Human IMP Dehydrogenase Catalyzes the Dehalogenation of 2-Fluoro- and 2-Chloroinosine 5′-Monophosphate in the Absence of NAD

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ABSTRACT: The ability of human type II inosine monophosphate dehydrogenase (IMPDH, EC 1.1.1.205) to catalyze the formation of xanthosine 5′-monophosphate (XMP) from C2 halogen-substituted analogs of IMP has been investigated. Adenosine deaminase was used to enzymatically synthesize 2-fluoroinosine and 2-chloroinosine from the 2-fluoro- and 2-chloroadenosine nucleoside analogs. Chemical phosphorylation yielded the corresponding 5′-nucleoside monophosphate derivatives. IMPDH catalyzes the conversion of both 2-fluoro- and 2-chloroinosine 5′-monophosphate (2-F- and 2-Cl-IMP) to XMP. The dehalogenation reactions proceed without nicotinamide adenine dinucleotide (NAD), the hydride acceptor required for the oxidation of IMP, the normal substrate of the enzyme. Formation of XMP from the 2-halo-IMPs was verified by UV absorption spectroscopy and by HPLC. Formation of XMP from 2-F-IMP yielded stoichiometric amounts of fluoride anion. IMP and XMP were competitive inhibitors toward 2-Cl-IMP in the dehalogenation reaction. Neither 2-F-IMP nor 2-Cl-IMP irreversibly inactivate IMPDH. Kinetic constants for the dehalogenation reactions have been determined and compared to the dehydrogenation reaction at 25 °C. (For 2-F-IMP: k_{cat} = 0.058 s^{-1}, K_m = 62 μM. For 2-Cl-IMP: k_{cat} = 0.049 s^{-1}, K_m = 48 μM. For the IMP dehydrogenation reaction: k_{cat} = 0.25 s^{-1}, K_m [IMP] = 4.1 μM, K_m [NAD] = 29 μM.) Hydrolytic dehalogenation of 2-halo-IMPs without a requirement for NAD demonstrates the formation of a tetrahedral intermediate in the catalytic mechanism of IMP dehydrogenase.

Inosine monophosphate dehydrogenase (IMPDH,[1] EC 1.1.1.205) catalyzes the nicotinamide adenosine dinucleotide (NAD) dependent oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP). This reaction is the committed step in the biosynthesis of guanine nucleotides. Because of the cellular requirements for guanine nucleotides, levels of IMPDH in many cells are elevated during rapid proliferation (Jackson *et al.*, 1975; Natsumeda *et al.*, 1988; Nagai *et al.*, 1991; Collart *et al.*, 1992). Inhibitors of IMPDH activity appear to suppress biological processes that depend on cellular proliferation and are being investigated for their effects on immunosuppression and as antiviral and antitumor agents (Streeter *et al.*, 1973; Jackson *et al.*, 1975; Weber, 1983; Liu *et al.*, 1984; Sidi *et al.*, 1988; Tricot *et al.*, 1989; Eugui *et al.*, 1991; Morris *et al.*, 1991). Two isozymes of IMPDH have been identified in human tissues (Natsumeda *et al.*, 1988). The type I enzyme appears to be constitutively expressed, whereas the type II isoform is induced during cellular proliferation (Nagai *et al.*, 1991, 1992).

The kinetic properties of IMPDH from a variety of sources have been studied (Anderson *et al.*, 1968; Brox & Hampton, 1968; Buzzee & Levin, 1968; Ishii & Shiibo, 1968; Yokosawa *et al.*, 1971; Wu & Scrimgeour, 1973; Holmes *et al.*, 1974; Heyde & Morrison, 1976; Heyde *et al.*, 1976; Jackson *et al.*, 1977; Gilbert *et al.*, 1979; Okada *et al.*, 1983; Hupe *et al.*, 1986; Collart & Huberman, 1987; Verham *et al.*, 1987; Carr *et al.*, 1993). IMPDH catalyzes an ordered bi–bi kinetic mechanism in which IMP binds prior to NAD, and NADH dissociates prior to XMP (Holmes *et al.*, 1974; Hedstrom & Wang, 1990; Carr *et al.*, 1993). This order of substrate binding is unusual among the NAD-dependent dehydrogenases, which in general display random kinetics or require NAD association with enzyme prior to the other substrate. Subsequent to binding of substrates, the reaction can proceed via one of two possible mechanisms. One mechanism involves the direct addition of a hydroxide at the C2 position, assisted by a catalytic group in the enzyme which functions as a general base, prior to hydride transfer to NAD (Hedstrom & Wang, 1990). After hydride transfer, removal of a proton from the hydroxyl oxygen or possibly at either the N1 or N3 nitrogen, also assisted by a general base at the enzyme's active site, leads to the final product, XMP. A second mechanism requires the formation of a covalent tetrahedral enzyme–substrate intermediate, formed by the attack at the C2 position by an enzymatic nucleophile, perhaps a cysteine sulfhydryl group, prior to hydride transfer. In the covalent mechanism, XMP formation follows the hydrolytic cleavage of the bond between C2 and the enzymatic nucleophile. These two mechanisms are summarized in Scheme 1. In both mechanisms, hydride transfer is facilitated by formation of a tetrahedral center at the C2 of IMP.

Formation of tetrahedral or covalent intermediates in enzymatic reactions has been demonstrated by means of halogen-substituted substrate analogs in many cases, for example, peptide cleavage by proteases, and methylation of pyrimidine nucleotides at the C5 position by thymidylate synthase and DNA-cytosine methylases. [For a review of enzyme inhibition by fluoro compounds, see Abeles and Alston (1990).] In the reaction catalyzed by IMPDH, alternative substrates with halogen substitutions on the purine ring may likewise be used to demonstrate the formation of a tetrahedral intermediate and are especially interesting in this respect. If a tetrahedral intermediate at the C2 position of the purine ring is indeed formed, then one would predict that replacement

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[1] Abbreviations: IMPDH, inosine 5′-monophosphate dehydrogenase; IMP, inosine 5′-monophosphate; 2-F-IMP, 2-fluoroinosine 5′-monophosphate; 2-Cl-IMP, 2-chloroinosine 5′-monophosphate; XMP, xanthosine 5′-monophosphate; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form, DTT, dithiothreitol.

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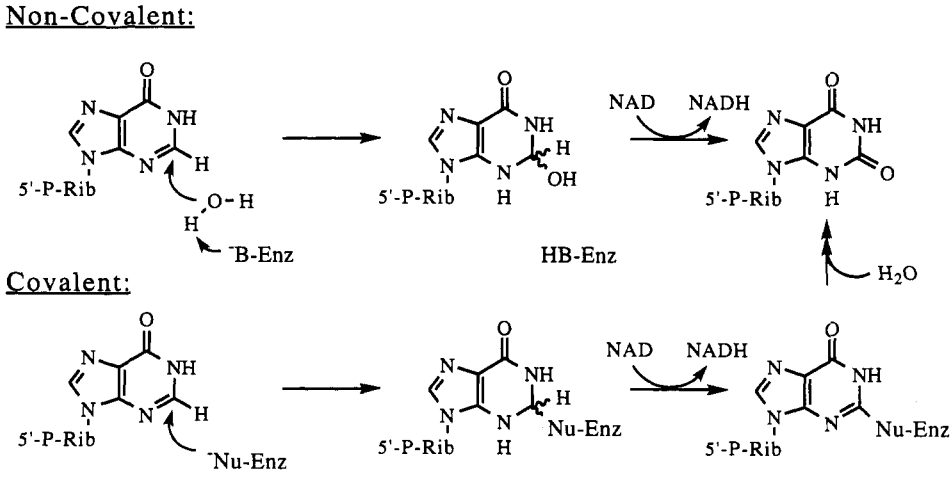
Scheme 1



of hydrogen at C2 by a halogen atom should yield an analog of IMP that may form a covalent adduct with the enzyme or be catalytically dehalogenated.

We have synthesized two IMP analogs, the C2 halo-substituted nucleotides[2] 2-F-IMP and 2-Cl-IMP in order to probe IMPDH for its mechanism of catalysis and its active site amino acids. Here we present studies on the ability of recombinant human type II IMPDH to catalyze turnover of 2-halo-IMPs as alternative substrates.

MATERIALS AND METHODS

Preparation of 2-F-IMP and 2-Cl-IMP. 2-Fluoroadenosine (107 mg, 318 μmol, 84.7% pure by HPLC, gift of John Montgomery, Southern Research Institute, Birmingham, AL) was deaminated to 2-fluoroinosine enzymatically by adenosine deaminase (1000 units, EC 3.5.4.4 from calf intestinal mucosa, obtained from Sigma) in H_2O (40 mL) at 37 °C for 24 h. The syntheses of 2-fluoroadenosine and 2-chloroadenosine have been reported previously (Montgomery & Hewson, 1964, 1968). Adenosine deaminase primarily catalyzes the reaction of adenosine to inosine (Brady & O'Connell, 1962; Murphy *et al.*, 1969). We have found the calf intestinal enzyme to be effective in the deamination of 2-fluoroadenosine to 2-fluoroinosine and 2-chloroadenosine to 2-chloroinosine, although it has been reported that 2-fluoroadenosine is a poor substrate of adenosine deaminase (Chilson & Fisher, 1963; Frederiksen, 1966; Agarwal *et al.*, 1975). The course of the reaction was monitored by HPLC using a Rainin Microsorb ODS "Short-One" column (C18-200, 3-μm particle size, 100-Å pore size, 4.6 × 10 mm) and a solvent system consisting of 9% acetonitrile and 91% 40 mM KH_2PO_4, pH 3.3, with a flow rate of 1 mL min^{-1}. The product was purified on a DEAE–Sepharose column. Phosphorylation of the 2-fluoroinosine by phosphoryl chloride was performed in triethyl phosphate at 4 °C according to the method described by Yoshikawa *et al.* (1967). The products of phosphorylation were analyzed by reversed-phase HPLC using a Rainin Microsorb ODS "Short-One" column with a solvent system containing an ion-pairing reagent (5 mM tetrabutylammonium phosphate in 0.1 M phosphate, pH 6) with a 0–50% MeOH gradient. The phosphorylation reaction was quenched by addition of a saturated bicarbonate solution, and the product was further purified by HPLC on a DYNAMAX semipreparative C18 column (Rainin) in the same solvent system. The 2-Cl-IMP was prepared in a similar manner using 2-chloroadenosine (98 mg, 325 μmol, Sigma) as the starting material. The syntheses of 2-F- and 2-Cl-IMP by other methods have been reported previously (Amarnath & Broom, 1982; Wong & Meyer, 1984).

Type II Human IMPDH. The enzyme preparation in these experiments is a recombinant fusion form of type II human IMPDH expressed in the *Escherichia coli* strain H 712, which is deficient in bacterial IMPDH activity (Nijkamp & De Haan, 1967; Collart & Huberman, 1988; Natsumeda *et al.*, 1990; Konno *et al.*, 1991). The enzyme was generated by *Bal*31 nuclease mediated truncation of the amino terminal sequence of a human type II cDNA inserted into the plasmid pBluescript SK^+ (Stratagene). The enzyme is constitutively expressed in H 712 cells and complements growth of H 712 cells in M9 minimal medium. Clones expressing active protein were selected from M9 agar. In the fusion enzyme a seven amino acid sequence (MTMITPS) of β-galactosidase replaces the terminal methionine of the sequence deduced for the native enzyme. The protein was purified to homogeneity as described by Carr *et al.* (1993). Analysis by Edman degradation and mass spectrometry verified the amino terminal sequence and indicated that the initiation methionine at the amino terminus is cleaved from the purified protein. The kinetic properties of the fusion enzyme are indistinguishable from the native enzyme purified from the human T-cell lymphoma cell line A3.01 (J. Wu, unpublished data). The protein concentration was determined by Bradford assay, and the calculated monomeric molecular weight is 56 304 g mol^{-1}. The native human type II IMPDH exists as a tetrameric protein in solution (Carr *et al.*, 1993).

Enzymatic Activity Assay. The standard assay solution contained 0.1 M Tris, 0.1 M KCl, 3 mM EDTA, 200 μM IMP, and 400 μM NAD at pH 8.0. The 2-F-IMP or 2-Cl-IMP assay mixture contained 200 μM 2-halo-IMP in 0.1 M Tris, 0.1 M KCl, 3 mM EDTA, pH 8.0, without NAD, unless otherwise specified. The 2-halo-IMP concentration was determined spectrophotometrically using an extinction coefficient of 12 300 M^{-1} cm^{-1} at 256 nm (λ_{max}). The IMPDH concentrations used for the IMP/NAD assay were 0.02–0.05 μM, and for the 2-halo-IMP assay, 0.3–0.5 μM. The assays were performed at 25 or 40 °C, as specified, using a Hewlett-Packard HP8452 diode array spectrophotometer with a water-jacketed multicell transport carriage, and the change in absorbance was measured either at 340 nm (NADH formation) or at 284 or 286 nm (XMP formation).

[2] 2-F-IMP and 2-Cl-IMP are collectively referred to as 2-halo-IMP.

The initial velocities of the dehalogenation reactions were also measured at varying concentrations of 2-halo-IMP (10–200 μM) in the absence of NAD at 25 °C with 0.3 μM IMPDH. These experiments were performed at 25 °C to optimize enzyme stability. Steady-state kinetic constants were calculated using Cleland's algorithms (1979).

Fluoride Release from 2-F-IMP. A fluoride electrode (Corning) and a double-junction reference electrode (Microelectrodes, Inc.) were used with a Corning Model 240 pH meter in the millivolt mode for the fluoride concentration measurements. The reaction was carried out in a 30-mL Pyrex beaker containing 4.73 mL of buffer (0.1 M Tris, 0.1 M KCl, 3 mM EDTA, pH 8.0) and 250 μL of a 4.3 mM aqueous solution of 2-F-IMP with the fluoride and reference electrodes submersed in the buffer. The reaction was initiated by the addition of 20 μL of IMPDH to give a final enzyme concentration of 0.5 μM. Readings of the millivolts were recorded as a function of time and were converted to [F^-] using the calibration data. In order to simultaneously measure the rate of XMP formation in the reaction, samples of the 2-F-IMP/IMPDH reaction mixture (above) were removed periodically and used to measure the [XMP] by

$$[\mathrm{XMP}]_t = \frac{[A_{276(t)} - A_{276(0)}]^*\mathrm{DF}}{[\epsilon_{276}(\mathrm{XMP}) - \epsilon_{276}(\text{2-F-IMP})]}$$

where $A_{276(t)}$ is the absorbance reading at time, t, at 276 nm, $A_{276(0)}$ is the absorbance reading at $t = 0$ and DF is the dilution factor. The product was analyzed by reversed-phase HPLC using a Rainin Microsorb ODS "Short-One" column with the same ion-pairing system used for 2-halo-IMP purification.

Inhibition of IMPDH by 2-Cl-IMP with IMP and NAD as the Substrates. Kinetic data for the dehydrogenation of IMP (5–80 μM) in the presence of varying concentrations of 2-Cl-IMP (0, 30, 60, and 90 μM) were determined at 40 °C in 0.1 M KH_2PO_4, pH 7.9, 0.5 M KCl, 2 mM EDTA, and 2 mM DTT with a fixed concentration of NAD (100 μM). The phosphate buffer containing a higher salt concentration was employed to insure stability of the enzyme at the higher temperature. The reactions were monitored by following the change in absorbance at 340 nm (NADH formation) and 284 nm (XMP formation) with time, and the inhibition constant, K_i, and K_m for 2-Cl-IMP were determined.

Inhibition of IMPDH by IMP and XMP with 2-Cl-IMP as the Substrate. Kinetic data for the dehalogenation of 2-Cl-IMP in the presence of varying concentrations of IMP or XMP were determined at 25 °C in 0.1 M Tris, 0.1 M KCl, 3 mM EDTA, pH 8.0, and 0.3 μM IMPDH. These experiments were performed at 25 °C to optimize enzyme stability. The reactions were monitored by following the change in absorbance at 284 nm with time, and the inhibition constants were determined.

RESULTS

Synthesis of 2-Cl-IMP and 2-F-IMP. The deamination of 2-chloroadenosine and 2-fluoroadenosine proceeded with 100% conversion to 2-haloinosine, as shown in the HPLC traces and accompanying spectra in Figure 1. The chemical phosphorylation step gave only a 12% yield for 2-F-IMP and 28% for 2-Cl-IMP. Some material was lost in the reactions as mixed diphosphates and unreacted starting material. Further losses were incurred during purification by DEAE–Sephacel ion exchange chromatography and preparative reversed-phase HPLC. The 2-F-IMP (16 mg) contained an inert byproduct and was approximately 87% pure as judged by HPLC (Figure 4A) and NMR (not shown). The 2-Cl-IMP (35 mg) was judged to be nearly 100% pure by HPLC (Figure 4C) and NMR (not shown).

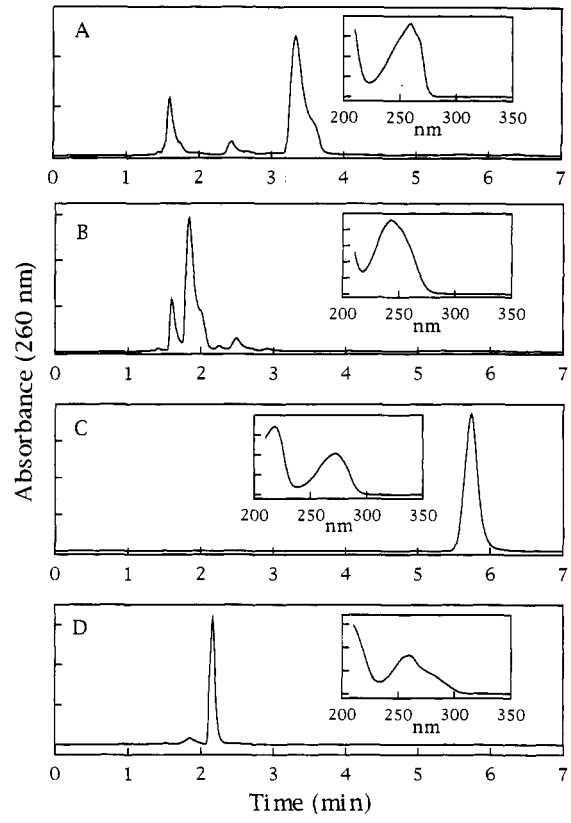


FIGURE 1: HPLC profiles and spectra (small inserts) of (A) 2-F-Ado, (B) 2-F-Ino, (C) 2-Cl-Ado, and (D) 2-Cl-Ino. The compounds were analyzed on a Rainin Microsorb ODS "Short-One" column using an isocratic solvent system consisting of 9% acetonitrile and 91% 40 mM KH_2PO_4, pH 3.3, and a flow rate of 1 mL min^{-1}. UV absorbances of HPLC time traces and spectra are relatively scaled.

IMPDH-Catalyzed Formation of XMP from 2-Cl-IMP. The formation of XMP from 2-Cl-IMP was monitored spectrophotometrically at 286 nm and compared to the IMP dehydrogenation reaction in the same conditions (Figure 2A). The time-dependent difference spectra for the 2-Cl-IMP to XMP reaction, shown in Figure 2, parts B and C, indicate the formation of a product with the characteristic absorption spectrum of XMP. Dehalogenation of 2-Cl-IMP required IMPDH (Figure 2A, reaction b vs a), and the reaction proceeded in the absence of NAD. Dehalogenation cannot be catalyzed by thiols in solution; DTT at 10 mM failed to generate XMP over extended times of incubation (>60 min). The enzyme is not irreversibly inactivated by 2-Cl-IMP under these conditions. The data indicate catalytic turnover of 2-Cl-IMP by IMPDH at approximately one-third the rate observed for the NAD-dependent dehydrogenation reaction at identical substrate concentrations.

Measurement of IMPDH-Catalyzed F^- Release from 2-F-IMP and Concomitant XMP Formation. The formation of XMP from 2-F-IMP was demonstrated by UV absorbance as in the case of 2-Cl-IMP. Moreover, the expected formation of fluoride ion upon the conversion of 2-F-IMP to XMP was confirmed by means of a fluoride electrode (Figure 3). The reaction shown in Figure 3 was analyzed continuously for F^- release. Samples from the same reaction were taken to simultaneously measure the rate of XMP formation by absorbance. The samples were diluted in 0.1 M NaOH, and the absorbance at 276 nm (λ_{max} for XMP in basic pH) was

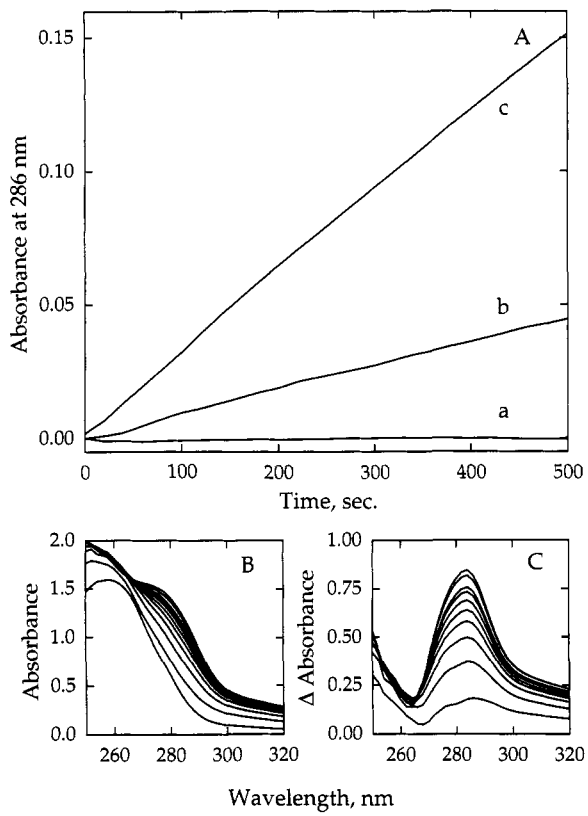


FIGURE 2: (A) Comparison of rates of IMPDH-catalyzed formation of XMP from IMP/NAD and from 2-Cl-IMP at 25 °C monitored by UV absorbance at 286 nm: (a) 2-Cl-IMP with no IMPDH, (b) 2-Cl-IMP with IMPDH, (c) IMP and NAD with IMPDH. The dehydrogenation reaction was performed in 200 μM IMP and 400 μM NAD in Tris buffer, pH 8.0, and the 2-Cl-IMP experiments were performed in the same buffer containing 200 μM 2-Cl-IMP. [IMPDH] was 0.3 μM. Part B shows the absorbance spectra of reaction of 2-Cl-IMP (200 μM) with IMPDH to form XMP taken at 5-min time intervals (in Tris, pH 8.0, 25 °C). Part C is the difference spectra of B obtained by subtracting the 2-Cl-IMP spectrum from spectra taken at time points.

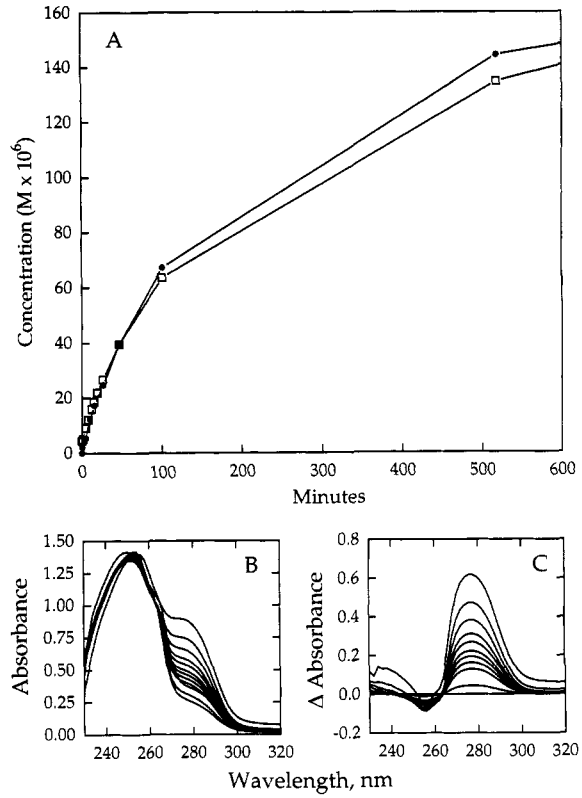


FIGURE 3: (A) Reaction of 2-F-IMP (200 μM) with IMPDH (0.5 μM in 0.1 M Tris, 0.1 M KCl, 3 mM EDTA, pH 8.0) as monitored by UV absorbance and F^- release: (●) XMP formation; (□) fluoride release. Part B shows the absorbance spectra of reaction of 2-F-IMP (200 μM) with IMPDH to form XMP. Part C is the difference spectra of B obtained by subtracting the 2-F-IMP spectrum from spectra taken at time points.

measured. The calculated rates of XMP formation, based on UV absorbance and fluoride ion release, are shown in Figure 3A. The time-dependent UV absorbance and difference spectra are shown in Figure 3, parts B and C, respectively. An initial rate of 1.02×10^{-6} M min^{-1} was determined for F^- release. The initial rate of XMP formation calculated by UV absorbance was 1.04×10^{-6} M min^{-1}, in agreement with the rate of F^- release. Thus both the rate and stoichiometry of the formation of fluoride ion correspond to those of XMP throughout the course of the reaction.

HPLC Analysis of Reaction Products. The formation of XMP in both dehalogenation reactions was verified by analysis on reversed-phase HPLC. HPLC profiles showing the conversion of 2-F-IMP or 2-Cl-IMP to XMP by IMPDH are shown in Figure 4. The 2-F-IMP sample contains an impurity which was not well-resolved by preparative HPLC. This peak eluted at 17.4 min and did not change in the course of the reaction. It is not known whether this impurity, which is calculated to be ~13% of the sample, inhibits this reaction or whether it affects the derived kinetic values for the defluorination reaction. No XMP was formed from 2-F-IMP or 2-Cl-IMP in the absence of IMPDH under these experimental conditions or in the presence of up to 10 mM DTT, as confirmed by UV absorbance as well as by HPLC.

Comparison of IMPDH-Catalyzed Formation of XMP from IMP/NAD, 2-F-IMP and 2-Cl-IMP, and Steady-State Kinetics. At identical substrate concentrations (200 μM), the rate of IMPDH-catalyzed dehalogenation of 2-F-IMP to XMP was approximately 3 times slower (not shown) and the reaction with 2-Cl-IMP was approximately 4 times slower than the reaction with the normal substrates, IMP and NAD (Figure 2A). Neither of the dehalogenation reactions required NAD. Inhibition of dehalogenation by NAD was examined at NAD concentrations up to 400 μM. At NAD concentrations above 300 μM, a modest decrease in the rate was observed (30% decrease with 400 μM NAD, 150 μM 2-Cl-IMP using 0.3 μM IMPDH at 25 °C). Due to interference of the dehalogenation assay by NAD because of its UV absorbance at 284 nm, an exact determination of the K_i of NAD in the dehalogenation reactions was not possible.

The steady-state kinetic parameters for all three reactions have been determined and are summarized in Table 1. The K_m values for the halo-IMPs are approximately 1 order of magnitude greater than that of IMP, while the k_{cat} values for the dehalogenation reactions are approximately 4-fold slower than that for IMP dehydrogenation at 25 °C. The k_{cat}/K_m values suggest a significant decrease in the catalytic specificity of the enzyme toward dehalogenation relative to dehydrogenation—a greater than 50-fold decrease was observed.

Inhibition of IMP Dehydrogenase Activity by 2-F-IMP and 2-Cl-IMP. When IMPDH was incubated with 2-Cl-IMP (100 μM to 1 mM, 25 and 40 °C, 1–25 min) and tested for activity at various time intervals with saturating concentrations of IMP and NAD at 40 °C (standard enzymatic assay procedure, see Materials and Methods), no loss of activity was observed. Thus the enzyme is not inactivated by 2-Cl-IMP under these conditions. However, when the rate of

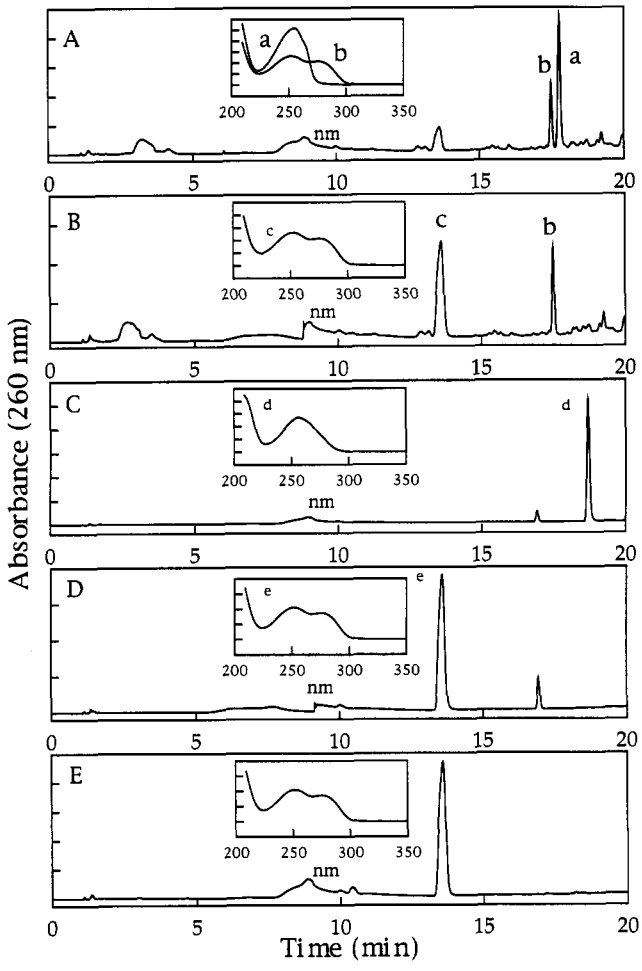


FIGURE 4: Reversed-phase HPLC profiles and spectra of (A) 2-F-IMP (elution time 17.4 min, spectrum a), (B) the reaction of 2-F-IMP with IMPDH demonstrating XMP formation (spectrum c), (C) 2-Cl-IMP (elution time 18.7 min, spectrum d), (D) the reaction of 2-Cl-IMP with IMPDH demonstrating XMP formation (the inert impurity elutes at 17.8 min, spectrum b), and (E) XMP standard (elution time 13.5 min) on Rainin Microsorb ODS "Short-One" column (Rainin) using ion-pairing buffer system. UV absorbances of HPLC time traces and spectra are relatively scaled. See text for further experimental details.

Table 1: Summary of the Kinetic Parameters for IMP, 2-F-IMP, and 2-Cl-IMP at 25 °C[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})
IMP	4.1 ± 0.5	0.25 ± 0.03	59
2-F-IMP	62 ± 5	0.06 ± 0.01	0.94
2-Cl-IMP	48 ± 5	0.05 ± 0.01	1.0

[a] In 0.1 M Tris, 0.1 M KCl, and 3 mM EDTA, pH 8.0, 25 °C.

catalysis of IMPDH with 200 μM IMP and 400 μM NAD was measured in the presence of varying concentrations of 2-F-IMP (25–250 μM) or 2-Cl-IMP (50 μM to 1.5 mM) in the reaction mixture, a decrease in the rate with increasing 2-halo-IMP concentration (as measured by NADH formation at 340 nm) was observed. This apparent inhibition is an expected behavior of alternative substrates. The mode of inhibition by 2-Cl-IMP, determined as described in the Materials and Methods, was competitive, and the K_i of 2-Cl-IMP at 40 °C was determined to be 57 ± 7 μM. The K_m of 2-Cl-IMP under these conditions was 115 ± 3 μM. The data used to calculate the K_m of 2-Cl-IMP was obtained by subtracting the rate of NADH formation (measured at 340 nm for the reaction of IMP and NAD to form XMP and NADH) from the total XMP formation (measured at 284 nm for the reaction of both IMP and 2-Cl-IMP with the enzyme). The resulting rate corresponds to the formation of XMP in the reaction of 2-Cl-IMP with IMPDH. The higher K_m at 40 °C (115 μM vs 48 μM at 25 °C) may be attributed to the temperature dependence of k_{cat}, which contributes to the value of K_m.

Inhibition of 2-Cl-IMP Dehalogenation by IMP and XMP. Both IMP and XMP were competitive inhibitors of the 2-Cl-IMP reaction. The K_i for IMP is 25 μM and for XMP, 27 μM. The results suggest that 2-Cl-IMP, IMP, and XMP bind to the same form of the enzyme, and the substrates probably react at the same site on the enzyme. The observation that NAD is a poor inhibitor of 2-Cl-IMP dehalogenation further supports the possibility of a common site for the 2-halo-IMPs and IMP. The reactivity of the halo-IMPs suggest that the IMP binding site may be occupied independently by an analog of IMP without affecting all of the essential catalytic functions of the enzyme.

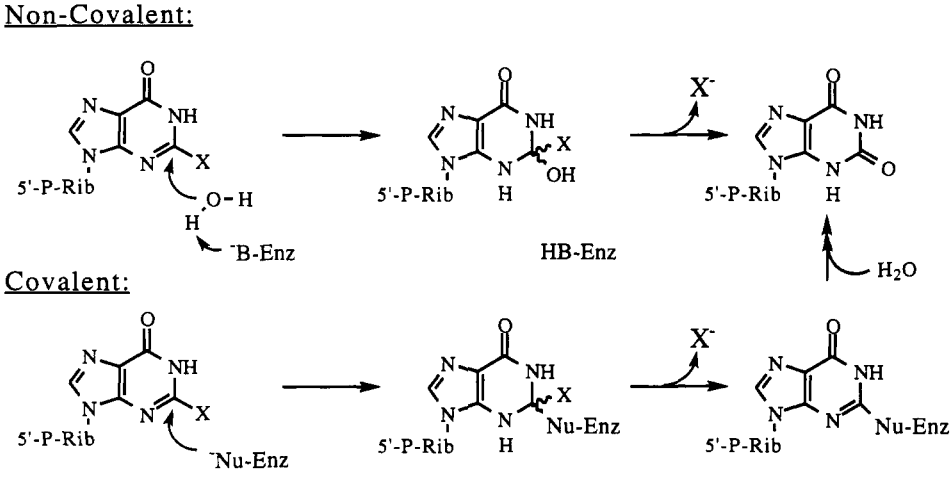
DISCUSSION

There were three possible outcomes for the interaction of the 2-halo-IMPs with IMPDH. The compounds might have behaved as reversible inhibitors, irreversible inhibitors, or alternative substrates. The experiments described herein demonstrate that human IMP dehydrogenase can catalyze the hydrolytic dehalogenation of 2-halo-IMPs without NAD. We have shown by spectral and HPLC analyses, and by measurements of fluoride release from 2-F-IMP, that human type II IMPDH catalyzes the formation of XMP and halide anions from 2-halo-IMPs (Figures 2–4). XMP is the product of the dehydrogenase reaction normally catalyzed by the enzyme, and its formation indicates that the enzyme mediates 2-dehalogenation via a hydrolytic mechanism similar to the mechanisms proposed for the normal dehydrogenation reaction.

The rates of the dehalogenation are nearly equivalent for 2-F-IMP and 2-Cl-IMP, with turnover rates of approximately 0.05 s^{-1} at 25 °C. These rates are within 1 order of magnitude of the rate observed for the dehydrogenation reaction under similar conditions (0.25 s^{-1}) (Table 1). The kinetics of dehalogenation indicate that the 2-halo-IMPs behave as IMP substitutes for the enzyme. Our results indicate that IMP is a competitive inhibitor of the dehalogenation reaction in the absence of NAD and suggest that the enzyme's active site for dehalogenation overlaps the site used for dehydrogenation. The converse, inhibition of IMP dehydrogenation by 2-Cl-IMP, also indicates competition for a single site on the enzyme. The K_i of 2-Cl-IMP for human type II IMPDH at 40 °C (57 μM) is within 1 order of magnitude of the K_i value (120 μM) reported for the *E. coli* IMPDH in a study of 2-substituted inosinic acids as inhibitors of IMPDH (Wong & Meyer, 1984). The ability of the enzyme to use 2-halo-IMPs as alternative substrates has not been reported.

NAD is not a required cofactor for the dehalogenation reaction and does not inhibit the reaction at concentrations below 300 μM. At 25 °C the K_m of NAD in the dehydrogenation reaction with IMP is approximately 30 μM. Thus dehalogenation is not inhibited at NAD concentrations that are saturating for dehydrogenation. We did not determine the accurate K_i for NAD because high concentrations (>400 μM) of NAD interfered with the UV absorbance assay for XMP formation. As in the dehydrogenation reaction, NAD concentrations above 300 μM are inhibitory in the dehalogenation reactions. The substrate inhibition at high concentrations of NAD could be due to a spill-over of NAD from

Scheme 2



the dinucleotide site into the IMP binding site, since both substrates are nucleotides. Thus the enzyme appears not to employ the NAD binding site for C2 dehalogenation, and binding interactions between NAD and IMPDH are separable from the IMP binding and some of the addition–elimination activities of the enzyme.

The absence of an NAD requirement for the formation of XMP by dehalogenation suggests that the enzyme can catalyze half of the overall dehydrogenation reaction without NAD involvement at the active site. Both mechanisms in Scheme 1 consist of multiple steps along the reaction pathway. The noncovalent mechanism requires the addition of hydroxide at C2, whereas the covalent mechanism involves attack of C2 by an enzymic nucleophile. If there were an E-S intermediate in the catalytic mechanism, then this complex might be trapped with the 2-halo-IMP compounds, as was found for the 6-Cl-IMP analog (Antonino *et al.*, 1994). However, we did not observe irreversible inactivation by the 2-halo compounds, nor were we able to trap an E-I intermediate with our experimental procedure. The compounds behaved as alternative substrates, with halide elimination accompanying the formation of XMP. This observation does not exclude the possibility of an E-S intermediate, because an E-S complex formed from the 2-halo-IMPs should be identical to the intermediate formed in the normal reaction if the covalent mechanism were operative in this reaction. Such an intermediate should be competent to generate XMP regardless of the substrate. Both the noncovalent and the covalent mechanisms can be invoked to explain the dehalogenation reaction (Scheme 2). In the noncovalent mechanism, hydroxylation at C2 forms a tetrahedral intermediate which should rapidly collapse to form XMP via halide elimination. In the covalent mechanism, attack of C2 by an enzymic nucleophile forms a similarly labile tetrahedral intermediate, which can collapse via halide elimination to form the enzyme-bound intermediate. Hydroxylation and elimination of the enzymic nucleophile to generate XMP completes the reaction. These transformations are most likely assisted by a basic group in the active site of the enzyme. The base which assists in the hydride-transfer step in the normal reaction may serve the same role in dehalogenation. The catalytic residues that activate H_2O for attack at C2 in either mechanism are not known, but there does not appear to be a cationic metal at the active site. The enzyme is active in the presence of high concentrations of EDTA and other chelating agents, and one likely candidate metal ion, zinc, has been ruled out by atomic absorption spectroscopy of the enzyme (unpublished observations).

The two mechanisms are distinguished by several important features which apply to both the dehalogenation and dehydrogenation reactions. In the noncovalent mechanism, hydroxylation at C2 precedes elimination of the halide or transfer of hydride, and carbonyl formation occurs concertedly or at least in the immediately succeeding step. In the covalent mechanism, halide elimination or hydride transfer is separated from XMP formation by an intervening hydroxylation step and a covalent intermediate common to both dehalogenation and dehydrogenation. Our results do not discriminate between the two mechanisms. The similar k_{cat} values suggest a common intermediate for the three substrates (Table 1), but the possibility of alternative steps that become rate determining in the dehalogenation reaction pathway cannot be ruled out. For example, in the covalent scheme, if the rate-determining step for dehydrogenation were hydrolysis of the covalent intermediate, then the smaller k_{cat} for dehalogenation may be due to a rate-determining addition to C2 or elimination of halide prior to the formation of the covalent intermediate.

The demonstration of dehalogenation in addition to the hydride-transfer activity of IMPDH suggests a significant degree of physical independence between the IMP and NAD binding sites. Thus, 2-Cl-IMP and 2-F-IMP may be used as substrates to distinguish between inhibitors or covalent modification reagents which affect exclusively the IMP site or the NAD site. The enzyme may employ the same active-site elements for addition and elimination at the C2 of both IMP and the 2-halo analogs. The observation that IMP is a competitive inhibitor in the 2-Cl-IMP reaction suggests that 2-Cl-IMP binds in the IMP binding site and utilizes the same amino acid residues for stabilization and catalysis. Studies to identify these amino acid residues are in progress, and recent results of those studies are presented in a companion article (Antonino *et al.*, 1994).

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REFERENCES

Abeles, R. H., & Alston, T. A. (1990) *J. Biol. Chem. 265*, 16705–16708.

Agarwal, R. P., Sagar, S. M., & Parks, R. E., Jr. (1975) *Biochem. Pharmacol. 24*, 693–701.

Amarnath, V., & Broom, A. D. (1982) *Nucleosides Nucleotides 1* (2), 163–171.
Anderson, J. H., & Sartorelli, A. C. (1968) *J. Biol. Chem. 243*, 4762–4768.
Antonino, L. C., Straub, K., & Wu, J. C. (1994) *Biochemistry* (following paper in this issue).
Brady, T. G., & O'Connell, W. (1962) *Biochim. Biophys. Acta 62*, 216–229.
Brox, L. W., & Hampton, A. (1968) *Biochemistry 7*, 2589–2596.
Buzzee, D. H., & Levin, A. P. (1968) *Biochim. Biophys. Res. Commun. 30*, 673–677.
Carr, S. F., Papp, E., Wu, J. C., & Natsumeda, Y. (1993) *J. Biol. Chem. 268*, 27286–27290.
Chilson, O. P., & Fisher, J. R. (1963) *Arch. Biochem. Biophys. 102*, 77–85.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–137.
Collart, F. R., & Huberman, E. (1987) *Mol. Cell. Biol. 7*, 3328–3331.
Collart, F. R., & Huberman, E. (1988) *J. Biol. Chem. 263*, 15769–15772.
Collart, F. R., Chubb, C. B., Mirkin, B. L., & Huberman, E. (1992) *Cancer Res. 52*, 5826–5828.
Eugui, E. M., Almquist, S. J., Muller, C. D., & Allison, A. C. (1991) *Scand. J. Immunol. 33*, 161–173.
Frederiksen, S. (1966) *Arch. Biochem. Biophys. 113*, 383–388.
Gilbert, H. J., Lowe, C. R., & Drabble, W. T. (1979) *Biochem. J. 183*, 481–494.
Hedstrom, L., & Wang, C. C. (1990) *Biochemistry 29*, 849–854.
Heyde, E., & Morrison, J. F. (1976) *Biochim. Biophys. Acta 429*, 635–644.
Heyde, E., Nagabhushanam, A., Vonarx, M., & Morrison, J. F. (1976) *Biochim. Biophys. Acta 429*, 645–660.
Holmes, E. W., Pehlke, D. M., & Kelley, W. N. (1974) *Biochim. Biophys. Acta 364*, 209–217.
Hupe, D. J., Azzolina, B. A., & Behrens, N. D. (1986) *J. Biol. Chem. 261*, 8363–8369.
Ishii, K., & Shiibo, I. (1968) *J. Biochem. (Tokyo) 63*, 661–669.
Jackson, R. C., Weber, G., & Morris, H. P. (1975) *Nature 256*, 331–333.
Jackson, R. C., Morris, H. P., & Weber, G. (1977) *Biochem. J. 166*, 1–10.
Konno, Y., Natsumeda, Y., Nagai, M., Yamaji, Y., Ohno, S., Suzuki, K., & Weber, G. (1991) *J. Biol. Chem. 266*, 506–509.
Lui, M. S., Faderan, M. A., Liepnieks, J. J., Natsumeda, Y., Olah, E., Jayaram, H. N., & Weber, G. (1984) *J. Biol. Chem. 259*, 5078–5082.
Montgomery, J. A., & Hewson, K. (1964) *J. Heterocycl. Chem. 1*, 213–214.
Montgomery, J. A., & Hewson, K. (1968) *J. Org. Chem. 33*, 432–434.
Morris, R. E., Wang, J., Blum, J. R., Flavin, T., Murphy, M. P., Almquist, S. J., Chu, N., Tam, Y. L., Kaloostian, M., Allison, A. C., & Eugui, E. M. (1991) *Transplant. Proc. 23*, 19–25.
Murphy, P. M., Noonan, M., Collins, P., Tully, E., & Brady, T. G. (1969) *Biochim. Biophys. Acta 171*, 157–166.
Nagai, M., Natsumeda, Y., Konno, Y., Hoffman, R., Irino, S., & Weber, G. (1991) *Cancer Res. 51*, 3886–3890.
Nagai, M., Natsumeda, Y., & Weber, G. (1992) *Cancer Res. 52*, 258–261.
Natsumeda, Y., Ikegami, T., Murayama, K., & Weber, G. (1988) *Cancer Res. 48*, 507–511.
Natsumeda, Y., Ohno, S., Kawasaki, H., Konno, Y., Weber, G., & Suzuki, K. (1990) *J. Biol. Chem. 265*, 5292–5295.
Nijkamp, H. J. J., & De Haan, P. G. (1967) *Biochim. Biophys. Acta 145*, 31–40.
Okada, M., Shimura, K., Shiraki, H., & Nakagawa, H. (1983) *J. Biochem. 94*, 1605–1613.
Sidi, Y., Panet, C., Wasserman, L., Cyjon, A., Novogrodsky, A., & Nordenberg, J. (1988) *Br. J. Cancer 58*, 61–63.
Streeter, D. G., Witkowski, J. T., Khare, G. P., Sidwell, R. W., Bauer, R. J., Robins, R. K., & Simon, L. N. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1174–1178.
Tricot, G. J., Jayaram, H. N., Lapis, E., Natsumeda, Y., Nichols, C. R., Kneebone, P., Heerema, N., Weber, G., & Hoffman, R. (1989) *Cancer Res. 49*, 3696–3701.
Verham, R., Meek, T. D., Hedstrom, L., & Wang, C. C. (1987) *Mol. Biochem. Parasitol. 24*, 1–12.
Weber, G. (1983) *Cancer Res. 43*, 3466–3492.
Wong, C. G., & Meyer, R. B., Jr. (1984) *J. Med. Chem. 27*, 429–432.
Wu, T.-W., & Scrimgeour, K. J. (1973) *Can. J. Biochem. 51*, 1391–1398.
Yokosawa, H., Tobita, T., & Yamada, T. (1971) *Biochim. Biophys. Acta 227*, 538–553.
Yoshikawa, M., Kato, T., & Takenishi, T. (1967) *Tetrahedron Lett. 50*, 5065–5068.