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RECOMBINANT HUMAN INOSINE MONOPHOSPHATE DEHYDROGENASE TYPE I AND TYPE II PROTEINS

PURIFICATION AND CHARACTERIZATION OF INHIBITOR BINDING

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Abstract—Inosine monophosphate dehydrogenase (IMPDH) activity results from the expression of two separate genes, and the resulting proteins (type I and type II) are 84% identical at the amino acid level. Although the type II mRNA is expressed at higher levels in proliferating cells, both mRNAs, and by extrapolation both proteins, are present in normal and malignant cells. Since IMPDH is an important target for the development of drugs with both chemotherapeutic and immunosuppressive activity, we have compared the kinetic and physical properties of the two human enzymes expressed in and purified from *Escherichia coli*. Type I and II IMPDH had k_{cat} values of 1.8 and 1.4 sec^{-1}, respectively, with K_m values for IMP of 14 and 9 μM and K_m values for NAD of 42 and 32 μM. The two enzymes were inhibited competitively by the immunosuppressive agent mizoribine 5′-monophosphate (MMP) with K_i values of 8 and 4 nM and inhibited uncompetitively by mycophenolic acid with K_i values of 11 and 6 nM. The association of MMP to either isozyme, as monitored by fluorescence quenching, was relatively slow with k_{on} values of 3–8 $\times$ 10^4 M^{-1} sec^{-1} and k_{off} values of 3 $\times$ 10^{-4} sec^{-1} (half-lives of 36–43 min). Thus, MMP is a potent, tight-binding competitive inhibitor that does not discriminate between the two IMPDH isozymes.

Key words: enzyme; kinetics; inhibition; mizoribine

IMPDH§ (EC 1.1.1.205) is the rate-limiting enzyme in the *de novo* purine biosynthetic pathway leading to the formation of guanine ribonucleotides and directly catalyzes the oxidation of IMP to XMP in the presence of NAD. The activity of this enzyme has been demonstrated to increase markedly in proliferating cells, and inhibitors of enzyme activity cause inhibition of cell growth that is reversible with the repletion of guanine nucleotides by exogenous guanine. Thus, the enzyme is a critical determinant of levels of guanine nucleotides that are essential for cellular proliferation, and inhibitors of the enzyme have been sought as potential pharmacologic agents for both malignancies and immunosuppression.

IMPDH enzymatic activity is encoded by two distinct genes, termed IMPDH I [1] and IMPDH II [2]. Although the type II enzyme appears to predominate in proliferating cells and to decrease in expression with cell differentiation [3–5], studies in human T lymphocytes have shown that both mRNAs corresponding to these proteins increase in amount in response to agents inducing cell division. Hence, the relative requirements for the two enzymes in dividing cells remain somewhat unclear. The proteins encoded by the cDNAs from these two genes each contain 514 amino acids and are 84% identical in sequence. The two proteins have been expressed in *Escherichia coli* [6–8], and recent studies have confirmed that both are tetramers with similar K_m values for IMP and NAD, as well as K_i values for XMP and NADH [6]. Both isozymes have ordered Bi–Bi kinetic mechanisms where IMP binding precedes the binding of NAD. One described distinction between the isozymes is a 5-fold difference in the K_i values for mycophenolic acid [6].

We have studied the relative inhibition of IMPDH I and II by a series of inhibitors and have further investigated the purified *E. coli*-expressed proteins for structural differences that could have relevance for the development of inhibitors that could be selective for either the type I or type II proteins. We are particularly interested in the 5′-monophosphate of mizoribine (Bredinin; 4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate), a nucleoside antibiotic that is a potent inhibitor of IMPDH and has immunosuppressive

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§ Abbreviations: 1-Alo-5′-P, 1-ribosyl-allopurinol-5′-phosphate; 6-aza-UMP, 6-aza-uridine 5′-monophosphate; DTT, dithiothreitol; IMPDH, inosine monophosphate dehydrogenase; IPTG, isopropyl-β-D-thiogalactopyranoside; β-Me-TAD, β-methylene thiazole-4-carboxamide adenine dinucleotide; 6-MeMPR-5′-P, 6-methyl mercaptopurine riboside 5′-phosphate; MMP, mizoribine 5′-monophosphate; and PMSF, phenylmethylsulfonyl fluoride.

activity in animal and human studies [9–12], and we have characterized its interaction with type I and type II purified IMPDH in detail.

MATERIALS AND METHODS

Materials. 6-Thio-GMP, 6-thio-IMP, 6-MeMPR-5′-P, and 1-Alo-5′-P were provided by Wayne Miller (Burroughs Wellcome Co., Research Triangle Park, NC). β-Me-TAD was provided by Dr. David Johns (NCI, Bethesda, MD). MMP (Bredinin monophosphate) was supplied by the Asahi Chemical Industry Co. (Tokyo, Japan). Ribavirin 5′-phosphate was supplied by Dr. Arnold Fridland (St. Jude Children's Research Hospital, Memphis, TN). Protein assay reagent was obtained from Bio-Rad (Richmond, CA).

Enzyme assays. IMPDH activity was measured at 37° using a spectrophotometric assay based on the reduction of NAD [13]. Assays were monitored at 340 nm except for assays with 6-thio-IMP, which were measured at 360 nm [14]. For the determination of K_m and K_i values, 0.4 to 4 mU of IMPDH was used per assay. K_m values were determined from Eadie–Hofstee plots of velocity versus V/S. K_i determinations for inhibitor binding were obtained from replots of K_m apparent versus the concentration of inhibitor, except for the tight-binding inhibitors mycophenolic acid and MMP. The K_i for mycophenolic acid was determined by the linear method of Henderson [15] for a noncompetitive inhibitor using Equation (1).

$$[I_t]/(1 - V_i/V_o) = [E_t] + K_i(V_o/V_i) \quad (1)$$

where I_t is the total concentration of inhibitor, E_t is the total concentration of enzyme, V_o is the initial velocity and V_i is the initial velocity in the presence of inhibitor.

The K_i for MMP was determined from the ratio of the individual dissociation and association rates. The individual rate constants were determined by fitting inhibition data to Equation (2), which is a transient velocity equation for a competitive inhibitor [16]. The kinetic scheme is diagrammed below. MMP competes with IMP, the first step in the pathway. Subsequent kinetic steps (binding of NAD and catalysis) have been simplified into the kinetic constant k'_{cat}. This analysis assumes that there is a rapid equilibrium between enzyme and IMP.

$$E + IMP \overset{K_m}{\leftrightarrow} E\text{–}IMP \overset{k'_{cat}}{\leftrightarrow} E + XMP$$

$$+ MMP$$

$$k'_1 \downarrow \uparrow k_2$$

$$E\text{–}MMP$$

$$[NADH] = \frac{[E_t]k^2k'_{cat}}{b}(t) + \frac{[E_t][I]k'_1k'_{cat}}{b^2}(1 - e^{-bt}) \quad (2)$$

where $b = [I]k'_1 + k_2$.

For Equation (2), E_t is the total concentration of enzyme, I is the total concentration of inhibitor, k'_1 is the observed association rate constant for inhibitor binding to enzyme, k_2 is the dissociation rate of the inhibitor enzyme complex, and k'_{cat} is the observed catalytic rate constant. The true association rate constant for inhibitor binding to the enzyme was obtained from Equation (3):

$$k_1 = \frac{K_m + [IMP]}{K_m} k'_1 \quad (3)$$

Fluorescence of IMPDH. Fluorescence data were collected on a Kontron SFM 25 spectrofluorimeter (excitation at 260 nm and emission at 310 nm). Initial experiments indicated that IMPDH is weakly fluorescent, as expected for a protein that has no tryptophan residues, although it does contain 11 tyrosine residues per monomer. MMP quenches the IMPDH fluorescence by approximately 50%. The fluorescence data were fitted to Equation (4) to determine the rate constant for the association of MMP with IMPDH type II:

$$\text{fluorescence} = ae^{-bt} + c + dt \quad (4)$$

where a is a correction factor, b is the observed rate constant for quenching, c represents the quenching end point, d is a correction for the intrinsic decrease in fluorescence with time (i.e. photobleaching), and t is time.

Data analysis. Nonlinear least squares fitting of data to the Michaelis–Menten equation and Equations 2 and 4 were performed using SigmaPlot for the MacIntosh (Jandel Scientific, Corte Madera, CA).

Expression of IMPDH protein in E. coli. IMPDH I and II were subcloned from pGEM derived vectors into pET24c(+) (Novagen) and expressed in *E. coli* BL-21 (DE3) [8]. Cells were grown at 37° in 10 L of YT + M-9 salts [17] in a New Brunswick fermentor to an OD_{600} of 1–2 and induced with 0.2 to 0.4 mM IPTG. Cells were harvested at 1.5 to 2 hr post-induction, washed with 10 mM Tris–Cl, 1 mM EDTA, 1 mM PMSF, and stored frozen at −70° (approximately 50 g of wet weight cells).

Protein purifications were carried out at 0–4°. Frozen cells (approximately 25 g) were suspended in 50 mL of 10 mM Tris–Cl, 1 mM EDTA, 2 mM DTT, 5 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM PMSF. The cells were sonicated in 20-sec bursts (6–8) in an ice bath until lysis was complete, as judged by the A_{260} of aliquots spun in a microfuge. The lysate was centrifuged for 20 min at 15,500 *g* in an SS-34 rotor. The pellet was extracted with 50 mL of 0.5 M NaCl, 4 M urea, 0.1 M Tris–Cl, pH 8, 2 mM DTT, 5 μg/mL leupeptin, 10 μg/mL aprotinin using a Dounce homogenizer to resuspend the cell debris and centrifuged at 15,500 *g* for 15 min.

The supernatant from the urea extraction was diluted with 4 vol. of 1 mM DTT and loaded onto a phosphocellulose column (18 cm^2 × 12 cm) that had been equilibrated with 10 mM potassium phosphate, 0.1 M KCl, 0.1 mM EDTA, pH 7.3. The column was washed with 10 mM potassium phosphate, 0.1 M KCl, 0.8 M urea, 1 mM DTT, 0.1 mM EDTA, pH 7.3, and eluted with a 0.1 to 1.0 M KCl gradient in the same buffer. Active fractions were pooled, diluted with an equal volume of 0.8 M urea, 1 mM DTT and loaded onto a Blue Sepharose column

Table 1. Purification summaries for the human IMPDH isozymes

Fraction	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
Type I				
Sonicate	50	125	1100	0.11
Supernatant	32	0	500	0.0
Urea extraction	45	420	760	0.55
Phosphocellulose	130	210	210	1.0
Blue Sepharose	225	230	150	1.5
Type II				
Sonicate	60	324	1600	0.21
Supernatant	50	50	850	0.06
Urea extraction	60	440	890	0.49
Phosphocellulose	205	370	340	1.1
Blue Sepharose	120	350	270	1.3

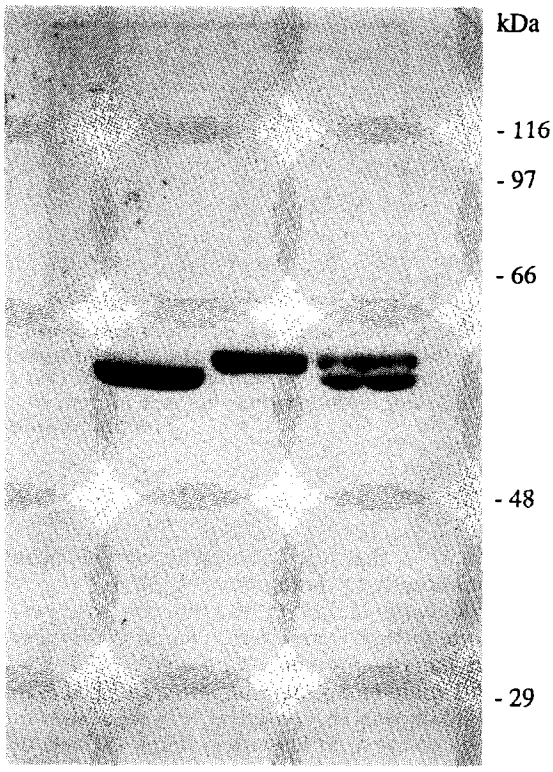

Fig. 1. SDS–PAGE of purified recombinant IMPDH isozymes. Lane 1, 5 μg of type I IMPDH. Lane 2, 5 μg of type II IMPDH. Lane 3, 2.5 μg each of types I and II.

(18 cm^2 × 12 cm) that had been equilibrated with 10 mM Tris–Cl, 0.15 M KCl, 0.1 mM EDTA, pH 7.5. The column was washed with 10 mM Tris–Cl, 0.15 M KCl, 0.8 M urea, 1 mM DTT, 0.1 mM EDTA, pH 7.5, and IMPDH was eluted with a 0.15 to 1.0 M KCl gradient in the same buffer.

N-Terminal sequence from purified type II IMPDH was determined by Dr. D. Klapper (University of North Carolina, Chapel Hill) using a gas phase automated sequencer.

Two-dimensional gel electrophoresis. Protein samples were dissolved in a solution (pH 9.5) containing 9.5 M urea, 1% DTT, 2% carrier ampholytes (LKB, pH 9–11), and 4% Nonidet P-40. Whole cell samples were centrifuged for 5 min at 435,000 *g* in a Beckman TL100 ultracentrifuge to pellet the DNA. Aliquots of the supernatants containing 0.5 to 200 μg of the protein samples were separated by isoelectric focusing (IEF) as described previously [18]. A 50:50 mixture of Biolytes pH 3–10 and Biolytes pH 5–7 was used to generate the pH gradient. The pI of the proteins was estimated from the pH profile of an IEF gel run on the same system. The second dimension SDS–polyacrylamide slab gels were run as described [19] with modifications that produce linear gradient gels containing 9–17% polyacrylamide [20].

For immunoblot analysis, the gel was equilibrated for 5 min in transfer buffer (10 mM 3-cyclohexylamino-1-propanesulfonic acid in 10% methanol) and transferred to Immobilon membrane. Membranes were blocked in 5% nonfat dry milk and incubated with an affinity purified IMPDH-specific monoclonal antibody [21]; immune complexes were visualized as described previously.

Metal analysis. Analysis for Zn, Mo, Cu, Fe, Mn, Mg, and Co was performed by Galbraith Laboratories (Knoxville, TN) using inductively coupled plasma emission spectroscopy.

RESULTS AND DISCUSSION

Cloning and purification. The human 1.5 kb IMPDH I and II cDNA coding regions were cloned into the *E. coli* expression vector pET24c, and the inserts were completely sequenced. As reported previously [8], the IMPDH I clone contained 11 base changes from the previously published sequence, but was confirmed as corresponding to IMPDH I mRNA by RNase protection assay. Upon induction by IPTG, *E. coli* BL21 (DE3) containing pET24-I or pET24-II produced large amounts of the IMPDH protein. The purification of the two proteins is summarized in Table 1. From these data, it is apparent that the expressed protein constituted roughly 15% of the bacterial cell sonicate. The final specific activities of 1.3 to 1.5 U/mg were similar to the specific activity of IMPDH enzyme purified from rat sarcoma cells [22], indicating the similarity of the *E. coli*-expressed enzyme to enzyme from mammalian cells. The purified proteins were compared on a 10% denaturing polyacrylamide gel, as shown in Fig. 1. The proteins were homogeneous, and the type II enzyme migrated slightly slower than the type I enzyme. This difference may be due to the slight difference in deduced molecular weights (55,263 for the type I enzyme, in contrast to 55,638 for the type II enzyme), differences in amino acid composition, post-translational modifications, or a combination of these factors. Direct amino acid sequencing demonstrated the absence of the N-terminal

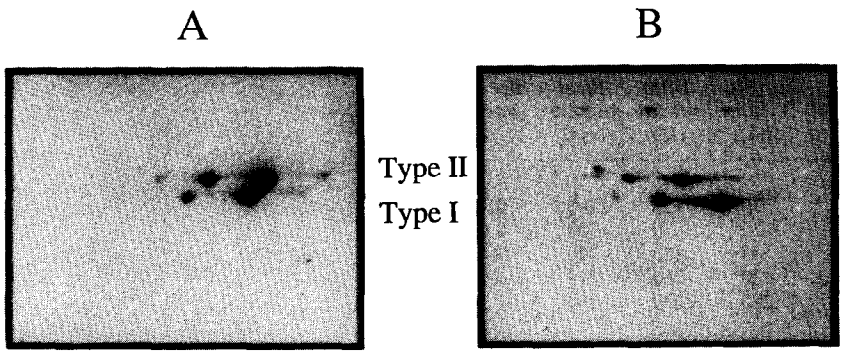

Fig. 2. Two-dimensional electrophoresis of native and recombinant IMPDH. (A) Coomassie-stained gel of purified IMPDH isozymes, 0.5 μg of types I and II. (B) Western blot of purified IMPDH type I (0.5 μg) and cell extract from human RE2 cells (200 μg). RE2 cells overexpress the type II isozyme. Following electrophoresis, the proteins were blotted to nitrocellulose and visualized with α-IMPDH, which recognizes both isozymes.

methionine in the type II protein, as has been reported recently for both isozymes [6].

Two-dimensional gel electrophoresis was performed to further characterize charge differences between the two proteins. As shown in Fig. 2A, IMPDH I could be distinguished from IMPDH II in the second dimension, consistent with the results in Fig. 1. In addition, at least two charge variants of both proteins could be distinguished in the first dimension. To determine whether similar variants could be detected in protein from mammalian cells, IMPDH I was run together with cell extract from RE2 melanoma cells that overexpress an amplified IMPDH II gene [23] (Fig. 2B). The RE2 type II protein (pI approximately 7.5) was charge-shifted from the *E. coli*-expressed type II protein when the migration of each protein was compared with that of type I. This difference can be accounted for by the blocked N-terminus of the mammalian protein, which leads to the loss of a positive charge. The distribution of isoforms may be attributed to different charge isoforms with analogous pIs. In this case, the difference in the two-dimensional gel pattern would reflect the relative proportion of the various charged isoforms in the recombinant and RE2 preparations. We conclude that the two bacterially expressed proteins are separable by denaturing gel electrophoresis and contain several charge variants similar to the corresponding mammalian proteins.

Kinetic studies. Table 2 confirms the similarity of K_m values for IMP and NAD and turnover numbers for the two enzymes. K_i values were then obtained for a number of known inhibitors to determine whether any would discriminate between the two isozymes. We tested inhibitors that are competitive with IMP (hence interact with the IMP binding site) and uncompetitive inhibitors with respect to IMP that interact with the NAD site. The results are summarized in Table 3. MMP was significantly more potent than the other competitive inhibitors tested with K_i values of 8.2 and 3.9 nM for the type I and type II enzymes, respectively (see below). Of the other competitive inhibitors, ribavirin 5′-monophosphate was the most potent by approximately 20-fold, but no significant differences could be detected between the type I and II enzymes with any compound. The K_i values for 6-thio IMP and 6-MeMPR-5′-P were similar to the values for IMPDH from mouse sarcoma cells [14]. 6-aza-UMP, a potent inhibitor of orotidylic acid decarboxylase, was also an inhibitor of the human IMPDH, as it is for the yeast enzyme [24]. β-Me-TAD is a phosphodiesterase-resistant analog of TAD [25] that interacts at the NAD site and showed no significant difference in inhibition between the isozymes. With respect to the uncompetitive inhibitor mycophenolic acid, our results were similar to those obtained by Carr *et al.* [6], although their observed difference in K_i values for mycophenolic acid (4.8-fold) was greater than in our study (2-fold). Whether this difference is of pharmacological significance is open to further study.

Table 2. Kinetic constants for human IMPDH I and II and relative inhibition by MMP

	IMPDH isoenzyme	
	Type I	Type II
K_m for IMP (μM)	14.2 ± 1.5	9.2 ± 0.7
K_m for NAD (μM)	42 ± 6	32 ± 3.6
k_{cat} (sec^{-1})	1.82 ± 0.04	1.40 ± 0.04
Inhibition by MMP		
k_{on} (M^{-1} sec^{-1})	3.3×10^4	8.2×10^4
k_{off} (sec^{-1})	2.7×10^{-4}	3.2×10^{-4}
K_i (nM)	8.2	3.9

MMP behaves as a classic tight-binding inhibitor where the order of addition of enzyme and substrates affects the initial velocity of the reaction [16]. Figure 3 illustrates the slow rate of inhibition of IMPDH type II with increasing concentrations of MMP. These data were directly fitted to a competitive model to determine on and off rates for MMP binding. MMP associated and dissociated relatively slowly from the enzyme with a $k_{on} = 8.2 \times$

Table 3. Inhibition constants for inhibitors of the human IMPDH isozymes

Inhibitor	Type of inhibition	Type I K_i	Type II K_i
MMP*	Competitive	8.2 nM	3.9 nM
Ribavirin-P	Competitive	0.65 μM	0.39 μM
6-aza-UMP	Competitive	15 μM	13 μM
6-thio-IMP	Competitive	15 μM	29 μM
6-MeMPR-5'-P	Competitive	520 μM	1070 μM
1-Alo-5'-P	Competitive	670 μM	1900 μM
Mycophenolic acid†	Uncompetitive	11 nM	6 nM
β-Me-TAD	Uncompetitive	95 nM	145 nM

The type of inhibition was determined from Eadie–Hofstee plots of steady-state reactions with IMP as the varied substrate. The K_i values were determined from replots of K_m apparent vs inhibitor concentration, except for MMP and mycophenolic acid.

* K_i values were determined from the ratio of k_{on} and k_{off} (see Table 2).

† K_i values were determined by the linear method of Henderson [15] (see Materials and Methods).

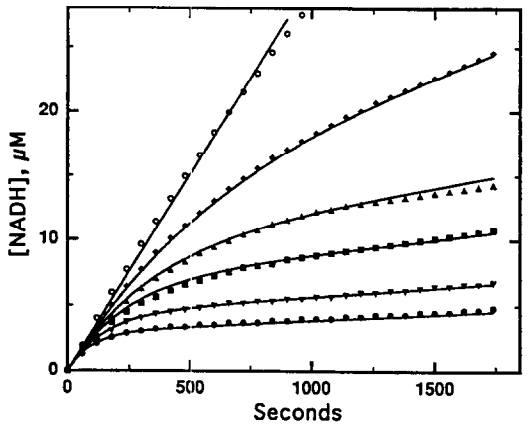

Fig. 3. Inhibition of IMPDH type II by MMP. Assays (37°, pH 8) included 0.1 M Tris–Cl, 0.1 M KCl, 3 mM EDTA, 0.25 mM NAD and 0.05 mM IMP. The concentrations of the inhibitor MMP were 0 nM (○), 100 nM (◆), 200 nM (▲), 300 nM (■), 500 nM (▼), and 750 nM (●). The reactions were initiated by adding IMPDH type II to 21 nM. Solid lines represent the fit to a competitive inhibition model, as described in Materials and Methods, yielding values for the association rate between MMP and IMPDH of $8.2 \times 10^4\,M^{-1}sec^{-1}$ and a dissociation rate of $3.2 \times 10^{-4}\,sec^{-1}$.

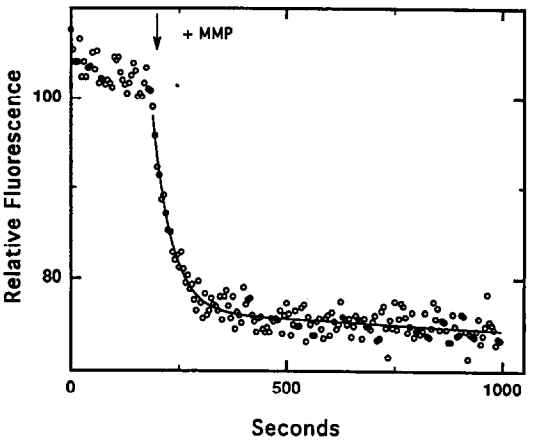

Fig. 4. Fluorescence quenching of 105 nM IMPDH type II upon addition of 400 nM MMP. Reactions contained 0.1 M Tris–Cl, 0.1 M KCl, pH 8, at 37°. Quenching by MMP was fitted as described in Materials and Methods. The fitted line corresponds to a second order rate constant of $6.6 \times 10^4\,M^{-1}sec^{-1}$.

$10^4\,M^{-1}sec^{-1}$ and a $k_{off} = 3.2 \times 10^{-4}\,sec^{-1}$. The dissociation rate corresponded to a half-life of 36 min. Similarly, the type I isozyme was found to have a $k_{on} = 3.3 \times 10^4\,M^{-1}sec^{-1}$ and $k_{off} = 2.7 \times 10^{-4}\,sec^{-1}$ (half-life of 43 min). The rate constants and K_i values determined from these rates are summarized in Table 2. We confirmed the relatively slow association rate for MMP binding to the type II enzyme using a fluorescence quenching assay, as shown in Fig. 4. We observed an association rate of $6.6 \times 10^4\,M^{-1}\,sec^{-1}$, in good agreement with our previous result. Since NIMPDH has an ordered Bi–Bi mechanism [6, 26, 27], the association rate for IMP as the first substrate was determined by the ratio of k_{cat}/K_m (IMP) [28]. For the type I and II isozymes the corresponding k_{cat}/K_m values ranged from 1.4 to $1.6 \times 10^5\,M^{-1}sec^{-1}$. The nonsteady-state inhibition of IMPDH by MMP reflected both the relatively slow association between MMP and IMPDH ($3–8 \times 10^4\,M^{-1}sec^{-1}$) and the stability of the complex (with half-lives of 36–43 min). The slow association is likely to be a general feature for inhibitors that are competitive with IMP, since the association rate for IMP is of similar magnitude. The fluorescence quenching of IMPDH upon binding of MMP is likely to be the result of the UV absorbance of MMP, since similar quenching did not occur upon addition of UV transparent ribavirin

5′-monophosphate (data not shown). It has been suggested that conformational changes in the enzyme–IMP complex are required to fit the inhibition studies of the *Tritrichomonas foetus* enzyme [27]. While the slow association rate for IMP or MMP is consistent with such a conformational change, a corresponding fluorescence change was not observed.

Several authors have described an inactivation of IMPDH by preincubation with an inhibitor [29–31]. We did not observe a time-dependent inactivation with ribavirin 5′-monophosphate with either isozyme, as described for rat IMPDH [29, 30]. The recently described inhibitor of IMPDH, EICAR (5-ethynyl-1-β - D - ribofuranosylimidazole - 4 - carboxamide) monophosphate [31], shows an apparent inactivation following preincubation. As we have demonstrated for MMP, this can be typical behavior for tight-binding inhibitors [16]. Alternatively, it is possible that EICAR monophosphate is irreversibly inactivating the enzyme. The very reactive Cys-331 at the active site [32] could react with the ethynyl moiety of EICAR, as described for dihydropyrimidine dehydrogenase and 5-ethynyluracil [33].

Physical properties. There are reports of mammalian IMPDH being unstable and sensitive to aggregation at low salt concentrations [22, 29, 34]. In our hands, both enzymes were stable at room temperature when concentrated or in the presence of bovine serum albumin ($T_{1/2}$ 2 to 2.5 hr), and the addition of DTT increased enzyme stability ($T_{1/2}$ >10 hr), consistent with the recent report that all eight cysteines in IMPDH type II are in the reduced form [32]. Both enzymes are inactivated by 6-thio-GMP with similar kinetics and are reactivated by DTT, consistent with modification of Cys-331, a cysteine residue at the nucleotide binding site that is conserved between IMPDH and GMP reductase and is crosslinked to 6-chloro-IMP [1, 2, 32, 35]. Both isozymes are susceptible to aggregation, although the presence of 0.8 M urea minimized precipitation in low salt solutions.

We tested purified type I IMPDH for the presence of Zn, Mo, Cu, Fe, Mn, Mg, or Co under the assumption that the mechanism of IMPDH catalysis involves an activation of a water molecule, similar to that described for alcohol dehydrogenase and the adenosine and cytosine deaminases [36–38]. The recent report of IMPDH catalyzing the conversion of 2-Cl-IMP to XMP in the absence of NAD is consistent with such a mechanism [39]. However, none of these metal ions were found in association with IMPDH type I or II proteins.

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