type IMPDH. If Hypothesis #1 is correct and the flap does not interact with the
inhibitors, then we expect to see a constant fold change in the same direction in IC$_{50}$ between
wild type and mutant IMPDH for every inhibitor tested as a result of a change in the equilibrium
between open-flap and closed-flap conformations. However, if Hypothesis #2 is correct and the
flap does interact with the inhibitors, then we predict that the fold change in IC$_{50}$ between wild
type and mutant IMPDH will not be constant between inhibitors, as different inhibitors will have
different interactions with the flap and thus cause the IC$_{50}$ to change by a different factor. If we
create a series of IMPDHs with point mutations in this dynamic flap, then we can pinpoint any
interactions we identify to a particular residue.

We selected the IMPDH from *Bacillus anthracis* (BaIMPDH) as our model system
because this ortholog is well-characterized and has been studied extensively. Specifically, we
worked with BaIMPDH$_{DL}$, a strain in which the CBS domain has been removed and replaced
with two glycine residues (Figure 3) (Makowska-Grzyska *et al.* 2015). We chose this because the
pre-steady state kinetics of this protein have been previously characterized (Wei *et al.* 2016). The
entire nucleotide and amino acid sequence of BaIMPDH$_{DL}$ is shown in Figure 10. The 6x-His-
tag, SGG connector that replaced the CBS domain, and the flap region are highlighted. We will
hereafter refer to this BaIMPDH$_{DL}$ strain as simply BaIMPDH.
Figure 10: BaIMPDHAL Sequence
Nucleotide and amino acid sequence of BaIMPDH. 6x-His-tag, which was incorporated to enable purification on a nickel column, is under the blue bar. The residues that comprise the dynamic flap are under the green bar. The nucleotides and amino acids that we altered in our point mutants are red.
Materials and Methods:

Mutant Selection:

Analysis of crystal structures of \textit{Ba}IMPDH in both the closed (3TSB) and open (4QM1) conformations using Chimera software informed mutation selection (Figure 11). The six point mutations that we designed are highlighted in Table 1. The positions of the mutations are the three residues immediately before and the three residues immediately after the conserved hydrolysis dyad. Mutations were designed to alter either the size, charge, hydrogen-bonding capacity, or hydrophobicity of the original residue in order to disrupt any potential interactions that may exist between the flap and the inhibitor or the cofactor site of the enzyme. The mutants are S401T, K402Q, D403N, F406E, Q407E, and E408K.

\textbf{Figure 11: Closed-Flap and Open-Flap Crystal Structures of \textit{Ba}IMPDH} (A) Ribbon structure of \textit{Ba}IMPDH with disordered open flap (light blue) complexed with inhibitor D67 (red) (4QM1) and aligned with apoenzyme with closed flap (tan) (3TSB). The black arrow is pointing to the the location of the disordered flap (dotted line). (B) Another view closed-flap IMPDH (3TSB). Residues in the flap region are colored orange.
Site-Directed Mutagenesis (SDM):

QuikChange polymerase chain reaction (PCR) was performed in order to incorporate the selected mutations into the guaB gene. The B. anthracis guaB gene in a pMCSG7 vector was used as the template in PCR. In addition to the gene that encodes wild type BalMPDH, the bacterial expression vector had an ampicillin resistance gene (as a selective marker for colonies that have been successfully transformed), a T7 promoter, T7 term terminator and LacI gene (in order to induce protein overexpression), and an N-terminal 6x-His-tag (in order to enable protein purification on a nickel column) (construction of the vector is described in Makowska-Grzyska et al. 2015). Overlapping mutant primers were designed and ordered from Integrated DNA Technologies (IDT) (Table 1). PCR was carried out in the Bio-Rad S1000 Thermal Cycler using the pMCSG7-guaB template, mutant primers, Phusion Hot Start DNA polymerase, dNTPs, phosphate buffer pH 8.0, DMSO, and nuclease free water. Phusion HS polymerase was added immediately before cycling began. PCR cycling conditions are outlined in Table S1.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Sequence</th>
<th>( T_m (^\circ \text{C}) )</th>
</tr>
</thead>
</table>
| S401T   | 5′ GCCATGGAAAAAGAACTAAAGATCG 3′  
          5′ GGAAGTAAGATCTTTAAGGCTTCCCTTTTCC 3′ | 56.3  
          55.9 |
| K402Q   | 5′ GGAAGTCAAGATCGTTACTTCAAG 3′  
          5′ GAAGTAAGATCTTTGACTTCCCTTTTCC 3′ | 55.4  
          56.0 |
| D403N   | 5′ GGAAGTAAATCGTACTTCCAAAGAAGG 3′  
          5′ GTAACGATTITTACTTCTTTTCCATCGC 3′ | 56.1  
          57.0 |
| F406E   | 5′ AAAAGGAAGTAAAGATCGTTACTGAGCAAGAAGGAAAATAAAAACTTG 3′  
          5′ CAAGTTTTTTATTTTCTTCCGACTGTAACGATTTACTTTCCCTTTT 3′ | 62.2 |
| Q407E   | 5′ AAAGGAAAGTAAGATCGTTACTTCAGAAGAAGGAAAATAAAAACTTGTTTCCGAAAGTAACGATTTACTTTCCCTTTT 3′  
          5′ GAACAAGTTTTTTATTTTCTTCCGACTGTAACGATTTACTTTCCCTTTT 3′ | 61.9 |
| E408K   | 5′ AGGAAGTAAAGATCGTTACTTCAAAAAGGAAAATAAAAACTTGTTTCCAGA 3′  
          5′ TCTGGAACAAAGTTTTATTTTCTTCCGACTGTAACGATTTACTTTCCCTT 3′ | 63.3 |

Table 1: Primer Sequences for BalMPDH Mutants
Primer sequences for BalMPDH mutants as well as the corresponding primer melting temperature, as calculated by IDT. For each mutant, the forward (top) and reverse (bottom) primer sequences are shown. The nucleotide mutations are in red.
The amplification product was digested with the restriction endonuclease DpnI to degrade all template DNA, leaving only mutant plasmid. XL10-Gold competent *E. coli* cells were transformed with the digested amplification product and incubated shaking with 1 mL Luria Broth (LB) for 1 hour at 37°C before plating on LB-amp plates (100 µg/mL ampicillin) and incubation at 37°C overnight. Plasmid DNA was extracted and purified from individual colonies with mini-prep procedure using the AxyPrep Mag Plasmid Protocol mini-prep kit. Plasmid concentration and purity (approximated by A$_{260}$/A$_{280}$) were assessed using the ThermoFisher NanoDrop 2000 to ensure that samples were appropriate to send for sequencing. Mutant sequences were confirmed by sequencing (Genewiz).

**Expression and Purification of Mutant BaIMPDHs:**

Mutant plasmids were transformed into BL21ΔguaB competent *E. coli* cells, which lack endogenous IMPDH due to deletion of the guaB gene. 1 mL of LB was added and the mixture was allowed to shake for 1 hour at 37°C. This was plated on LB-amp plates and incubated at 37°C overnight.

Starter cultures were created from a single colony of each mutant in 20 mL LB-amp solution (100 µg/mL ampicillin) and incubated at 37°C overnight. These cultures were diluted into 2 L LB-amp solution and allowed to shake at 37°C for about three hours. OD$_{600}$ was monitored using the Thermo Fisher Spectronic 200 and IMPDH overexpression was induced by adding IPTG to a final concentration of 0.5 mM when the OD reading was between 0.6 and 0.8. Cultures were allowed to shake at 20°C for 24 hours.

Cells were harvested by centrifugation at 4°C at 4,900 x g for 15 minutes. Supernatant was discarded and pellet was re-suspended in minimal lysis buffer (10 mM imidazole, 50 mM
HEPES pH 8.0, 500 mM KCl, 10% glycerol, 5 mM 2-mercaptoethanol, and 100 µM IMP). Cells were lysed by sonication with the Fisher Sonic Dismembrator for 5 cycles of 45-second sonication and 2-minute rest at 4°C. After centrifugation at 4°C at 9,000 x g for 30 minutes, supernatant was carefully aspirated and pellet was discarded.

IMPDH was further purified using nickel affinity chromatography. Nickel-NTA beads were equilibrated by washing with water and buffer #1 (10 mM imidazole, 50 mM HEPES pH 8.0, 500 mM KCl, 10% glycerol, 5 mM 2-mercaptoethanol, and 100 µM IMP). Cell lysate was incubated with approximately 5 mL nickel-NTA beads on the rotator overnight at 4°C to allow His-tagged protein to bind nickel beads. The column was washed with 250 mL of buffer #1, 200 mL of buffer #2 (same as buffer #1, but 30 mM imidazole), and 100 mL of buffer #3 (same as buffer #1, but 70 mM imidazole). IMPDH was eluted from the column with 50 mL of buffer #4 (same as buffer #1, but 200 mM imidazole) and was collected in 2 mL fractions.

SDS-PAGE was used to identify which fractions contained IMPDH and assess fraction purity, and kinetic analysis (procedure described in the following section) was used to compare the relative activity of the fractions (results in Supporting Information). Fractions with high IMPDH content, high purity, and high activity were combined and concentrated in the Millipore Amicon Ultra-4 30K centrifugal filtration device by spinning at 4°C at 3,000 x g for about 20 minutes. Excess imidazole was removed through three rounds of dialysis in 4 liters of fresh IMPDH buffer (50 mM Tris pH 8, 100 mM KCl, 3 mM EDTA, and 1 mM DTT) at 4°C for 12 hours per round.

Protein concentration was measured with the Bradford Assay using IgG as a standard and dividing by 2.6, and was confirmed using the ThermoFisher NanoDrop 2000. Protein was flash-frozen in aliquots using liquid nitrogen and stored at -80°C for future use.
Steady-State Kinetics:

Mutants K402Q BalIMPDH and E408K BalIMPDH were selected for more in-depth analysis because of their superior enzymatic activity based on preliminary kinetic screens (data not shown).

The rate of the reaction, which corresponds to NADH production, was measured by monitoring change in absorbance at 340 nm using a Shimadzu UV-2600 spectrophotometer. All assays were performed in IMPDH buffer (50 mM Tris pH 8, 100 mM KCl, 3 mM EDTA, and 1 mM DTT) at 25°C. To characterize the dependence of IMPDH activity on NAD\(^+\), we varied the concentration of NAD\(^+\) (12.5 µM to 5 mM) in the presence of fixed enzyme (75 nM) and fixed, saturating IMP (1 mM). Because high concentrations of NAD\(^+\) have been shown to inhibit IMPDHs, initial velocity data were fit to the uncompetitive substrate inhibition equation (Equation 1) using DynaFit software and the kinetic parameters \(K_m\) with respect to NAD\(^+\) and \(K_{ii}\) were calculated (DynaFit script adapted from Wei et al. 2016). Assays that investigated the dependence of IMPDH activity on IMP used varying concentrations of IMP (12.5 µM to 5 mM), fixed enzyme concentration (75 nM) and fixed NAD\(^+\) concentration equal to 1.4 x \(K_m\) with respect to NAD\(^+\), which was experimentally determined to be optimally between the \(K_m\) and \(K_{ii}\). The data was fit to the Michaelis-Menten equation (Equation 2) using SigmaPlot software and the kinetic parameters \(K_m\) with respect to IMP, \(V_{max}\), and \(k_{cat}\) were calculated.

\[
\nu = \frac{V_{max} [S]}{K_m + [S]} \quad \text{(Equation 1)}
\]

\[
\nu = \frac{V_{max} [S]}{K_m + [S]} \quad \text{(Equation 2)}
\]
IC$_{50}$ Analysis:

Evaluation of inhibitor sensitivity was accomplished by monitoring the production of NADH by fluorescence in the presence of varying inhibitor concentrations. We monitored fluorescence at 445 nm with the Synergy 4 BioTek plate reader machine for 10 minutes at 25°C. We set up the assay in a 96-well assay plate. Each well had IMPDH buffer (100 mM Tris pH 8, 50 mM KCl, 3 mM EDTA, and 1 mM DTT), a concentration of IMP equal to 5 x $K_m$, a concentration of NAD equal to 1.4 x $K_m$. For each row, several inhibitor concentrations were tested. Inhibitors were dissolved in DMSO. Additionally, 10 nM enzyme was used for WT and K402Q and 50 nM enzyme was used for E408K. Data was analyzed with the Gen5 program. Data reduction was completed such that only points 1-6 were considered. Structures of the inhibitors tested are shown in Figure 12.
Figure 12: Inhibitor Structures
Structures of the inhibitors (A110, C91, D41, D67, P19, P24, Q11, and Q67) tested against WT BtIMPDH, K402Q BtIMPDH, and E408K BtIMPDH.
Results:

Expression and Purification of Mutant *Ba*IMPDHs:

Sequencing confirmed that site-directed mutagenesis worked and the selected mutations were successfully incorporated into each plasmid without any spontaneous mutations.

Preliminary expression and kinetic analysis confirmed that all lysates containing mutant enzymes produce NADH in a reaction that requires IMP (data not shown). This suggests that despite the drastic mutations, the enzymes all still have activity. For this reason, large-scale expression was carried out for all mutants.

SDS-PAGE gels from this larger scale expression confirmed that induction worked for each mutant, as there were thick bands at the molecular weight of IMPDH in all of the lanes after induction. Additionally, the gels showed that purification was relatively successful, as there weren’t many contaminating bands in the elution fractions. Furthermore, kinetic analysis done at this point showed that all of the fractions that contained IMPDH were active (Figure S1-6).

However, after concentration and dialysis, there was no activity in the S410T, D403N, F406E, or Q407E mutants, even at very high enzyme concentrations (data not shown). We hypothesize that there was a problem with the dialysis buffer and that the structure of the protein may have been compromised during this step, rendering it inactive. It is likely that too little DTT caused oxidation of the catalytic cysteine. However, expression and purification of K402Q and E408K were successful.
Kinetic Parameters of *Ba*IMPDH Mutants are Similar to Those of Wild Type *Ba*IMPDH:

Kinetic parameters with respect to NAD$^+$ were determined by measuring initial reaction rates at the constant, saturating concentration of 1 mM IMP and enzyme concentration of 75 nM for varying concentrations of NAD$^+$ for one replicate each. Reaction velocity was plotted against concentration of NAD$^+$ and the data was analyzed with the DynaFit program as in [Wei et al. 2016](#). The data was fit to single-substrate Michaelis-Menten kinetic curve that considers the uncompetitive inhibition by NAD$^+$ at high concentrations (Equation 1). Kinetic parameters $K_m$ and $K_{ii}$ were calculated by DynaFit, as well as $R^2$ values, which indicate how well the data fits the model. The results are summarized graphically in Figure 13 and numerically in Table 2.

Kinetic parameters with respect to IMP were determined by measuring initial reaction rates at a concentration of NAD$^+$ equal to $1.4 \times K_m$ for each enzyme and enzyme concentrations of 75 nM for varying concentrations of IMP for one replicate each. $1.4 \times K_m$ was chosen as it allowed for peak activity with minimal inhibition and to be consistent for each enzyme. Reaction velocity was graphed against concentration of IMP and the data was analyzed with the SigmaPlot program. The data was fit to single-substrate Michaelis-Menten kinetic curve (Equation 2). Kinetic parameters $K_m$ and $V_{max}$ were calculated by SigmaPlot, as well as $R^2$ values, which indicate how well the data fits the model. The results are summarized graphically in Figure 14 and numerically in Table 2.

Turnover number, $k_{cat}$, was calculated based on data from Activity vs. [IMP] trials, but it could have been calculated from the trials that varied [NAD$^+$] as well. This was chosen arbitrarily. $V_{max}$ was calculated by SigmaPlot and $k_{cat}$ was calculated as $k_{cat} = V_{max}/[E]$. Results are summarized in Table 2.
Assays were performed in IMPDH buffer (50 mM Tris pH 8, 100 mM KCl, 3 mM EDTA, and 1 mM DTT) with 1 mM IMP (saturating) and 75 nM enzyme at 25°C. Plots and data fitting were produced with DynaFit. All three enzymes exhibit the characteristic inhibition of IMPDHs at high concentrations of NAD⁺.
Figure 14: Enzyme Activity vs. [IMP]
Enzyme activity vs. [IMP] for WT BalMPDH, K402Q BalMPDH, and E408K BalMPDH. Assays were performed in IMPDH buffer (50 mM Tris pH 8, 100 mM KCl, 3 mM EDTA, and 1 mM DTT) with 490µM NAD⁺ for WT, 1150 µM NAD⁺ for K402Q and 500 µM NAD⁺ for E408K, and 75 nM enzyme at 25°C. Plots and data fitting were produced with SigmaPlot.
Table 2: Kinetic Parameters for Wild Type and Mutant *Bal*IMPDHs

<table>
<thead>
<tr>
<th></th>
<th>WT <em>Bal</em>IMPDH</th>
<th>K402Q <em>Bal</em>IMPDH</th>
<th>E408K <em>Bal</em>IMPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (NAD$^+$) (μM)</td>
<td>351 ± 7 (0.996)</td>
<td>810 ± 18 (0.994)</td>
<td>358 ± 19 (0.970)</td>
</tr>
<tr>
<td>$K_{ii}$ (NAD$^+$) (μM)</td>
<td>1420 ± 30 (0.996)</td>
<td>1500 ± 30 (0.994)</td>
<td>969 ± 52 (0.970)</td>
</tr>
<tr>
<td>$K_m$ (IMP) (μM)</td>
<td>107 ± 5 (0.997)</td>
<td>123 ± 8 (0.995)</td>
<td>79 ± 11 (0.971)</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>1.57 ± 0.02 (0.997)</td>
<td>2.50 ± 0.05 (0.995)</td>
<td>0.57 ± 0.02 (0.971)</td>
</tr>
</tbody>
</table>

As expected, we saw characteristic inhibition of IMPDHs at high concentrations of NAD$^+$. Although there is a bit of variation in the measured $K_m$’s for the different enzymes, they are within a factor of 2.5 with respect to NAD$^+$ and within a factor of 2 with respect to IMP. Again, although there is slight variation in $k_{cat}$ between the different enzymes, these values are within an order of magnitude. Any discrepancies may be due to changes caused by the mutation, or due to inaccuracy in measuring enzyme concentration. Since all of the kinetic parameters of K402Q and E408K are similar to those of wild type, we believe that these mutants are useful to test inhibitor effects.
IC\textsubscript{50} Fold Changes Were Consistent for Some Inhibitors and Different for Others for Each Mutant Enzyme:

IC\textsubscript{50} analysis was done for two replicates per combination of inhibitor and enzyme at the same time on the same day. Enzyme and inhibitor were added together first and substrates were added last, so as to give the enzyme a chance to interact with the inhibitor before measurements began. The slope was taken from the initial rates of fluorescence over time, averaged, and was normalized on a scale from 0 to 1. This was plotted against the log of the inhibitor concentration (Figure 15). Data was fit to the IC\textsubscript{50} model using Sigma Plot. The tight-binding model was used if the predicted IC\textsubscript{50} was lower than the enzyme concentration. IC\textsubscript{50} values are shown in Table 3 and fold changes between the IC\textsubscript{50} values of the mutant enzymes and the wild type enzyme are shown in Table 4.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>BalMPDH IC\textsubscript{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>A110</td>
<td>68.4 ± 8.6 (0.987)</td>
</tr>
<tr>
<td>C91</td>
<td>71.2 ± 9.1 (0.984)</td>
</tr>
<tr>
<td>D41</td>
<td>106.9 ± 11.1 (0.989)</td>
</tr>
<tr>
<td>D67</td>
<td>689.1 ± 78.4 (0.981)</td>
</tr>
<tr>
<td>P19</td>
<td>4.5 ± 1.5 (0.989)</td>
</tr>
<tr>
<td>P24</td>
<td>53.6 ± 5.0 (0.992)</td>
</tr>
<tr>
<td>Q11</td>
<td>199.6 ± 13.8 (0.995)</td>
</tr>
<tr>
<td>Q67</td>
<td>8.6 ± 1.5 (0.994)</td>
</tr>
</tbody>
</table>

Table 3: IC\textsubscript{50} values for WT, K402Q, and E408K for several inhibitors. IC\textsubscript{50} ± standard error, as calculated in SigmaPlot. R\textsuperscript{2} for fit of data is shown in parentheses.
Figure 15: IC₅₀ curves for WT, K402Q, and E408K BαIMPDH
IC₅₀ curves for WT (black), K402Q (blue), and E408K (red) BαIMPDH and (A) A110, (B) C91, (C) D41, (D) D67, (E) P19, (F) P24, (G) Q11, and (H) Q67. X-axis is inhibitor concentration on a log scale and Y-axis is relative activity, normalized to a scale of 0 to 1.
Table 4: Fold Change between WT and Mutant Ba1MPDH IC$_{50}$ values
Fold changes were calculated by dividing mutant IC$_{50}$ by WT IC$_{50}$ for each inhibitor.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mutant IC$_{50}$ Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K402Q</td>
</tr>
<tr>
<td>A110</td>
<td>0.34</td>
</tr>
<tr>
<td>C91</td>
<td>0.34</td>
</tr>
<tr>
<td>D41</td>
<td>0.32</td>
</tr>
<tr>
<td>D67</td>
<td>0.30</td>
</tr>
<tr>
<td>P19</td>
<td>0.10</td>
</tr>
<tr>
<td>P24</td>
<td>0.38</td>
</tr>
<tr>
<td>Q11</td>
<td>0.32</td>
</tr>
<tr>
<td>Q67</td>
<td>0.09</td>
</tr>
</tbody>
</table>

For mutant K402Q, all eight inhibitors showed a decrease in IC$_{50}$, representative of tighter binding. The IC$_{50}$ for six of the eight inhibitors tested (A110, C91, D41, D67, P24, and Q11) decreased by the same factor. This result is consistent with the hypothesis that changing the amino acid composition of the flap would alter the conformational equilibrium between the open-flap and closed-flap conformations. Interestingly, the two inhibitors with IC$_{50}$ values that did not differ by a factor of about 0.3 (P19 and Q67) did differ by the same factor as each other: about 0.1.

Similarly, the IC$_{50}$ values of all eight inhibitors decreased with mutant E408K. While the pattern of inhibition is not as obvious as it was for K402Q, the IC$_{50}$ fold changes segregate into distinct groups: inhibitors A110, P19, and P24 have IC$_{50}$ fold changes around 0.04, and inhibitors C91, D41, D67, Q11, and Q67 have IC$_{50}$ fold changes around 0.15.
Discussion:

IMPDH is an attractive target for antimicrobial drugs because prokaryotic IMPDHs have cofactor binding sites that are highly diverged from eukaryotic IMPDHs, and because many bacteria have poor salvage pathways for guanine nucleotide biosynthesis. While several hundred inhibitors of bacterial IMPDHs have been developed, different bacterial IMPDH orthologs have very different inhibitor sensitivity profiles, despite little variation in inhibitor binding sites. We propose that the amino acid composition of a mobile flap region controls inhibitor sensitivity. We hypothesize that if the flap does not interact directly with inhibitors but does change the equilibrium between the closed-flap and open-flap conformations, then all inhibitors will show a uniform decrease in IC\textsubscript{50} fold change for IMPDH mutants that were designed to disrupt evolved interactions. However, if inhibitors do interact with the flap, then the IC\textsubscript{50} fold changes will not be the same for all inhibitors of a mutant enzyme.

It is initially surprising that the IC\textsubscript{50} values decreased significantly for both mutants, yet the value of k\textsubscript{cat} remained relatively the same. However, this observation can be explained if the closed flap conformation dominates. For example, if in WT \textit{Bd}IMPDH, 99% of the enzyme is in the closed conformation and only 1% is in the open conformation, then in a mutant in which the IC\textsubscript{50} decreases by a factor of 3 (the open conformation triples, from 1% to 3%), then the closed conformation only experiences a decrease from 99% to 97%, which does not drastically change the equilibrium between the open and closed state. This scenario can explain why the IC\textsubscript{50} and k\textsubscript{cat} values do not intuitively seem to agree.

Note that for all of the inhibitors except for P19 and Q67, the IC\textsubscript{50} for wild type is about 3-fold higher than for K402Q. This suggests that the K402Q mutation changes the equilibrium between closed flap and open flap conformations for these inhibitors. However, the fact that P19
and Q67 differ by different factors suggests that these inhibitors interact directly with the flap in a manner different than the other inhibitors.

While a mutation that only changes the conformational equilibrium between the closed and open flap conformations will change the IC$_{50}$ values of all inhibitors by the same factor and in the same direction, this is not the case if inhibitors interact with the flap. If inhibitors interact with the flap, then the fold change in IC$_{50}$ between wild type $Ba$MPDH and a flap mutant would be dependent on the inhibitor and could, in theory, go in either direction. We observed in K402Q that even after accounting for the decrease in IC$_{50}$ based on a change in the conformational equilibrium, the IC$_{50}$ values for the ones that don’t fit the pattern decrease even further, that is, the inhibitor binds even tighter.

One explanation for this is that our K402Q mutation introduced a new favorable or stabilizing interaction with these inhibitors, causing them to bind better. Although this is unlikely, we cannot rule out this possibility.

A stronger hypothesis is that there are many “open” conformation of the flap and all of these conformations are in equilibrium with one another (Figure 16A). We hypothesize that inhibitors preferentially bind to certain “open” conformations. We propose that the amino acid composition of the flap changes the equilibrium between open conformations, which would, in turn, change the proportion of enzyme in the appropriate binding conformation for particular inhibitors.
Figure 16: Hypothesis #2: Dynamic Flap Interacts with Inhibitors and Influences Sensitivity

(A) Model that shows the equilibrium between open flap and closed flap conformation \( (K_c) \), the equilibria between several open flap conformations, and the fact that different inhibitors may bind preferentially to certain open conformations. The dark green circle is covalently bound XMP. The pink, purple, and yellow shapes are different inhibitors that bind preferentially to open conformations #1, #2, and #3, respectively. (B) Hypothetical explanation of the data we observed based on the model. In this case, the pink inhibitor represents A110, C91, D41, D67, P24, and Q11, while the purple inhibitor represents P19 and Q67.
We can illustrate this principle hypothetically (Figure 16B). Let Flap #1 be the wild type enzyme and Flap #2 be the K402Q mutant. For simplicity, let us also assume that those inhibitors with IC$_{50}$ fold change of about 0.3 bind only to open conformation #1 and those with IC$_{50}$ fold change of about 0.1 bind only to open conformation #2. Thus, the fold changes in this hypothetical situation match the data we observed. If a change in the equilibrium between the various open conformations was the only thing happening, then the scenario above could explain the data for K402Q.

Importantly, the model, as illustrated above, predicts that inhibitors that bind preferentially to open conformation #3 will see an increase in IC$_{50}$, that is, they will not bind as tightly because proportion of enzyme in open conformation #3 decreases. We can reconcile this prediction with our data with several explanations. For example, maybe inhibitors don’t bind to open conformation #3. Alternatively, maybe some inhibitors do, but we didn’t test any of them. This leaves work to be done.

The best explanation for the data combines Hypothesis #1 and Hypothesis #2. Our working model is that residues in the flap both influence conformational equilibrium and interact with inhibitors. Again, we can use a hypothetical situation to model the data (Figure 17). The hypothetical numbers in this scenario are such that the total amount of enzyme in the open conformation triples, but the factors by which certain open conformations change is different. In this model, all of the inhibitors that exhibited a fold change of about 0.3 bind equally well to all open conformations, but those that exhibited a fold change of about 0.1 bind only to open conformation #1. In this way, the amount of open conformation #1 increases by a factor of 10 (so IC$_{50}$ would decrease by a factor of 10), so the effects of the conformational equilibrium change $K_c$ are compounded. According to this model, the effects of the change in conformational
equilibrium are so strong that no inhibitors would experience an increase in IC$_{50}$, as there are no open conformations that have a decreased population as a result of the flap mutation.

<table>
<thead>
<tr>
<th></th>
<th>Closed</th>
<th>Open #1</th>
<th>Open #2</th>
<th>Total Open</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT BaIMPDH</td>
<td>99%</td>
<td>0.2%</td>
<td>0.8%</td>
<td>1%</td>
</tr>
<tr>
<td>K402Q BaIMPDH</td>
<td>97%</td>
<td>2.0%</td>
<td>1.0%</td>
<td>3%</td>
</tr>
</tbody>
</table>

**Figure 17: Model Based on Combined Hypotheses**

(A) Model that describes (B) mathematically matches the data observed for K402Q. This model takes into account that residues in the flap both influence conformational equilibrium and interact with inhibitors. A more detailed description is in the text.

The existence of multiple open conformations is also supported by the crystallography data (Figure 18). Although most of the dynamic flap is disordered in crystal structures, the hinge regions on either end are discernible. When the crystal structures of BaIMPDH complexed with inhibitors from five different classes are overlayed, it is clear that the flaps begin to exist in different locations. Since the only difference between these structures is the inhibitor, so it is likely that identity of the inhibitor affects the conformation of the flap.
**Figure 18: Open-Flap Conformations are Different with Different Inhibitors**

All crystal structures are *BalMPDH* with different inhibitors. Orange is with inhibitor D67 (PDB: 4QM1), purple is with inhibitor C91 (PDB: 4MY9), blue is with inhibitor A110 (PDB: 4MYA), pink is with inhibitor Q21 (PDB: 4MY8), and green is with inhibitor P200 (PDB: 5UUZ). The dotted lines are the places where the flap is disordered. The arrows indicate where the flaps diverge for each crystal structure, suggesting the existence of multiple open conformations.

We’d like to be able to identify a pattern between the structure of the inhibitors and the fold changes they exhibit. Thus far, we see that the inhibitors in the P-series and Q-series experience the same IC$_{50}$ fold changes in E408K but not for K402Q. It is interesting that the inhibitors segregated in this manner. In order to elucidate any patterns, we will need to test more inhibitors against our mutants. We should select inhibitors that have similar structures in order to identify functional groups that may cause IC$_{50}$ fold change to be different, and thus interact with the flap.

Additionally, it would be worthwhile to re-express and purify S401T, D403N, F406E, and Q407E. Initial screenings indicate that these mutants are active, so activity must have been lost somewhere along the purification process, likely during dialysis, if too little DTT caused the catalytic cysteine to oxidize. Testing our inhibitors against these four other mutants will provide more evidence in support of our hypothesis.
Furthermore, it would be interesting to create several mutants with different point mutations at the same residue. This would allow us to see if all inhibitors experience the same $IC_{50}$ fold changes for all point mutants at a single position, or if the identity of the mutated residue makes a difference. These experiments may allow us to determine the relative importance of each residue in the flap. Finally, we would like to perform stopped-flow experiments to characterize the pre-steady state conditions of the mechanism.

The search for novel antimicrobial drugs is more imperative now than ever before, and IMPDH is a promising target. Understanding how the composition of the dynamic flap influences inhibitor sensitivity will aide drug design. Our experiments have provided us with initial evidence that the composition of the flap influences inhibitor sensitivity by both altering conformational equilibrium and interacting directly with some inhibitors. This area of research merits additional study.
Supplemental Information

<table>
<thead>
<tr>
<th>Step</th>
<th>Sample Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98.0°C for 1:00</td>
</tr>
<tr>
<td>2</td>
<td>98.0°C for 0:25</td>
</tr>
<tr>
<td>3</td>
<td>65.2°C for 0:30</td>
</tr>
<tr>
<td>4</td>
<td>72.0°C for 4:30</td>
</tr>
<tr>
<td>5</td>
<td>Go to step 2 (49 repeats)</td>
</tr>
<tr>
<td>6</td>
<td>72.0°C for 2:00</td>
</tr>
<tr>
<td>7</td>
<td>4.0°C hold</td>
</tr>
</tbody>
</table>

**Table S1: PCR Cycling conditions**
Example PCR cycling conditions for mutant 406. Steps 1 and 2 are denaturation. Step 3 is annealing of primers to template. Annealing temperature was set to 3°C higher than the melting temperature as calculated by IDT. Step 4 is elongation. Elongation time was calculated as 30 seconds for every 1,000 base pairs (pGMCSG7-GuaB is about 7,000 bps) plus an extra minute to ensure elongation is complete. Step 5 allows for linear amplification of mutant plasmid.
**S401T BaIMPDH**

**Figure S1**: S401T BaIMPDH SDS-PAGE Gel and Activity Analysis

SDS-PAGE gel and activity analysis for elution fractions from nickel affinity chromatography column for S401T BaIMPDH. Based on IMPDH content, purity, and activity, fractions 1-5 were combined.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>lysate</td>
<td>0.04614</td>
</tr>
<tr>
<td>load</td>
<td>0.00101</td>
</tr>
<tr>
<td>10 mM wash</td>
<td>0.00052</td>
</tr>
<tr>
<td>30 mM wash</td>
<td>0.00029</td>
</tr>
<tr>
<td>fraction 1</td>
<td>0.00593</td>
</tr>
<tr>
<td>fraction 2</td>
<td>0.00856</td>
</tr>
<tr>
<td>fraction 3</td>
<td>0.00671</td>
</tr>
<tr>
<td>fraction 4</td>
<td>0.01025</td>
</tr>
<tr>
<td>fraction 5</td>
<td>0.01446</td>
</tr>
<tr>
<td>fraction 6</td>
<td>0.01246</td>
</tr>
<tr>
<td>fraction 7</td>
<td>0.01709</td>
</tr>
<tr>
<td>fraction 8</td>
<td>0.0202</td>
</tr>
<tr>
<td>fraction 9</td>
<td>0.01331</td>
</tr>
<tr>
<td>fraction 10</td>
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</tr>
<tr>
<td>fraction 11</td>
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</tr>
<tr>
<td>fraction 12</td>
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</tr>
<tr>
<td>fraction 13</td>
<td>0.01322</td>
</tr>
<tr>
<td>fraction 14</td>
<td>0.01054</td>
</tr>
<tr>
<td>rest</td>
<td>0.00823</td>
</tr>
</tbody>
</table>
**Figure S2: K402Q BaIMPDH SDS-PAGE Gel and Activity Analysis**

SDS-PAGE gel and activity analysis for elution fractions from nickel affinity chromatography column for K402Q BaIMPDH. Based on IMPDH content, purity, and activity, fractions 1-5 were combined.
Figure S3: D403N *Ba*IMPDH SDS-PAGE Gel and Activity Analysis
SDS-PAGE gel and activity analysis for elution fractions from nickel affinity chromatography column for D403N *Ba*IMPDH. Based on IMPDH content, purity, and activity, fractions 1-5 were combined.
**F406E BaIMPDH**

**Figure S4: F406E BaIMPDH SDS-PAGE Gel and Activity Analysis**

SDS-PAGE gel and activity analysis for elution fractions from nickel affinity chromatography column for F406E BaIMPDH. Based on IMPDH content, purity, and activity, fractions 1-5 were combined.
Figure S5: Q407E BaIMPDH SDS-PAGE Gel and Activity Analysis
SDS-PAGE gel and activity analysis for elution fractions from nickel affinity chromatography column for Q407E BaIMPDH. Based on IMPDH content, purity, and activity, fractions 1-5 were combined.
**Figure S6: E408K BaIMPDH SDS-PAGE Gel and Activity Analysis**

SDS-PAGE gel and activity analysis for elution fractions from nickel affinity chromatography column for E408K BaIMPDH. Based on IMPDH content, purity, and activity, fractions 1-5 were combined.
Acknowledgements:

I would like to begin by expressing my deep gratitude for Joe Arciprete, without whom this project would not have been possible. Thank you for lifting the rotor, unscrewing bottle caps, and running approximately a million gels. Thank you also for your help with experimental design, for troubleshooting through problems when things didn’t go as planned, and for making my time in lab this year a truly pleasant experience.

I would also like to thank Masha Rosenberg for being my first mentor in the lab. The word “patient” doesn’t even begin to describe Masha. Masha demonstrated techniques (and re-demonstrated them when I forgot to take notes), showed me where everything in lab was, and always made herself available to answer each and every single one of my questions.

Thank you to the rest of the Hedstrom lab, particularly Devi Gollapalli, Ann Lawson, Minjia Zhang, and Runhan Yu. Each of you has played a significant role in my training throughout my time in the Hedstrom lab. The techniques you’ve taught, the critical thinking skills you’ve tested, and the advice you’ve given have made me a better scientist and will serve me well throughout the rest of my scientific career.

Also, I would like to thank the bio lab staff, Melissa Kosinski-Collins, Kene Piasta, and Lindsay Mehrmanesh, for the constant guidance and advice, for the pep-talks when I wasn’t feeling confident in myself, and for providing me with a home away from lab. Dr. KC has been a mentor to me since I was in high school and I am incredibly grateful for everything she’s done for me over the years.

Thank you to Kene Piasta, Lindsay Mehrmanesh, Emily Greenwald, and Sofia Lavrentyeva for listening to me practice my defense and for your insightful comments. Thank you also to Kene for review of this manuscript. Thank you to Sabrina McDonnell, Jessie Kirshner, and Anisha Khemlani for keeping me sane throughout this process.

Last but certainly not least, thank you to Dr. Liz Hedstrom for taking a chance on me. There’s so much I could say, but I’ll be brief and say that you are the kind of scientist, teacher, and mentor I hope to be one day. Thank you for everything.
References:


