Isolation and Characterization of Mycophenolic Acid-resistant Mutants of Inosine-5'-monophosphate Dehydrogenase*

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Mycophenolic acid (MPA) is a potent and specific inhibitor of mammalian inosine-monophosphate dehydrogenases (IMPDH); most microbial IMPDHs are not sensitive to MPA. MPA-resistant mutants of human IMPDH type II were isolated in order to identify the structural features that determine the species selectivity of MPA. Three mutant IMPDHs were identified with decreased affinity for MPA. The mutation of Gln277 → Arg causes a 9-fold decrease in the $K_m$ for MPA, and a 5–6-fold decrease in the $K_m$ for NAD, and a 3-fold decrease in $k_{cat}$ relative to wild type. The mutation of Ala462 → Thr causes a 3-fold decrease in the $K_m$ for MPA, a 2.5-fold increase in the $K_m$ for NAD, and a 1.5-fold increase in $k_{cat}$. The combination of these two mutations does not increase the $K_m$ for MPA, but does increase the $K_m$ for NAD 3-fold relative to Q277R and restores $k_{cat}$ to wild type levels. Q277R/A462T is the first human IMPDH mutant with increased $K_m$ for MPA and wild type activity. The third mutant IMPDH contains two mutations, Phe464 → Ser and Asp470 → Gly. $K_m$ for MPA is increased 3-fold in this mutant enzyme, and $K_m$ for IMP is also increased 3-fold, while the $K_m$ for NAD and $k_{cat}$ are unchanged. Thus increases in the $K_m$ for MPA do not correlate with changes in $K_m$ for either IMP or NAD, nor to changes in $k_{cat}$. All four of these mutations are in regions of the IMPDH that differ in mammalian and microbial enzymes, and thus can be structural determinants of MPA selectivity.

Inosine-monophosphate dehydrogenase (IMPDH)* catalyzes the oxidation of IMP to XMP with the concomitant conversion of NAD to NADH (Fig. 1). This reaction is the rate-limiting step in guanine nucleotide biosynthesis, and rapidly growing cells have increased levels of IMPDH (1). Inhibitors of IMPDH have antiproliferative activity and are used clinically for cancer, viral, and immunosuppressive chemotherapy (2–4). Moreover, differences in the properties of microbial and mammalian IMPDHs suggest that species-selective IMPDH inhibitors can be designed, which will be useful for anti-infective chemotherapy (5–7). Two human IMPDH isozymes exist; type I is constitutively expressed, while type II is expressed in rapidly proliferating cells (8–11). The IMPDH reaction involves attack of Cys311 (human type II numbering) at the 2-position of IMP, followed by expulsion of the hydride to NAD (Fig. 1). The resulting covalent E-XMP intermediate is subsequently hydrolyzed to XMP (12, 13).

Mycophenolic acid (MPA) is a potent and specific inhibitor of mammalian IMPDHs, and a MPA derivative, mycophenolate mofetil, is a promising immunosuppressive drug (14, 15). MPA affinity varies greatly among microbial and mammalian IMPDH, for example $K_m$ = 22 nM for human IMPDH, 500 nM for the Bacillus subtilis, 20 μM for the Escherichia coli and 14 μM for the Trichromonas foetus enzymes (16–18). MPA traps the E-XMP intermediate in mammalian IMPDHs (Fig. 1), and the crystal structure of the E-XMP-IMP complex of IMPDH from Chinese hamster has recently been solved (12, 19, 20). MPA stacks against E-XMP in the likely nicotinamide binding site, as predicted by multiple inhibitor studies (17). Of the residues that contact MPA, only Arg322 and Gln441 differ in mammalian and microbial IMPDHs; Arg322 is replaced by Lys, and Gln441 is replaced by Glu (21–23). While the mutation of Gln441 → Ala decreases MPA sensitivity by 20-fold, activity is also decreased 20-fold. Mutations at Arg322 have not been reported. Moreover, both B. subtilis and E. coli IMPDHs contain Lys322 and Glu441, although the $K_v$ values of MPA for these enzymes vary by 40-fold. Thus residues 322 and 441 cannot be the only structural determinants of MPA selectivity.

Random mutagenesis followed by selection for the ability to grow in the presence of MPA can identify mutations in IMPDH that confer MPA resistance. Selection for MPA resistance has previously been reported in both mammalian and parasite systems. In most cases MPA resistance resulted from increased expression of IMPDH, usually via gene amplification (24–26). Mutant IMPDHs with altered sensitivity to MPA have been reported in murine lymphoma and leukemia cells, although identity of these mutations and their effect on enzyme activity were not characterized (27, 28). A MPA-resistant neuroblastoma cell line has been isolated in which a mutant IMPDH is overexpressed by gene amplification (29). While this mutant IMPDH is less sensitive to MPA, it is also much less active than wild type. This MPA-resistant IMPDH contains two mutations: Thr333 → Ile and Ser351 → Tyr. Recent mutagenesis experi-

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1 The abbreviations used are: IMPDH, inosine-monophosphate dehydrogenase; MPA, mycophenolic acid; IPTG, isopropyl-1-thio-β-D-galactopyranoside; kb, kilobase pair(s); Mes, 4-morpholineethanesulfonic acid.

2 N. Benfield and L. Hedstrom, unpublished observations.
ments suggest that resistance results from the alteration of Thr<sup>333</sup> (20). This residue is strictly conserved in all IMPDHs sequenced to date, and therefore cannot be a determinant of species selectivity. In addition to these examples, MPA resistance can also result from alterations in purine salvage pathways or in enzymes that utilize guanine nucleotides and from inactivation of MPA via glucuronidation (30–32).

We have isolated and characterized MPA-resistant mutants of human type II IMPDH in order to explore the structural basis of the species selectivity of MPA. We have identified three mutant enzymes that have a decreased sensitivity to MPA. These enzymes are 3–8-fold less sensitive to MPA. Four substitutions are identified in regions of the protein that are conserved in mammalian IMPDHs, but different in microbial enzymes. Thus these residues may be structural determinants of MPA sensitivity. Interestingly, these substitutions are in residues that do not contact MPA.

**MATERIALS AND METHODS**

IMP, NAD, and mycophenolic acid were purchased from Sigma. Plasmid pHIMP containing human IMPDH type II cDNA was the generous gift of Dr. Frank Collart (8).

**Construction of an Expression System for Human IMPDH Type II**—The 1.5-kb Ncol/SacI fragment containing the IMPDH coding sequences from a partial digest of pHIMP was ligated to the Ncol/SacI fragment of the pBluescript II KS<sup>−</sup>“based vector pSt (33) (pBluescript II KS<sup>−</sup> from Stratagene). This construct (pStI) was used as a template for Kunkel mutagenesis (34). Four silent mutations were inserted into the IMPDH coding sequences, creating four unique restriction sites (pStI).

The following oligonucleotides were used (mismatched bases are underlined): Ncol at position 1, AAC AAA CAC CAT ATG GCC GAC TAC; XhoI at position 473, ATC ATC TCG AGC AGG GAC ATT ATG; KpnI at position 750, GCC ATT GGTT ACC CAT GAG GAC ATT; SacII at position 1232, AAG AAA TAC CGC GTG ATG GTT.

This construct was sequenced in its entirety to confirm that only the desired mutations were introduced. One difference from the published cDNA sequence was noted (8); we found that the sequence of bases 608–611 is GCA GGC, not CGC AGC as reported previously. As a consequence, residues 190 and 191 are Ala and Gly, respectively, rather than Arg and Ser. This sequence was also found in pHIMP, indicating that this discrepancy did not arise during the construction and mutagenesis of pStI. Our results agree with the cDNA sequence reported by Natsume and collaborators (34). Four silent mutations were inserted into the IMPDH coding sequences, creating four unique restriction sites (pStI).

**Construction of L30F, Q277R, and Q277R/A462T**—The plasmid pHIMA17, which expresses L30F IMPDH, was digested with XhoI and HindIII. The 1.2-kb fragment containing the L30F mutation was ligated to the 5.0-kb XhoI/HindIII fragment of pHIMA51, which expresses IMPDH. The 5.0-kb fragment of pHIMA17 was ligated to the 1.2-kb fragment of pHIMA51 to produce pHIMA52, which expresses Q277R IMPDH. Similarly, the 0.7-kb XhoI/SacII fragment of pHIMA18 containing the A462T mutation was ligated to the 5.5-kb XhoI/SacII fragment of pHIMA17 to produce pHIMA53, which expresses Q277R/A462T IMPDH.

**DNA Sequencing**—DNA sequencing was performed using either <sup>35</sup>S-dATP with a Sequenase kit (U.S. Biochemical Corp.) or a PRISM DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc.) and an Applied Biosystems 373A DNA sequencer at the Brandeis Sequencing Facility.

**IMPDH Purification**—Wild-type enzyme was purified in two steps using affinity chromatography. H712 cells carrying pHIA5 were grown overnight in LB broth containing 1 mA IPTG and 100 μg/ml ampicillin. The cells were harvested by centrifugation and resuspended in 50 mM Tris, pH 7.5, 1 mM dithiothreitol, 10% glycerol (Buffer A). The cells were disrupted by sonication and cell debris removed by centrifugation at 12,000 × g for 20 min. The crude lysate was applied to a Cibacron Blue Sepharose column and eluted as above. This preparation was homogeneous as judged by SDS-polyacrylamide gel electrophoresis analysis. IMPDH was concentrated using Centriprep filters (Amicon). IMP was removed by dialysis against buffer A as needed. Protein was quantified using the Bio-Rad assay with IgG as a standard. This assay overestimates the IMPDH concentration by a factor of 2.6 (7), and measurements were adjusted accordingly.

The concentration of IMPDH determined by this method agreed with that determined in the MPA inhibition experiments.

**Purification of Mutant IMPDHs**—F456S/D470G, D255G, M482I, and G340E were purified as described above. A462T did not bind to the IMP affinity column. Therefore crude lysate of A462T was chromatographed on a Bio-Gel A5m column. A462T containing fractions were applied to a Cibacron Blue Sepharose column and eluted as above. This preparation was homogeneous as determined by SDS-polyacrylamide gel electrophoresis. L30F/Q277R, L30F, and Q277R are unstable in the absence of IMP. Therefore fractions from the Cibacron Blue Sepharose column were collected in tubes containing IMP such that the final IMP concentration was 0.5 mM. Activity was lost upon further purification of these enzymes. No NADH oxidase or phosphatase activity was detected in these preparations, which suggests that the partially purified enzymes are suitable for kinetic studies. It should be noted that the specific activity of wild type IMPDH does not increase after the Blue Sepharose column, which indicates that this single purification step yields > 80% purity.

**Isolation of MPA-resistant Clones**—A library of randomly mutagenized pHIA5 was created by transforming pHIA5 into the mutDS E. coli strain NM9072 (40). The plasmid was reisolated from a culture grown on LB broth containing 100 μg/ml ampicillin. This mutagenized plasmid was used to transform E. coli strain H712, which carries a partial deletion in the guaB gene (41).3 MPA-resistant colonies were selected by growth on minimal media containing 200 μg mycophenolic acid, 9.6 μg/ml tryptophan, 48 μg/ml histidine, 5.0 μg/ml tyrosine, 0.1 μg/ml thymine, 2% glucose, and 25 μg/ml ampicillin. Plasmid was isolated from these colonies and used to transform H712 cells. The new transformants were tested for the ability to grow on minimal medium in the presence of MPA as described above.

**Construction of L30F, Q277R, and Q277R/A462T**—The plasmid pHIMA17, which expresses L30F/Q277R IMPDH, was digested with XhoI and HindIII. The 1.2-kb fragment containing the L30F mutation was ligated to the 5.0-kb XhoI/HindIII fragment of pHIA5 to produce pHIMA51, which expresses L30F/Q277R IMPDH. The 5.0-kb fragment of pHIMA17 was ligated to the 1.2-kb fragment of pHIA5 to produce pHIMA52, which expresses Q277R IMPDH. Similarly, the 0.7-kb XhoI/SacII fragment of pHIMA18 containing the A462T mutation was ligated to the 5.5-kb XhoI/SacII fragment of pHIMA17 to produce pHIMA53, which expresses Q277R/A462T IMPDH.

**Isolation of MPA-resistant Clones**—A library of randomly mutagenized pHIA5 was created by transforming pHIA5 into the mutDS E. coli strain NM9072 (40). The plasmid was reisolated from a culture grown on LB broth containing 100 μg/ml ampicillin. This mutagenized

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3 K. Kerr and L. Hedstrom, unpublished observations.
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Homogeneous enzyme preparations. The concentrations of L30F/Q277R, L30F, and Q277R were determined in the MPA inhibition experiment. Q277R/A462T is also unstable in the absence of IMP. The Cibacron Blue Sepharose fractions of Q277R/A462T were further purified on a POROS CM cation exchange column using a BioCAD Sprint perfusion chromatography system (PerSeptive Biosystems). The column was equilibrated in 7 mM Hepes, 7 mM Mes, 7 mM acetic buffer, pH 6.0 (Buffer B). Enzyme was eluted in a linear gradient of 0–1 mM NaCl in Buffer B into test tubes containing IMP.

**Enzyme Assays**—The standard assay solution contains 50 mM Tris, pH 8.0, 100 mM KCl, 1 mM dithiothreitol, 3 mM EDTA, 125 μM IMP, and 100 μM NAD. The production of NADH is monitored by the change in absorbance at 340 nm on a Hitachi U-2000 spectrophotometer. For Km determinations, IMP and NAD are varied as appropriate. Enzymes were dialyzed to remove IMP prior to Km determinations. MPA affinity was determined by varying NAD and MPA concentration in the presence of 1 mM IMP. All assays were performed at 25°C.

**Data Analysis**—Michaelis-Menten parameters were determined by fitting initial rate data to a sequential mechanism (Equation 1) using KinetAsyst software.

\[
v = V_m AB/(K_a K_c + K_B + K_A + AB)
\]

(Eq. 1)

v is the initial velocity, Vm is the maximal velocity, A is the concentration of IMP, B is the concentration of NAD, K_a is the dissociation constant for IMP from the binary EA complex, K_c is the Km for NAD, and K_B is the Km for IMP. MPA affinity was determined by fitting initial rate data to Equation 2, which describes a tight binding uncompetitive inhibitor (43, 44).

\[
v = (v_0/2E)[(E - I)/(1 + K_c + K_B)] + [(1 + K_c + K_B + E^2 + 4K_c[1 + K_B]E^0.5)] (Eq. 2)
\]

I is the concentration of MPA, v_0 is the initial velocity in the absence of I, E is the concentration of IMPDH active sites, and K_c is dissociation constant for MPA.

**Results**

**Expression of Human IMPDH Type II in E. coli**—The cDNA (1.5 kb) of human IMPDH type II was modified by silent mutations to insert restriction sites at the following bases: −1 (Ndel), 473 (XhoI), 750 (KpnI), and 1232 (SacI). The modified cDNA was cloned into the E. coli expression vector pKK233-2 as described under “Materials and Methods” (37). The Ndel site was lost in the cloning process. This construct, pHI5A, expresses human IMPDH type II under the control of the trc promoter. pHI5A was transformed into E. coli strain H712, which contains a partial deletion in gluB, the gene encoding IMPDH (41). H712 cells carrying pHI5A can grow on minimal medium in the absence of guanine, which indicates that IMPDH is expressed in the absence of IPTG. These cells cannot grow on minimal medium in the presence of 200 μM MPA (data not shown).

**Isolation of MPA-resistant Clones**—A library of randomly mutagenized IMPDH was generated using a mutD5 strain of E. coli. This library was transformed into E. coli strain H712. MPA-resistant clones were selected by growth on minimal medium containing 200 μM MPA. Thirty clones were selected from 10^6 colonies screened in two separate experiments. Plasmids were isolated from these clones and used to transform H712 cells. All of the retransformed clones could grow on minimal medium in presence of 200 μM MPA, which indicates that MPA resistance is contained on the plasmid. Twenty-three of these clones were chosen for further characterization.

**Screen of IMPDH Activity and MPA Resistance**—MPA resistance can result either from a decrease in the affinity of IMPDH for MPA or from an increase in IMPDH levels. Therefore, crude extracts were prepared from the MPA-resistant clones in order to screen for clones that might contain altered IMPDHs. Crude extracts were prepared from cultures grown in LB broth in the presence of IPTG. These growth conditions were chosen in order to maximize the amount of IMPDH in the crude extracts.

**TABLE I**

| Clone    | Specific activity (relative) | IC_{50} (relative) | Mutation          |
|----------|-----------------------------|-------------------|------------------|
| Wild type| 1.0                         | 1.0               | None             |
| MA1      | 1.5                         | 1.2               | None             |
| MA2      | 1.5                         | 1.0               | 6A → C          |
| MA3      | 2.0                         | 2.0               | Ala_{462} → Thr |
| MA4      | 2.7                         | 1.2               | Asp_{255} → Gly |
| MA5      | 1.6                         | 1.0               | Ala_{462} → Thr |
| MA6      | 2.6                         | 2.0               | Ala_{223} → Thr |
| MA7      | 2.1                         | 1.4               | None             |
| MA8      | 1.1                         | 1.5               | Met_{482} → Ile |
| MA9      | 1.1                         | 15                | None             |
| MA10     | 2.3                         | 1.4               | 6A → G          |
| MA11     | 1.7                         | 1.0               | None             |
| MA12     | 1.8                         | 2.0               | Ala_{462} → Thr |
| MA13     | 2.9                         | 1.6               | None             |
| MA14     | 1.5                         | 4.0               | 6A → G          |
| MA15     | 0.3                         | 0.4               | 6A → C          |
| MA16     | 2.3                         | 3.0               | 6A → G          |
| MA17     | 0.2                         | 4                 | Leu_{30} → Phe/Gln_{377} → Arg |
| MA18     | 1.9                         | 15                | Ala_{462} → Thr |
| MA4A     | 1.7                         | 0.9               | None             |
| MA11A    | 0.4                         | >4                | Phe_{256} → Ser/Asp_{470} → Gly |
| MA12A    | 1.7                         | 1.1               | None             |
| MA13A    | 1.0                         | 0.8               | Gly_{340} → Glu |
| MA15A    | 2.1                         | 1.1               | None             |

Crude extracts were prepared from LB cultures containing 100 μg/ml ampicillin and 1 mM IPTG. Assays contained 125 μM IMP, 300 μM NAD, 100 μM MPA, which indicate that MPA resistance may result from alteration of IMPDH rather than an increase in IMPDH concentrations.

**Sequencing of MPA-resistant IMPDHs**—Plasmids isolated from the MPA-resistant colonies were sequenced in order to identify mutations in IMPDH. Of the six clones identified above, four express mutant IMPDHs. MA5, MA9, MA14, MA17, MA18, and MA11 all of these mutants have specific activities less than 1.5-fold of wild-type, which suggests that MPA resistance may result from alteration of IMPDH rather than an increase in IMPDH concentrations.

The remaining 17 clones were also sequenced. Overall, 17 mutations were identified in the IMPDH coding and adjacent sequences of the 23 plasmids (Tables I and II). Only two of the mutations were transversions, which is consistent with previous observations of mutD5-mediated mutagenesis in rich media (40). Five clones contained substitutions at −6, which could
increase expression of IMPDH and thus cause MPA resistance. A total of five clones were isolated containing the mutation of Ala<sup>462</sup> → Gly. The MPA IC<sub>50</sub> values of these clones varied from 2- to 15-fold of wild type. This variability could result from the presence of mutations elsewhere on the plasmid. Alternatively, this variability might result from the difficulties inherent in working with crude extracts. In addition to the mutations described above, one clone was isolated with each of the following single mutations: Met<sup>482</sup> → Ile, Gly<sup>340</sup> → Glu, and Asp<sup>385</sup> → Gly. No mutations could be identified in the IMPDH coding sequences of the remaining eight clones.

**Characterization of Mutant IMPDHs**—The mutant proteins were purified as described under "Materials and Methods." A<sub>462</sub>T did not bind to an IMP affinity column, which indicates a change in IMP binding. L30F/Q<sub>277</sub>R is unstable in the absence of IMP. The remaining mutant IMPDHs have purification characteristics similar to wild type.

The K<sub>i</sub> values for MPA inhibition of wild type and mutant IMPDHs are summarized in Table III. MPA is a tight binding inhibitor of IMPDH under the conditions in these assays. In all cases, the data best fit an uncompetitive inhibition mechanism versus NAD. However, while a competitive mechanism can be definitively eliminated, the fit to a noncompetitive mechanism is only marginally worse than the competitive fit. The parameters for human IMPDH type II are similar to those reported elsewhere (18, 39). The K<sub>i</sub> for MPA inhibition is increased relative to wild type in the three mutant enzymes originally identified in the crude extract screen. The K<sub>i</sub> of MPA is increased 3-fold in A<sub>462</sub>T. This mutation was isolated five times; although these isolations do not necessarily represent independent mutational events, this observation suggests that MPA resistance can result from this modest decrease in MPA affinity. A 3-fold increase in K<sub>i</sub> is also observed for F<sub>456S</f/d> D<sub>470G</sub>. The K<sub>i</sub> of MPA increased 8-fold in L30F/Q<sub>277</sub>R. No increase in the K<sub>i</sub> for MPA is observed in the other mutant IMPDHs, in agreement with the crude extract experiments.

Alterations are also observed in the steady state kinetic parameters of the MPA-resistant mutants (Table III). In A<sub>462</sub>T, K<sub>m</sub> for IMP is increased by 2.5-fold relative to wild type, and k<sub>c</sub> is increased 1.5-fold, while no significant change is observed in the K<sub>m</sub> for NAD. In L30F/Q<sub>277</sub>R, K<sub>m</sub> for NAD is increased by 4.5-fold relative to wild type, and k<sub>c</sub> is decreased 5.5-fold. Unfortunately, the K<sub>m</sub> for IMP could not be measured because this enzyme is unstable in the absence of IMP. In F<sub>456S</f/d> D<sub>470G</sub>, neither the K<sub>m</sub> for IMP nor the K<sub>m</sub> for NAD are significantly changed, although k<sub>c</sub> is decreased 2.4-fold. The K<sub>m</sub> values and k<sub>c</sub> values of D<sub>255G</f/d> M<sub>482</sub>I, and G<sub>340E</f/d> are indistinguishable from wild type.

**Characterization of Single Mutants L30F and Q<sub>277</sub>R—L30F/Q<sub>277</sub>R is the only mutant with a dramatic increase in the K<sub>i</sub> of MPA. Therefore the single mutants L30F and Q<sub>277</sub>R were constructed, purified, and characterized in order to determine if both mutations are necessary for MPA resistance. The K<sub>i</sub> for MPA inhibition of L30F is similar to wild type, while the K<sub>i</sub> for MPA inhibition of Q<sub>277</sub>R is 6-fold greater than wild type. Thus the Gln<sup>277</sup> → Arg mutation is sufficient to confer MPA resistance. The K<sub>m</sub> for NAD of L30F is also similar to wild type, although k<sub>c</sub> is decreased by 5-fold. The K<sub>m</sub> values of both IMP and NAD are increased 5–6-fold in Q<sub>277</sub>R, while k<sub>c</sub> is decreased by 3-fold.

**Characterization of Q<sub>277</sub>R/A<sub>462</sub>T—A double mutant was constructed in order to determine if the two mutations that confer MPA resistance, Gln<sup>277</sup> → Arg and Ala<sup>462</sup> → Thr, function independently. The K<sub>i</sub> for MPA inhibition of Q<sub>277</sub>R/A<sub>462</sub>T is similar to Q<sub>277</sub>R; thus the effects of the mutations are not additive and the mutations are not independent. Interestingly, the K<sub>m</sub> for IMP of Q<sub>277</sub>R/A<sub>462</sub>T is similar to Q<sub>277</sub>R; however, the K<sub>m</sub> for NAD is increased 3-fold and k<sub>c</sub> is increased 4-fold. Thus the Ala<sup>462</sup> → Thr mutation restores the defect in k<sub>c</sub> caused by the Gln<sup>277</sup> → Arg mutation. Q<sub>277</sub>R/A<sub>462</sub>T is the first mutant IMPDH with wild type levels of activity and decreased sensitivity to MPA.

**DISCUSSION**

**Isolation of MPA-resistant Mutants of Human IMPDH**—We have used a random mutagenesis and selection approach to identify mutations in human IMPDH type II that can confer MPA resistance. Human IMPDH type II was expressed in an E. coli strain containing a deletion in guaB. This strain cannot grow in the presence of MPA and was used to select MPA-resistant clones. Unlike previous approaches, this method can screen large numbers of clones and other mutations in the purine biosynthetic pathways can be readily eliminated.

Twelve MPA-resistant clones were identified, which contained mutations in IMPDH. Three mutant IMPDHs were isolated with decreased K<sub>m</sub> values for MPA: L30F/Q<sub>277</sub>R, A<sub>462</sub>T, and F<sub>456S</f/d> D<sub>470G</sub>. The greatest increase in the K<sub>i</sub> for MPA (8-fold) is observed in L30F/Q<sub>277</sub>R. This increase can be attributed to the substitution of Arg for Gln<sup>277</sup>. A 3-fold increase in the K<sub>i</sub> for MPA is observed in A<sub>462</sub>T. A 3-fold increase in MPA K<sub>i</sub> is also observed in F<sub>456S</f/d> D<sub>470G</sub>. Since this increase is modest, no attempt has been made to determine if both mutations are
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required for the change in MPA affinity. Three additional mutant IMPDHs were selected in the screen for MPA resistance: D255G, M482I, and G340E. These mutations had no measurable effect on the $K_{M}$ of MPA, or on the steady state kinetic parameters. It is possible that these mutations are fortuitous (like the Leu$^{462} \rightarrow$ Phe mutation) and that additional mutations, present elsewhere on the plasmid, confer MPA resistance. Alternatively, the in vitro assay conditions may not mimic in vivo conditions closely enough to reveal differences in enzyme function.

Effects of the MPA-resistant Mutations on the Function of IMPDH—MPA binds in the nicotinamide site and traps the E-XMP$^+$ intermediate (12, 20). Therefore MPA resistance can result from a change in the MPA/nicotinamide site and might be manifest in the $K_{M}$ for NAD. Since MPA binding involves a stacking interaction with E-XMP$^+$, changes in the IMP site can also increase the $K_{M}$ for MPA. In addition, the $K_{M}$ for MPA will be increased if E-XMP$^+$ no longer accumulates during the IMPDH reaction. For example, a mutation that changed the rate of a conformational change could change the accumulation of E-XMP$^+$. These mechanisms need not be mutually exclusive; indeed, while the $K_{M}$ values of both NAD and IMP are higher in IMPDH from T. foetus and E. coli than in the human enzyme, the $k_{cat}$ values are also higher in the microbial enzymes, which suggests that both mechanisms are important. A462T, Q277R, D255G, M482I, and G340E. These mutations had no measurable effect on the $K_{M}$ values for NAD, while the substrate $k_{cat}$ values are also higher in the microbial enzymes, which is possible that these mutations are fortuitous (like the Leu$^{462} \rightarrow$ Phe mutation) and that additional mutations, present elsewhere on the plasmid, confer MPA resistance. Alternatively, the in vitro assay conditions may not mimic in vivo conditions closely enough to reveal differences in enzyme function.

Structural Context of Mutations That Confer MPA Resistance—Unfortunately, the coordinates of the structure of the E-XMP-IMP complex are not yet available (20), so it is difficult to evaluate the effects of these mutations on the structure of IMPDH. In addition, while the residues that contact MPA and the E-XMP intermediate appear to be clearly delineated, the residues that contact NAD have yet to be identified. These residues are also expected to influence MPA sensitivity. IMPDH is an $\alpha/\beta$ barrel; the active site is located in loops on the C-terminal ends of the $\beta$ strands. The active site Cys$^{331}$ is found in the loop between $\beta6$ and $\alpha6$ (residues 325–342), and additional active site residues are in the loops between $\beta4$ and $\alpha4$ (residues 275–280) and $\beta8$ and $\alpha8$ (residues 400–450). The large loop after $\beta8$ forms a flap over the active site; the flap residues interact with both E-XMP and MPA.

Glu$^{277}$ is in the loop between $\beta4$ and $\alpha4$. The adjacent residues, Asp$^{274}$ Ser$^{275}$ and Ser$^{276}$ contact MPA in the structure. Thus, while Glu$^{277}$ does not contact MPA directly, substitutions at position 277 can easily affect the residues that do contact MPA. While residues 274–276 are conserved among IMPDHs, 277 is His and Asp in the IMPDHs from E. coli and T. foetus, respectively (21, 23). Thus residue 277 may be a determinant of species selectivity. In addition, residues 279–281 are involved in intersubunit contacts in the IMPDH tetramer. Therefore, substitutions at 277 may also affect the function of the adjacent subunit. Ala$^{462}$ is located in the middle of $\alpha8$ and would appear to be removed from the active site. Phe$^{466}$ is also located in $\alpha8$, at the beginning of the helix. Interestingly, residue 462 is the $n + 6$ residue from 456, and thus would be on the opposite side of the helix. These clusters of mutations suggest an important role for $\alpha8$ in the IMPDH reaction. It is possible that $\alpha8$ may influence the flap, and thus modulate the accumulation of E-XMP$^+$ and MPA affinity. Helix $\alpha8$ is highly conserved in mammalian IMPDH, but varies widely in IMPDHs from other sources (Fig. 2). Therefore, $\alpha8$ may be a structural determinant of MPA selectivity.

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