Acid−Base Catalysis in the Chemical Mechanism of Inosine Monophosphate Dehydrogenase†

George D. Markham,* Cindy L. Bock,‡ and Céline Schalk-Hihi§

Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111

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ABSTRACT: Inosine-5′-monophosphate dehydrogenase (IMPDH) catalyzes the K^+-dependent reaction IMP + NAD + H_2O → XMP + NADH + H^+ which is the rate-limiting step in guanine nucleotide biosynthesis. The catalytic mechanism of the human type-II IMPDH isozyme has been studied by measurement of the pH dependencies of the normal reaction, of the hydrolysis of 2-chloro-IMP (which yields XMP and Cl^- in the absence of NAD), and of inactivation by the affinity label 6-chloro-purine-ribotide (6-Cl-PRT). The pH dependence of the IMPDH reaction shows bell-shaped profiles for k_{cat} and the k_{cat}/K_m values for both IMP and NAD, illustrating the involvement of both acidic and basic groups in catalysis. Half-maximal k_{cat} values occur at pH values of 7.2 and 9.8; similar p*K* values of 6.9 and 9.4 are seen in the k_{cat}/K_m profile for NAD. The k_{cat}/K_m profile for IMP, which binds first in the predominantly ordered kinetic mechanism, shows p*K* values of 8.1 and 7.3 for acidic and basic groups, respectively. None of the kinetic p*K* values correspond to ionizations of the free substrates and thus reflect ionization of the enzyme or enzyme−substrate complexes. The rate of inactivation by 6-Cl-PRT, which modifies the active site sulfhydryl of cysteine-331, increases with pH; the p*K* of 7.5 reflects the ionization of the sulfhydryl in the E·6-Cl-PRT complex. The p*K*s of the acids observed in the IMPDH reaction likely also reflect ionization of the cysteine-331 sulfhydryl which adds to C-2 of IMP prior to NAD reduction. The k_{cat} and k_{cat}/K_m values for hydrolysis of 2-Cl-IMP show a p*K* value of 9.9 for a basic group, similar to that seen in the overall reaction, but do not exhibit the ionization of an acidic group. Surprisingly, the rates of 2-Cl-IMP hydrolysis and of inactivation by 6-Cl-PRT are not stimulated by K^+, in contrast to the >100-fold K^+ activation of the IMPDH reaction. Apparently the enigmatic role of K^+ lies in the NAD(H)-dependent segment of the IMPDH reaction. To evaluate the importance of hydrogen bonding in substrate binding, several deamino- and deoxy-analogues of IMP were tested as substrates and inhibitors. Only 2′-deoxy-IMP was a substrate; the other compounds tested were competitive inhibitors with K_i values at most 10-fold greater than the K_D for IMP, illustrating the greater importance of hydrogen-bonding interactions in the chemistry of the IMPDH reaction than simply in nucleotide binding.

Inosine-5′-monophosphate dehydrogenase (IMPDH)[1] catalyzes the NAD-dependent biosynthesis of xanthine-5′-monophosphate in the reaction shown below (*1*):

$$\text{IMP} + \text{NAD} + \text{H}_2\text{O} \xrightarrow{\text{K}^+} \text{XMP} + \text{NADH} + \text{H}^+$$

The IMPDH-catalyzed reaction is the rate-limiting step in the biosynthesis of guanine nucleotides in mammals (*2*−*4*). IMPDH is a well-established target for the development of chemotherapeutic agents as antineoplastic, antiviral, antiparasitic, and immunosuppressive agents (*7*−*13*). In humans the tetrameric IMPDH occurs as two isozymes with 84% amino acid sequence identity over the entire 514 residues (*5*, *6*). The type 2 isozyme has historically been regarded as the most promising target for isozyme selective drug development (*14*) because of variation in levels in different cells (*15*, *16*) and has been characterized most extensively. IMPDH from various sources displays a predominantly ordered steady-state kinetic mechanism with IMP binding before NAD, and XMP is the last product released (*17*−*20*). Recent studies of primary kinetic isotope effects from [2-deutero]IMP demonstrated that IMP can dissociate from the E·IMP·NAD complex and thus established the presence of a random component to the mechanism (*21*, *22*). The chemical mechanism of the reaction appears to proceed through a covalent linkage between C2 of IMP and the sulfhydryl of cysteine-331 (*23*, *24*); hydride transfer would occur to NAD from this adduct, and then the resulting covalent enzyme-oxidized IMP complex, E-IMP_{ox}, would hydrolyze to give XMP (Figure 1). NADH dissociates before E-IMP_{ox} hydrolysis and the rate-limiting step occurs later in the pathway (*21*). The reaction scheme shown in Figure 1

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* To whom correspondence should be addressed. Tel: 215-728-2439. Fax: 215-728-3574. E-mail: GD_MARKHAM@fccc.edu.

‡ Present address: DuPont Pharma, Wilmington, DE.

§ Present address: 3-D Pharmaceuticals, Exton, PA.

[1] Abbreviations: IMPDH, inosine 5′-monophosphate dehydrogenase; 2-Cl-IMP, 2-chloro-IMP; 6-Cl-PRT, 6-chloro-purine-riboside-5′-monophosphate; BTP, Bis-Tris-propane.

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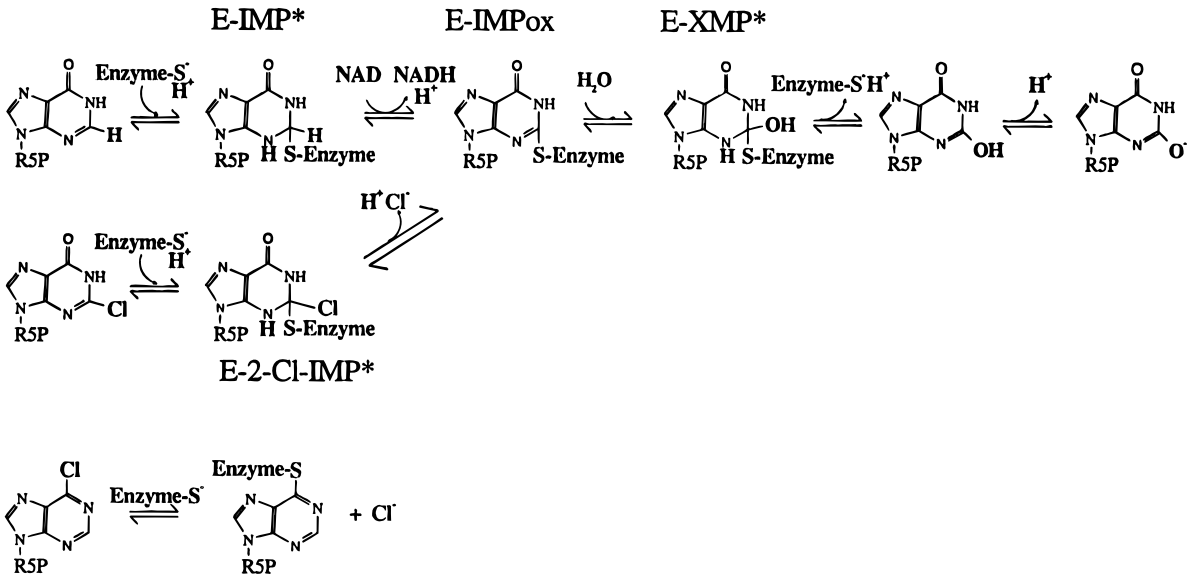

FIGURE 1: Reactions catalyzed by IMPDH studied in this work. Top: The IMPDH reaction. Middle: The 2-Cl-IMP hydrolysis reaction. Bottom: The 6-Cl−IMP affinity labeling reaction. The results herein suggest localization of the K^+-dependent step to an event in the NAD-dependent segment of the IMPDH reaction. The probable confluence of the IMPDH and 2-chloro-IMP hydrolytic reactions is illustrated.

also illustrates that at neutral pH XMP is an enolate at the 2-position (the p*K* of the xanthine base is ~5.5 but whether XMP is bound as an anion in the E·XMP complex is unknown (*25*)). The rate-limiting step in the mechanism occurs after hydride transfer and could be either hydrolysis of E-IMP_{ox} or XMP release (*21*, *22*); the lack of a solvent deuterium isotope effect on k_{cat} favors the latter possibility (*22*).

In addition to the overall IMPDH reaction, the enzyme catalyzes the hydrolysis of 2-chloro and 2-fluoro-IMP to yield XMP and the halide anion in reactions that do not require NAD and which have 20% and 23%, respectively, of the k_{cat} of the IMPDH reaction (*26*). Thus, the rate-determining step for these reactions precedes any step shared with the IMPDH reaction sequence. The similarity in rates for the chloro- and fluoro-IMPs suggests that carbon−halogen bond cleavage is not rate-limiting; rather the rate-limiting step might be carbon−sulfur bond formation or a conformational change (see Figure 1). Furthermore, the enzyme has long been known to be inactivated by the affinity label 6-Cl-purine riboside-5′-monophosphate (6-Cl-PRT) (*27*−*30*), which was recently shown to react with the sulfur of the active site residue cysteine-331 to give a stable thioether adduct (*31*−*32*).

While the chemical steps involved in these reactions involve numerous proton transfers, the ionization properties of the groups involved in these steps remain unknown. Of particular interest are the p*K* values of the nucleophilic cysteine-331 sulfhydryl and the kinetic dependencies on the ionization state of IMP. IMP has two ionizations of potential significance, those of the N1 proton of the hypoxanthine moiety and the secondary ionization of the phosphate. The present work has investigated the pH dependencies of various kinetic and binding parameters to probe these processes. The pH dependencies of catalytic properties of other dehydrogenases have been effectively used to elucidate groups important in enzyme function (*33*−*37*). The results of these studies are particularly useful in the context of the three-dimensional protein structure. The three-dimensional X-ray structure of hamster IMPDH in the covalent E-IMP_{ox} complex with the inhibitor mycophenolic acid has been reported at 2.6 Å (*38*); the structure of the homologous enzyme from *Tritrichomonas foetus* has been reported as both the apoenzyme (at 2.3 Å resolution) and as a noncovalent E·XMP complex (at 2.6 Å resolution) (*39*). Although large portions of the protein are disordered in all of these structures, it is clear that the active site is located between subunits, and numerous interactions between the protein and the nucleotide have been described (see Figure 2). Interestingly, while waters were observed near C2 and N3 of the purine, no candidates for a catalytic base were identified. Structures of complexes containing NAD(H) or closely related inhibitors have not been reported.

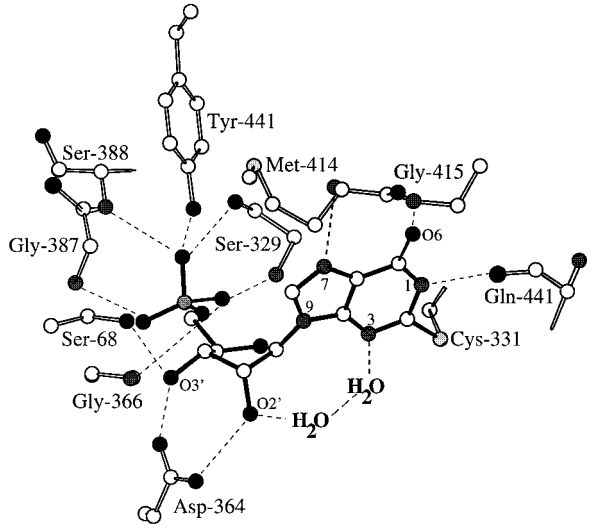

FIGURE 2: The substrate binding site of IMPDH from the crystal structure of the hamster enzyme (*38*). Hydrogen bonds deduced by Sintchak et al. (*38*) are shown as dotted lines. The positions of the nucleotides modified in substrate analogues are numbered. This figure was prepared with MolScript (*43*).

The IMPDH reaction is stimulated more than 100-fold by K^+ or other monovalent cations of similar ionic radius (*1*, *20*), but in common with most other K^+-stimulated enzymes the mechanistic basis for this rate enhancement is unknown

(*40*). While K^+ binds before either substrate in the predominant kinetic pathway, equilibrium binding studies showed that IMP, XMP, and NADH can bind in the absence of K^+ with affinities comparable to those observed in the presence of the activator; NAD binding in the absence of IMP was not detectable (*20*). The location of a K^+ ion was reported in the crystal structure of the hamster IMPDH, with coordination to the carbonyl of cysteine-331, a water molecule, and four other unspecified main chain carbonyl groups (*38*). The reasons for assigning this density to a K^+ ion were not given. The location of this apparent K^+ suggested that a K^+-coordinated water might be the nucleophile in the hydrolytic phase of the reaction. We thus investigated the role of the monovalent cation in the reactions of 2- and 6-chloro-IMP. Surprisingly, neither of these reaction rates is affected by K^+. These results imply that the role of K^+ is uniquely in the NAD^+-dependent segment of the IMPDH reaction.

MATERIALS AND METHODS

IMP, 2′-deoxy-IMP, 3′-deoxy-AMP, NAD, 2-Cl-adenosine, 6-Cl-purine-riboside-5′-monophosphate (6-Cl-PRT), inosine and other nucleosides, Tris, and Bis-Tris propane (BTP) were purchased from Sigma. Recombinant human type-II IMPDH was prepared as described previously (*20*) and had a specific activity of 0.68 μmol/min/mg in the standard assay (0.1 M Tris/Cl, 20 mM KCl, 0.2 mM IMP, 0.2 mM NAD, pH 8.0) at 37 °C. NMR spectra were recorded on a Bruker AM300 spectrometer operating at 300 MHz for ^{1}H, 121.1 MHz for ^{31}P, and 75.45 MHz for ^{13}C. Solutions for ^{31}P and ^{13}C measurements contained 5% D_2O for field-frequency locking while ^{1}H NMR samples were in >99% D_2O.

2-Cl-IMP (ϵ(256 nm) = 12.3 mM^{-1} cm^{-1} (*26*)) was prepared from 1 mequiv of 2-Cl-adenosine in two steps. In the first step, 2-Cl-adenosine was phosphorylated with $POCl_3$ as described by Sowa and Ouchi (*41*); the 2-Cl-AMP was purified by chromatography on a 2.6 cm × 21 cm column of Q-Sepharose (Pharmacia) that was eluted at 4 mL/min with a 1.5 L gradient from H_2O to 0.7 M triethylamine bicarbonate, pH 9.0. Fractions with the expected ultraviolet and ^{31}P NMR spectra were pooled. The 2-Cl-AMP was converted to 2-Cl-IMP by treatment with 0.4 M $NaNO_2$ in 0.8 M HCl for 2 days at room temperature in the dark (*22, 42*), and the product was isolated by chromatography on Q-Sepharose as described above. The ultraviolet, ^{31}P NMR, and ^{1}H NMR spectra were consistent with the anticipated structure. 1-Methyl-inosine, purine nucleoside, 8-aza-9-deaza-adenosine (formycin), 3-deaza-adenosine, and 7-deaza-adenosine (tubercidin) were phosphorylated, purified, and analyzed in the same fashion. 7-Deaza-AMP and 3-deaza-AMP were converted to the corresponding IMP analogues as described above.

3′-Deoxy-IMP was prepared from 3′-deoxy-AMP (cordecypin monophosphate) by enzymatic deamidation of 3 mL of a 5 mM solution in 0.1 M potassium phosphate buffer, pH 6.5, with 1 mg of *Aspergillis* AMP deaminase (Sigma) overnight at room temperature. The product was purified on a Mono-Q column (5 × 5, Pharmacia) with elution at 1 mL/min by a 15 mL gradient of 0−1 M triethylammonium bicarbonate, pH 8.9. The ultraviolet absorption spectrum was consistent with that expected for an inosine monophosphate. Formycin monophosphate was converted to the IMP analogue formycin(B) monophosphate by the same method.

The p*K* values of the phosphate groups of IMP, XMP, and 2-Cl-IMP were determined by ^{31}P NMR. The p*K* values of the hypoxanthine moieties were determined by ^{13}C NMR for [2-^{13}C]IMP, by UV spectroscopy for XMP, and potentiometrically for 2-Cl-IMP. The following values were obtained: IMP (5.8 phosphate; 9.1 hypoxanthine); XMP (6.1 phosphate; 5.5 xanthine); 2-Cl-IMP (5.8 phosphate; 6.9 2-Cl-hypoxanthine).

Kinetic studies were conducted at 25 °C in 50 mM BTP/Cl, 20 mM KCl, and varying concentrations of substrate. In pH−rate profile studies solutions of BTP were adjusted to the desired pH with HCl. Experiments with 10, 20, and 30 mM KCl at the pH values of the activity maximum and extremes for the IMPDH reaction verified that the 20 mM K^+ concentration was saturating and the lack of a significant ionic strength dependence. Reactions were monitored using an HP8452A diode array spectrophotometer. The IMPDH reaction was monitored by NADH absorbance at 340 nm ($\Delta\epsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}) at varying concentrations of NAD and IMP (or an analogue thereof). 2-Cl-IMP hydrolysis was monitored by XMP production at 286 nm ($\Delta\epsilon = 6.2 \times 10^3$ M^{-1} cm^{-1}) (*26*). The inactivation of the enzyme by 6-Cl-PRT was studied by incubating enzyme with the affinity label at 25°; 40 μL aliquots of the reaction were removed at various times and added to 1 mL of the standard assay solution both to stop the reaction by dilution into excess IMP and NAD and to allow residual IMPDH activity to be determined. Kinetic data were analyzed using the equations of Cleland (*33*) as implemented in the program SCIENTIST (MicroMath, Inc.) or with the program ENZFITTER (Elsevier Biosoft). For solvent deuterium isotope effect measurements solutions containing the appropriate amounts of buffer and substrate were dried under vacuum and redissolved in either H_2O or 99% D_2O.

In order to examine whether the enzyme remains a tetramer over the pH range in which kinetic studies were conducted, a series of gel filtration experiments were performed. Enzyme was chromatographed on a 1.0 cm × 30 cm column of Superose 12 (Pharmacia). The column was eluted at a flow rate of 1 mL/min with 50 mM BTP/Cl, 50 mM KCl, and 1 mM DTT at various pH values. Elution was monitored by absorption at 280 nm. From pH 6.0 to 10.1 the protein chromatographed solely as a tetramer. Below pH 6.0, tailing of the tetramer peak indicated that dissociation was occurring during the ca. 12 min of chromatography. The kinetic data that might be affected by dissociation are the pH 5.2 data for the overall reaction; however, these data fall on the fitted lines (Figure 3), suggesting that the short reaction times and/or the presence of substrate provided reliable results.

Coordinates of the *T. foetus* IMPDH apoenzyme complex (*39*) were obtained from the PDB (code 1AK5). Coordinates of the hamster enzyme•IMP•MPA complex (*38*) were obtained from M. Sintchak, Vertex Inc., and from international patent application WO97/41211 of Vertex Inc.; these coordinates are not deposited with the PDB.

RESULTS

pH Dependence of the IMPDH Reaction. The pH dependencies over a 5 pH unit range for k_{cat}/K_m for IMP and NAD,

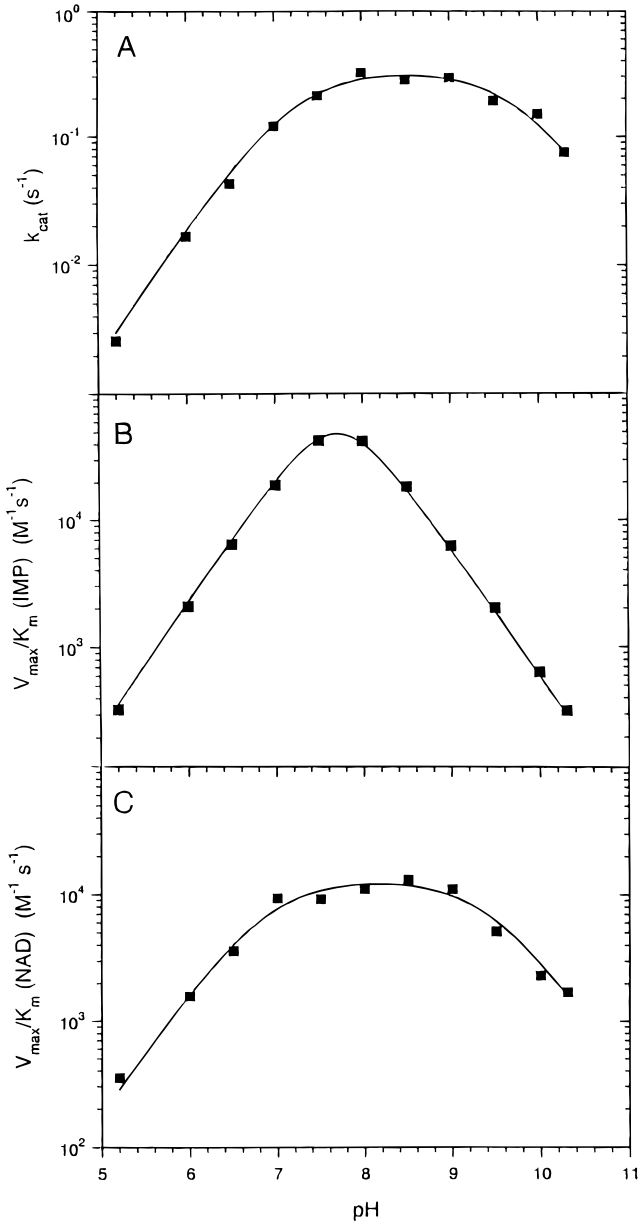

FIGURE 3: The pH dependencies of k_{cat} (A), k_{cat}/K_m for IMP (B), and k_{cat}/K_m for NAD (C). Solutions contained 50 mM BTP/Cl and 20 mM KCl. The lines are theoretical curves drawn with p*K* values for the acidic and basic groups of 7.2 and 9.8 (part A), 8.1 and 7.3 (part B), and 6.9 and 9.4 (part C).

as well as the k_{cat} profile, are all bell-shaped curves (Figure 3), indicating requirements for both protonated and deprotonated groups for maximal activity. The steady-state kinetic mechanism for IMPDH has random binding of IMP and NAD with a kinetically preferred route of IMP binding first (*21*, *22*). The major pathway has the following binding order: K^+ before IMP before NAD, and NADH release before XMP (*20*). The data here are interpreted in terms of this major kinetic pathway.

The k_{cat}/K_m profile for IMP displays p*K* values of 8.1 ± 0.2 and of 7.3 ± 0.2 for groups that must be deprotonated and protonated, respectively (Figure 3B and Table 1). Since these p*K* values are less than one unit apart they are in fact indistinguishable as noted by Cleland (33). The pH-independent k_{cat}/K_m value is only 3×10^5 M^{-1} s^{-1}, far below the diffusion limit. These p*K* values might reflect groups

Table 1: Ionizations Seen in Reactions Catalyzed by IMPDH[a]

parameter	pK_a	pK_b
IMPDH Reaction		
k_{cat}	7.2 ± 0.1	9.8 ± 0.1
k_{cat}/K_m (IMP)	8.1 ± 0.2	7.3 ± 0.2
k_{cat}/K_m (NAD)	6.9 ± 0.2	9.4 ± 0.1
2-Cl-IMP Dehalogenation Reaction		
k_{cat} (2-Cl-IMP)	NA	9.9 ± 0.2
k_{cat}/K_m (2-Cl-IMP)	NA	9.9 ± 0.3
6-Cl-IMP Inactivation Reaction		
k_{inact} (6-Cl−IMP)	7.5 ± 0.1	NB

[a] Solutions contained 50 mM BTP/HCl and 20 mM KCl. Reactions were carried out at 25 °C. NA = no decrease in rate at low pH was observed, pH 6.0 being the lowest pH tested. NB = no decrease in rate at high pH was observed.

either on the free enzyme or the substrate. Variations in NMR resonances of IMP with pH show that under these conditions IMP has p*K* values of 5.8 for the secondary ionization of the phosphate group and 9.1 for deprotonation of the hypoxanthine ring. Thus, neither p*K* value seen in the k_{cat}/K_m profile corresponds to that for IMP. The interpretation of the k_{cat}/K_m data for IMP is not straightforward since kinetic isotope effect measurements have shown that IMP is a slightly "sticky" substrate (e.g., binding is not in rapid equilibrium) and thus the kinetic p*K* values could be perturbed from the equilibrium values (*33*). Further characterization of the consequences of IMP ionization is described below from experiments in which IMP was used as a competitive inhibitor of the 2-Cl-IMP hydrolytic reaction.

The k_{cat}/K_m profile for NAD displays p*K* values of 6.9 ± 0.2 and 9.4 ± 0.1 for groups that must be deprotonated and protonated, respectively. These groups include p*K* values for the interaction of NAD with the enzyme·IMP complex. Since NAD does not have p*K* values in this range, the ionizing groups are located in the enzyme·IMP complex. The k_{cat} profile displays p*K* values of 7.2 ± 0.1 and 9.8 ± 0.1 for the E·IMP·NAD complex; these values probably reflect perturbations of the p*K* values of the same groups seen in the k_{cat}/K_m(NAD) profile. In light of studies with the affinity label 6-Cl-PRT (see below), it is probable that the p*K* values seen for the acidic group in these three profiles all reflect the ionization of the reactive cysteine-331 thiol group in altered environments. The p*K* value near 9.8 can plausibly be attributed to a requirement for a neutral purine moiety for the reaction to proceed.

The Role of Ionizable Groups in 2-Cl-IMP Hydrolysis. In order to characterize a simpler reaction catalyzed by IMPDH, the pH dependence of the 2-Cl-IMP hydrolysis reaction was determined. At pH 8.0 the reaction has a k_{cat} of 25% of the overall reaction and a K_m of 80 μM in accord with literature values (K_m of 48 μM, k_{cat} = 20% of the IMPDH reaction (*26*)). As is illustrated in Figure 4, the pH dependence of k_{cat} shows a basic p*K* of 9.9 ± 0.2, similar to the p*K* of 9.8 ± 0.1 seen in the k_{cat} profile for the overall reaction. The K_m for 2-Cl-IMP is pH-independent over the pH range studied (6.0 to 10.6); therefore, the k_{cat}/K_m profile shows the same dependence as the k_{cat} profile. Thus, neither the secondary ionization of the phosphate group nor ionization of the hypoxanthine moiety of 2-Cl-IMP (p*K* values of 5.8 and 6.9, respectively) is seen in the kinetic data, in agreement with IMP in the IMPDH reaction.

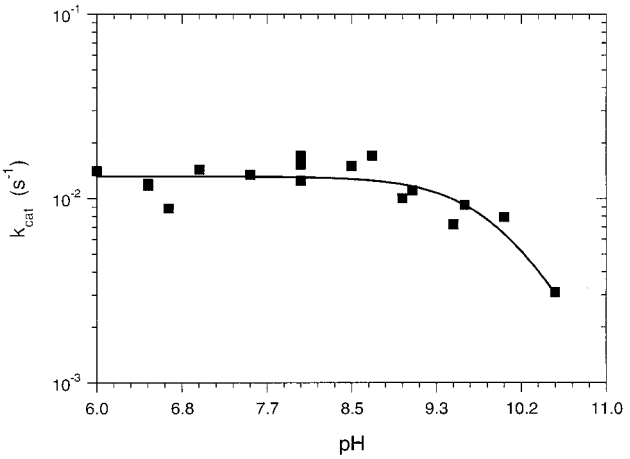

FIGURE 4: The pH dependence of k_{cat} for the 2-Cl-IMP hydrolytic reaction. Solutions contained 50 mM BTP/Cl and 20 mM KCl. The line is a theoretical curve drawn for a p*K* value of a basic group of 9.9.

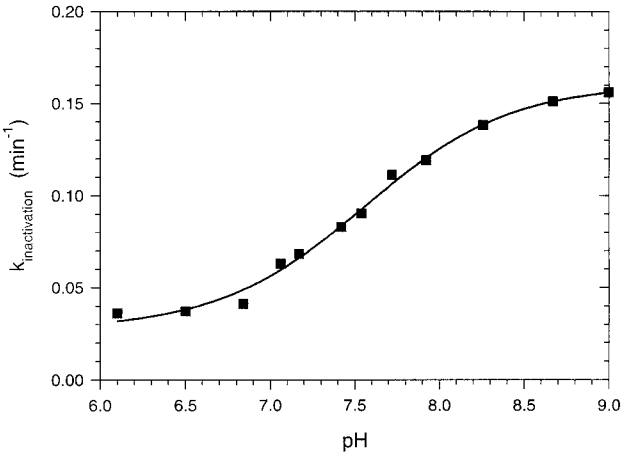

FIGURE 5: Rate of inactivation of IMPDH by 6-Cl-purine ribotide at various pH values. The line is a best fit curve for a p*K* of 7.5. Solutions contained 50 mM BTP/HCl and 20 mM KCl at the indicated pH values. Concentrations of 1.0 and 0.5 mM 6-Cl-PRT gave equivalent inactivation rates at each pH.

To further investigate the mechanism of the dehalogenation reaction, the potential presence of a primary solvent deuterium isotope effect was assessed in 99% D_2O at pH 8.0; there were no significant deuterium kinetic isotope effects on k_{cat} or k_{cat}/K_m (<10%). The k_{cat} result is analogous to the null D_2O effect reported for the IMPDH reaction (*22*); in contrast, k_{cat}/K_m effects were reported for both IMP (1.4 ± 0.1) and NAD (2.0 ± 0.2) in the overall reaction (*22*). Since 2-Cl-IMP has a higher K_m than IMP and the reaction has a lower k_{cat}, it is unlikely that the lack of a k_{cat}/K_m isotope effect solely reflects a high commitment to catalysis. Thus, the lack of a solvent kinetic isotope effect implies that proton transfer steps are relatively fast in the hydrolytic reaction.

To obtain more readily interpretable data regarding the influence of the ionization state of IMP on its affinity, the K_i for IMP inhibition of 2-Cl-IMP hydrolysis was measured at several pH values since p*K* values for competitive inhibitors are always intrinsic (33). IMP is a competitive inhibitor of 2-Cl-IMP hydrolysis, just as 2-Cl-IMP is competitive with IMP in the IMPDH reaction. The K_i for IMP was constant at 0.15 mM in the pH range 7−9.5, surprisingly suggesting that ionization of the hypoxanthine moiety (p*K* 9.1) does not dramatically affect the binding and that the ionizations seen in the k_{cat}/K_m profile for IMP reflect steps after binding.

Crystallographic results suggested that a K^+-coordinated solvent molecule might be a source of the oxygen that becomes incorporated into XMP (*38*). Thus, the monovalent cation dependence of 2-Cl-IMP hydrolysis was examined at pH 8.0 in both 0.1 M Tris/Cl and 0.1 M Bis-Tris-propane/Cl buffers; the triethylammonium salt of 2-Cl-IMP was used. Surprisingly, the 2-Cl-IMP hydrolysis reaction had the same rate in the presence of either 10 mM KCl or 10 mM $(CH_3)_4N^+ \cdot Cl^-$, even at K_m concentrations of substrate (80 μM), in contrast to the overall reaction for which the maximal rate is enhanced by more than 2 orders of magnitude by K^+. The same results were obtained at pH 5.3 and 10.5, making it unlikely that H^+ was substituting for K^+. These results indicate that the oxygen that is incorporated from solvent into XMP is unlikely to arise from a water that is activated by being a ligand to the monovalent cation. Since both the IMPDH reaction and the 2-Cl-IMP hydrolytic reaction are proposed to proceed through a common covalent thioimidate intermediate (E-IMP_{ox}) and to share the hydrolytic sequence of steps (see Figure 1), it seems that the monovalent cation facilitates a step between IMP binding and the hydrolysis of E-IMP_{ox}.

pH Dependence of the Rate of Inactivation by 6-Cl-PRT. The IMP analogue 6-chloro-purine ribotide (6-Cl-PRT) is a long known affinity label for IMPDH from a variety of species (*26*−*31*). 6-Cl-PRT has been shown to react with cysteine-331 of the human IMPDH even in the absence of NAD, and there was no detectable reduction of NAD when it was present (*31*). Under our conditions the concentration dependence of the rate of inactivation exhibited saturation behavior with a K_i of 80 μM at pH 8.7. To determine the p*K* value of the thiol of cysteine-331, the rate of inactivation of the enzyme at saturating concentrations of the inactivator was determined as a function of pH. Pseudo-first-order rates of inactivation were observed at each pH. The results show an increase in rate with pH with a p*K* of 7.5 ± 0.1 (Figure 5). This p*K* value resembles that seen in the low pH region of the k_{cat} profile for the IMPDH reaction, suggesting that the IMPDH reaction profiles reflect the p*K* for deprotonation of the thiol of cysteine-331. Previous studies of the inactivation of *Escherichia coli* IMPDH by nonsaturating concentrations of 6-Cl-PRT indicated a cysteinyl p*K* of 8.4 (*30*), which would reflect the p*K* in the free enzyme. Unfortunately other detailed pH dependencies of reactions catalyzed by the *E. coli* enzyme have not been reported.

The dependence of the inactivation rate on K^+ was investigated, using the noninteracting $(CH_3)_4N^+$ cation as a control. No effects of K^+ on the reaction rate were detected, indicating that the monovalent cation activator is not involved in activating the nucleophilic sulfhydryl group.

IMP Analogue Studies of Hydrogen-Bonding Interactions. The IMPDH active site in the crystal structure of the E-IMP_{ox}·mycophenolic acid complex of the highly homologous hamster enzyme (six of the 514 residues differ from the human enzyme) shows both side chain and main chain group hydrogens near the nucleotide (Figure 2). The ionizable residues are the side chains of the (presumably) deprotonated nucleophile cysteine-331 and aspartate-364 which interacts with both the 2′- and 3′-hydroxyls (OH2′, OH3′) of the

Table 2: Inhibition of IMPDH by IMP Analogues[a]

	K_m (mM)	K_i[b] (mM)
IMP	0.015	0.15[c,d]
2′-deoxy IMP	0.025	0.34[d]
3′-deoxy IMP		1.0
1-methyl IMP		0.23
3-deaza-IMP		0.11
7-deaza-IMP		0.35
8-aza-9-deaza-IMP[e]		0.16
purine-MP		0.97
2-Cl-IMP	0.080	0.29
inosine		>10[f]

[a] Measured in 50 mM Bis-Tris-propane/HCl and 20 mM KCl, pH 8.0, 25 °C. The uncertainties in K_i values are within 20%. [b] Measured as a competitive inhibitor with respect to IMP in the IMPDH reaction unless noted. [c] A dissociation constant of 0.1 mM was previously measured for the enzyme·IMP·K^+ complex (*20*). [d] Measured as a competitive inhibitor of 2-Cl-IMP hydrolysis. [e] Formycin (B) monophosphate. [f] No inhibition at 10 mM.

substrate ribose (*38*). The groups that are protonated are the side chains of serine-68, which interacts with OH3′, as well as serine-329 and tyrosine-411, which interact with the phosphate group. Mutagenesis studies of *E. coli* IMPDH indicate that the conserved aspartate corresponding to aspartate-364 in the human enzyme is important for IMP binding and catalysis (*44*). The importance of the interactions with the specific parts of the ribose moiety was examined using modified substrates (Table 2). At pH 8.0, 2′-deoxy-IMP is a good substrate with a k_{cat} of 55% of IMP and a K_m of 25 μM, compared to the K_m value of 15 μM for IMP, suggesting a minor importance of protein interactions with the 2′-hydroxyl group. Protein interactions with the 3′-hydroxyl group are important since 3′-deoxy-IMP has no detectable substrate activity (<0.1% of IMP) and is a weak competitive inhibitor with a K_i of 1.0 mM. Inosine at 10 mM is neither a substrate nor an inhibitor when IMP is present at its K_m value, showing the importance of interactions with the 5′-phosphate group. Studies of phosphate-substituted IMP analogues (e.g., inosine-5′-thiophosphate, inosine-5′-monofluorophosphate) with IMPDH from *Aerobacter aerogenes* led to the suggestion that the substrate preferentially binds as a dianion (*45*, *46*). Titration calorimetry studies have indicated that proton uptake is not associated with IMP binding at pH 8.0 (*47*), implying that the major solution species is the form that binds.

The crystal structures of both the hamster and *T. foetus* IMPDH show hydrogen bonds from the protein backbone to the purine at positions N1, N7, and O6, and from water to N3 (Figure 2). Since the importance of these interactions is not testable by conventional mutagenesis techniques, the influence of these interactions was evaluated using a series of IMP analogues. The compounds used for this evaluation (N1-methyl-IMP, 3-deaza-IMP, 7-deaza-IMP, 8-aza-9-deaza-IMP, and purine-riboside-5′-monophosphate) were not substrates for IMPDH (<0.1% of the activity of IMP when tested at 1 mM) (Table 2). All were competitive inhibitors with K_i values at most 10-fold larger than that of IMP, and none exhibited slow binding behavior as might occur if adducts analogous to E-IMP* formed. It is necessary to compare the K_i values with the dissociation constant for IMP rather than its K_m value since the K_m has contributions from steps after binding. The K_i values for IMP and 2′-deoxy-IMP were obtained by using them as competitive inhibitors of 2-Cl-IMP hydrolysis. The dissociation constants reflect the energetic differences between hydrogen bonding to water in the free compound and the hydrogen bonds formed at the active site (*48*). The relatively high K_i values for 3′-deoxy-IMP and purine monophosphate, both of which will be less hydrated in solution than IMP as well as lacking certain hydrogen bonds when bound, suggest that the protein forms stronger hydrogen bonds to the 3′OH and O6 of IMP than exist between both water and IMP. This is consistent with the interaction of OH3′ with the charged carboxylate of aspartate-364. For the other compounds tested, the similarity of their K_i values and that of IMP suggests similar hydrogen bonding at the altered positions in the active site and in solution. The failure of all of these compounds to act as substrates suggests that enzyme·IMP interactions that are particularly important in transition state stabilization have been lost (*49*).

Unfortunately, no crystal structures of the enzyme complexed with bound NAD or NADH have been reported, and thus detailed interpretation of results with NAD analogues would not be possible. Mutagenesis studies of the *E. coli* IMPDH indicate a role for a conserved aspartate in NAD binding (corresponding to aspartate-274 in human IMPDH) (*44*), but the p*K* of this group is probably not seen in our data.

DISCUSSION

The results of this study indicate that the p*K* of the reactive cysteine-331 sulfhydryl of human type-II IMPDH is ~7.5 in the enzyme−nucleotide complex and ~8.1 in the free enzyme. These p*K*s are within the range observed for cysteine in peptides (*50*) and near the value of 8.4 reported for the reactive cysteine of *E. coli* IMPDH (*30*). While the ionization of an acidic group is seen in all the pH profiles for the IMPDH reaction, surprisingly it is not seen in the profiles for the 2-Cl-IMP hydrolytic reaction although the two presumably share much of the reaction sequence (Figure 1). Perhaps the enhanced electrophilicity of carbon-2 upon chlorine substitution renders formation of the E-2-Cl-IMP* intermediate a rapid step. Although seemingly unlikely, it is possible that the hydrolysis of 2-Cl-IMP does not proceed through a covalent E-2-Cl-IMP intermediate but rather a direct displacement of Cl^- by water takes place. The p*K* of 7.3 in the k_{cat}/K_m profile for IMP reflects a requirement for a protonated group that is important in a step before hydrolysis of E-IMP_{ox}. This group could be N1 or N3 of the purine in E-IMP*. The p*K* near 9.8 seen in the k_{cat} and k_{cat}/K_m (NAD) profiles for the IMPDH reaction, and for 2-Cl-IMP hydrolysis, can be attributed to the requirement of the purine in E-IMP_{ox} or E-XMP* to be protonated at N1, and hence uncharged, for the reaction to proceed. The results of spectroscopic studies investigating the protonation states of enzyme-bound substrate and product complexes should resolve these uncertainties.

Our studies with a variety of purine-altered IMP analogues were designed to probe the importance of crystallographically observed hydrogen-bonding interactions between the substrate and enzyme, many of which come from the peptide backbone (*38*, *39*, *51*). The majority of the IMP analogues tested were not substrates for the IMPDH reaction and all tested inhibitors had affinities at most 10-fold weaker than IMP. The effects of substitutions remote from the reactive

C2−N3 region of IMP are consistent with the greater importance of enzyme·substrate interactions in these regions in transition state stabilization than in initial complex formation, as has been inferred in the case of cytidine deaminase for example (*49*).

The role of K^+ in activating the IMPDH reaction has been an enigma. An unexpected finding of this study is that there are no significant K^+ effects on either the rate of 2-Cl-IMP hydrolysis or of inactivation by 6-Cl-IMP, in contrast to the overall reaction. Thus the nucleotide can bind productively to the enzyme in the absence of K^+; this is in accord with previous equilibrium binding studies which showed the presence of one binding site per subunit for IMP and XMP even in the absence of a monovalent cation activator (*20*). Apparently K^+ is not responsible for increasing the nucleophilicity of the sulfhydryl of cysteine-331. Furthermore, a K^+-coordinated water is apparently not the nucleophile in the hydrolysis of the covalent E-IMP_{ox} intermediate.

Kinetic studies of IMPDH from *A. aerogenes* led Heyde et al. to propose that K^+ induces a conformational change which is required for NAD binding (*17, 18*). More recently, circular dichroism and proteolytic susceptibility studies (*52*), as well as titration calorimetry studies (*47*), have indicated the presence of a conformational change when IMP or XMP binds to hamster type-II IMPDH; effects of K^+ were not reported in those studies. No evidence for NAD binding in the absence of IMP has been obtained for either hamster or human IMPDH. The present results are consistent with important K^+-induced structural alterations, specifically in the NAD-dependent segment of the IMPDH reaction. The structural nature of the probable K^+-provoked conformational change remains to be elucidated.

NOTE ADDED IN PROOF

The crystal structure of human type-2 IMPDH has recently been solved as a complex of the 6-Cl-PRT-modified enzyme with the NAD analogue selenazofurin adenine dinucleotide (*53*).

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