Kinetic Mechanism of Human Inosine 5′-Monophosphate Dehydrogenase Type II: Random Addition of Substrates and Ordered Release of Products†

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ABSTRACT: IMP dehydrogenase (IMPDH) catalyzes the oxidation of IMP to XMP with the concomitant reduction of NAD to NADH. This reaction is the rate-limiting step of guanine nucleotide biosynthesis. IMPDH is a target of immunosuppressive, antiviral, anticancer, and antiparasitic chemotherapy. We have determined a minimal kinetic mechanism for human IMPDH type II using NAD analogs, isotope effects, hydride exchange, and presteady state kinetics. The values of k_{cat} for the NAD analogs are similar despite a great variation in the structure and reactivity of the compounds. This observation suggests that a common step is rate-limiting, i.e., either hydrolysis of the E−XMP* intermediate or release of XMP. No V_m isotope effect is observed when 2-^{2}H-IMP is the substrate, which indicates that hydride transfer is fast. This conclusion is confirmed by the observation of a burst of NADH production under presteady state conditions. These observations further suggest that either E−XMP* hydrolysis or XMP release is rate-limiting. V/K_m deuterium isotope effects are observed for both substrates (1.9 for IMP and 2.5 for NAD), which indicates that substrate association is random. This result contradicts previous conclusions based on product inhibition studies. No NADH consumption is observed in the presence of XMP and IMPDH, which indicates that the overall reaction is irreversible. NADH consumption is observed in the presence of thio-NAD, IMP, and enzyme. These observations indicate that NADH traps the E−XMP* intermediate and demonstrates that hydride transfer is reversible. At infinite NADH, all of E−XMP* is trapped by NADH, as indicated by the equivalence of the rates of consumption of thio-NAD and NADH. Therefore the release of products is ordered, with NADH release preceding hydrolysis of E−XMP*.

IMP dehydrogenase (IMPDH)[1] controls a critical junction in the metabolism of nucleotides: the conversion of IMP to XMP is the rate-limiting step in de novo guanine nucleotide biosynthesis. IMPDH has long been recognized as a target for cancer and viral chemotherapy and has recently emerged as a target for immunosuppressive drugs (Weber, 1983; Robins, 1982; DeClerq, 1993; Allison et al., 1993). The activities of the antitumor drug tiazofurin, the antiviral drug ribavirin, and the immunosuppressive drugs mizoribine and mycophenolic acid (MPA) are attributed to the inhibition of IMPDH (Cooney et al., 1982; Smith et al., 1974; Franklin & Cook, 1969). Two human isozymes exist: type I, which is constitutively expressed, and type II which is amplified in proliferating cells (Collart & Huberman, 1988; Natsumeda et al., 1990; Collart et al., 1992; Nagai et al., 1992). Thus human IMPDH type II is a major target for cancer and immunosuppressive chemotherapy. In addition, bacterial and parasitic IMPDH's differ significantly from mammalian enzymes, which indicates that IMPDH is also a target for antiinfective chemotherapy (Franklin & Cook, 1969; Hupe et al., 1986; Verham et al., 1987).

The IMPDH reaction involves attack of Cys331 (human type II numbering) on the 2 position of IMP (Figure 1). This E−IMP intermediate transfers a hydride to NAD, resulting in an E−XMP* intermediate, which subsequently hydrolyzes to XMP (Link & Straub, 1996; Huete-Perez et al., 1995; Sintchak et al., 1996). The reaction is irreversible. In the presence of mycophenolic acid, all of the enzyme is trapped as E−XMP* (Link & Straub, 1996; Fleming et al., 1996). The IMPDH reaction is generally believed to follow an ordered bi bi kinetic mechanism where IMP is the first substrate bound and XMP is the last substrate released (Scheme 1A). This conclusion derives from product inhibition studies of enzymes from various sources, including both human isozymes, *Tritrichomonas foetus*, *Eimeria tenella*, sarcoma 180 cells (Holmes et al., 1974; Carr et al., 1993; Verham et al., 1987; Anderson & Sartorelli, 1968; Xiang et al., 1996), as well as ligand binding studies (Nimmesgern et al., 1996). In contrast, a dead end inhibitor study suggests that IMPDH from *Aerobacter aerogenes* follows a random kinetic mechanism (Heyde et al., 1976) (Scheme 1B).

We report a minimal kinetic mechanism for human IMPDH type II as derived from primary deuterium isotope effects, hydride exchange reactions, and presteady state kinetic studies. In contrast to previous reports, these studies demonstrate that substrates associate randomly, although

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[1] Abbreviations: IMPDH, inosine 5′-monophosphate dehydrogenase; IMP, inosine 5′-monophosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; XMP, xanthosine 5′-monophosphate; thio-NAD, thionicotinamide adenine dinucleotide; thio-NADH, reduced thionicotinamide adenine dinucleotide; acetylpyridine-NAD, acetylpyridine adenine dinucleotide; pyridinealdehyde-NAD, 3-pyridinealdehyde adenine dinucleotide; NHD, nicotinamide hypoxanthine dinucleotide; aminopyridine-NAD, 3-aminopyridine adenine dinucleotide; pyridinealdehyde-NHD, 3-pyridinealdehyde hypoxanthine dinucleotide; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; EICARMP, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide 5′-monophosphate.

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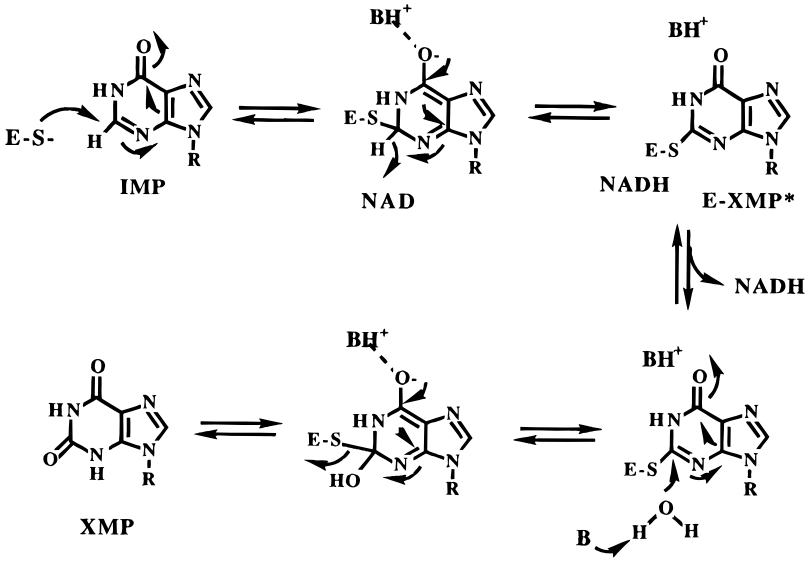
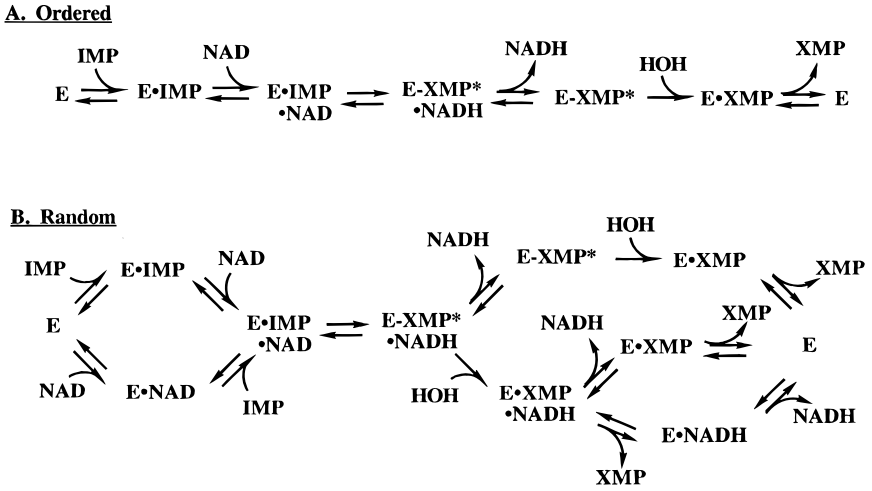


FIGURE 1: Mechanism of the IMPDH reaction.

Scheme 1: Putative Kinetic Mechanisms for Human IMPDH Type II



product dissociation is ordered with NADH release preceding XMP hydrolysis. In addition, these results show that hydride transfer is rapid and reversible and indicate that either product release or hydrolysis of E−XMP* is rate-limiting in the IMPDH reaction.

MATERIALS AND METHODS

Materials. IMP, NAD, and NAD analogs were purchased from Sigma Chemical Co.

Synthesis of 2-^{2}H-IMP. 2-^{2}H-Inosine was synthesized from AICAR and ^{2}H-ethyl formate and phosphorylated with phosphorus oxychloride in trimethyl phosphate using known procedures (Wong & Meyer, 1984; Yoshikawa et al., 1967). 2-^{2}H-IMP was desalted by absorption onto charcoal and purified by DEAE cellulose chromatography with a gradient of triethylammonium bicarbonate. The IMP-containing fractions were lyophilized and chromatographed on a DIONEX anion exchange column with a gradient of ammonium bicarbonate. The sodium salt was obtained by treating with Dowex50(H^+) followed by Dowex50(Na^+). The concentration of IMP was determined by A_{248} and verified by enzymatic assay using IMPDH and excess NAD.

Enzyme Purification. Recombinant human IMPDH type II was purified from an *Escherichia coli* expression system using Cibacron blue affinity resin and cation exchange chromatography as previously described (Farazi et al., 1997).

Enzyme Assays. Standard assay solutions contained 100 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, and 50 mM Tris, pH 8.0. Assays were performed at 25 °C. Production of NADH was monitored spectrophotometrically at 340 nm ($\epsilon = 6.22$ mM^{-1} cm^{-1}), using a Hitachi U2000 spectrophotometer or a Hewlett-Packard 8453 diode array spectrophotometer. The following wavelengths were used to monitor NAD analogs: thio-NADH: 395 nm, $\epsilon = 11.3$ mM^{-1} cm^{-1}; 3-acetylpyridine-NAD: 363 nm, $\epsilon = 9.1$ mM^{-1} cm^{-1}; 3-pyridinealdehyde-NAD and pyridinealdehyde-NHD: 358 nm, $\epsilon = 9.3$ mM^{-1} cm^{-1}; NHD: 340 nm, $\epsilon = 6.22$ mM^{-1} cm^{-1}; aminopyridine-NAD: 331 nm, $\epsilon = 3.09$ mM^{-1} cm^{-1}. In the absence of substrate inhibition, data was fit to a sequential mechanism (eq 1) using KinetAsyst software:

$$v = V_m[A][B]/(K_{ia}*K_b + K_a*[B] + K_b*[A] + [A]*[B]) \quad (1)$$

where v is the initial velocity, A is IMP, B is NAD or analog, V_m is the maximal velocity, K_a and K_b are the Michaelis constants of IMP and NAD, respectively, and K_{ia} is the dissociation constant of E·IMP (nomenclature of Cleland, 1963). When substrate inhibition is observed, steady state

Table 1: Michaelis−Menten Parameters for Human IMPDH Type II[a]

dinucleotide substrate	$E^{0\prime b}$ (V)	IMP K_m (μM)	dinucleotide K_m (μM)	dinucleotide K_i (μM)	k_{cat} (s^{-1})	IMP V/K_m (M^{-1} s^{-1})	dinucleotide V/K_m (M^{-1} s^{-1})
NAD	−0.320	4 ± 1	6 ± 1	590 ± 20	0.39 ± 0.01	(9.8 ± 1.4) × 10^4	(6.5 ± 0.5) × 10^4
thio-NAD	−0.285	4 ± 1	120 ± 20	490 ± 10	0.34 ± 0.03	(7.9 ± 1.3) × 10^4	(2.8 ± 0.5) × 10^3
acetylpyridine-NAD	−0.258	34 ± 5	30 ± 5	na[c]	0.62 ± 0.05	(2.3 ± 0.2) × 10^4	(2.1 ± 0.2) × 10^4
pyridinealdehyde-NAD	−0.262	41 ± 10	270 ± 55	na[d]	0.48 ± 0.05	(1.2 ± 0.2) × 10^4	(1.8 ± 0.2) × 10^3
NHD	−0.320	8 ± 3	590 ± 140	na[e]	0.34 ± 0.03	(5.6 ± 1.7) × 10^4	(5.7 ± 0.9) × 10^2

[a] Reactions were performed in 100 mM KCl, 50 mM Tris, pH 8.0, 1 mM dithiothreitol and 2 mM EDTA at 25 °C. Absorbance was monitored as described in Materials and Methods. n.a., not applicable. [b] From Hermes *et al.*, 1984. [c] No substrate inhibition observed at [dinucleotide] ≤ 50 μM. [d] No substrate inhibition observed at [dinucleotide] ≤ 400 μM. [e] No substrate inhibition observed at [dinucleotide] ≤ 2000 μM.

Table 2: Isotope Effects[a]

dinucleotide	^DV	$^DV/K_m$ (IMP)	$^DV/K_m$ (NAD)
NAD	1.0 ± 0.1	1.9 ± 0.3	2.5 ± 0.2
thio-NAD	1.1 ± 0.1	3.4 ± 0.5	3.3 ± 0.5

[a] Reactions were performed in 100 mM KCl, 50 mM Tris, pH 8.0, 1 mM dithiothreitol and 2 mM EDTA at 25 °C.

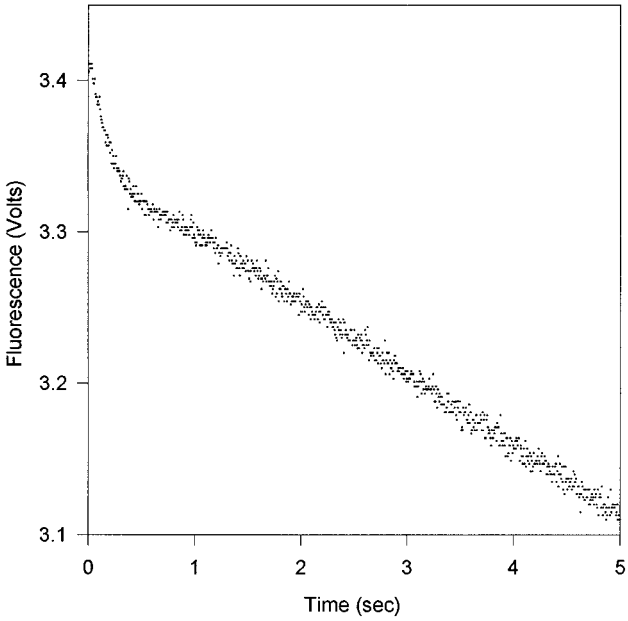


FIGURE 2: Presteady state burst of NADH production. Conditions: 1 μM human IMPDH type II, 400 μM IMP, 200 μM NAD, 100 mM KCl, 1 mM dithiothreitol, 50 mM Tris, pH 8.0, 25 °C. Production of NADH was monitored by fluorescence with an Applied Photophysics stopped flow spectrofluorimeter, excitation wavelength 340 nm, emission monitored with a 420 nm cutoff filter.

parameters were derived by first determining the apparent values of V_m for the initial velocity versus IMP plots (eq 2) and replotting these values against NAD concentration. This data was fit to eq 3, which describes uncompetitive substrate inhibition. The value of K_m (IMP) was derived by first determining the apparent values of V_m for the initial velocity versus NAD plots using eq 3 and replotting these values against IMP concentration (eq 2).

$$v = V_m[\text{IMP}]/(K_a + [\text{IMP}]) \quad (2)$$

$$v = V_m\,[\text{dinucleotide}]/(K_b + [\text{dinucleotide}] + [\text{dinucleotide}]^2/K_i) \quad (3)$$

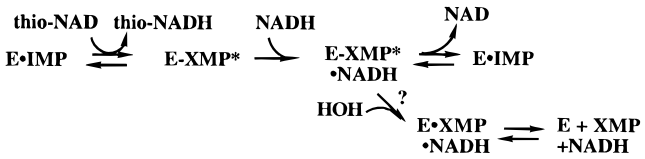
Isotope effects were determined by fitting data from the reactions of IMP and ^{2}H-IMP to eq 1 (for the NAD reaction) or to eqs 2 and 3 (for the thio-NAD reaction). Presteady state experiments were performed on an Applied Photophysics SX.17MV stopped flow spectrophotometer by monitoring fluorescence of NADH, excitation wavelength 340 nm, 420 nm cut-off emission filter.

Table 3: Hydride Exchange Reaction[a]

reaction	ΔA_{340} s^{-1} (×10^7)	ΔA_{395} s^{-1} (×10^7)
IMP + thio-NAD	−230	6200
XMP + NADH	1.3	nd
IMP + NADH	3.9	−4.3
IMP + thio-NAD + NADH	−1800	5800

[a] The reactions contained 0.3 μM IMPDH, 250 μM IMP, 200 μM thio-NAD, 200 μM NADH, and/or 250 μM XMP as indicated in 100 mM KCl, 50 mM Tris, pH 8.0, 1 mM dithiothreitol, and 2 mM EDTA at 25 °C. Absorbance was monitored simultaneously at 340 nm (NADH consumption) and 395 nm (thio-NADH production) using a Hewlett Packard 8453 diode array spectrophotometer. nd, not determined.

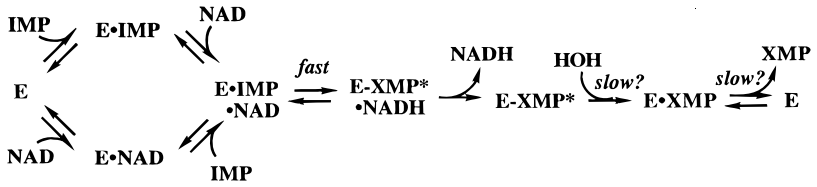
Scheme 2: Hydride Exchange Reaction



RESULTS AND DISCUSSION

Dinucleotide Specificity. Thio-NAD, acetylpyridine-NAD, pyridinealdehyde-NAD, and NHD are substrates for human IMPDH type II, as summarized in Table 1. No reaction could be observed for aminopyridine-NAD or pyridinealdehyde-NHD (less than 2% of the reaction with NAD was observed). The values of K_m for the NAD analogs are 10- to 200-fold greater than the K_m of NAD, while the values of K_m for IMP vary by no more than 7-fold. High concentrations of both NAD and thio-NAD inhibit IMPDH. Substrate inhibition is often observed when product release is ordered (Cleland, 1977). NAD inhibition is uncompetitive in *T.*

Scheme 3: Kinetic Mechanism of Human IMPDH Type II



foetus IMPDH, and is consistent with NAD binding to E−XMP* (Hedstrom & Wang, 1990; Wu et al., 1995). The value of K_i for NAD is 100-fold greater than the value of K_m, while the value of K_i for thio-NAD is 4-fold greater than K_m. No substrate inhibition is observed for the other dinucleotides. Despite the great variation in reduction potentials and structures of the NAD analogs, the k_{cat}'s are comparable. This observation suggests that neither hydride transfer nor NADH release is rate-limiting. A common step, either hydrolysis of E−XMP* or release of XMP, may be rate-limiting in the reactions of the NAD analogs.

Primary Deuterium Isotope Effects. The primary deuterium isotope effects were measured by varying the concentrations of both 2-^{2}H-IMP and either NAD or thio-NAD (Table 2). For NAD, substrate inhibition could be ignored since the value of K_i for NAD is 100-fold greater than the value of K_m. Therefore, the isotope effects for the reaction were determined by comparing the values of V_m and V/K_m determined in the fit of the data to eq 1. No V_m isotope effect is observed when NAD is the substrate, which indicates that hydride transfer is not rate-determining for this dinucleotide. This observation further supports the conclusion that E−XMP* hydrolysis or XMP release is rate-limiting in the IMPDH reaction. Both $^D(V/K_{IMP})$ and $^D(V/K_{NAD})$ are significantly greater than 1, which indicates that substrate addition must be random (Cook & Cleland, 1981). The difference between $^D(V/K_{IMP})$ and $^D(V/K_{NAD})$ suggests that IMP is the "stickier" substrate. Similar ^DV_m and $^D(V/K)_{NAD}$ have been reported (Wu et al., 1995). For thio-NAD, the values of K_m and K_i are similar. Therefore isotope effects were determined from the fit of the data to eqs 2 and 3. No V_m isotope effect is observed when thio-NAD is the substrate, which further suggests that a common step, either E−XMP* hydrolysis or XMP release, may be rate-limiting in the IMPDH reaction. Like NAD, significant isotope effects are observed for $^D(V/K_{IMP})$ and $^D(V/K_{thio\text{-}NAD})$, which further confirms random addition of substrates.

Burst of NADH Production. The failure to observe a V_m isotope effect in the NAD reaction indicates that hydride transfer is not rate-limiting under steady state conditions. This conclusion is confirmed by the observation of a presteady state burst of NADH production in the presence of saturating concentrations of IMP and NAD (Figure 2). The magnitude of the burst of NADH, measured by fluorescence, is linearly dependent on enzyme concentration, as expected (data not shown). These observations further confirm that hydrolysis of E−XMP* and/or XMP release is rate-limiting in the IMPDH reaction.

Hydride Exchange. A hydride exchange reaction was performed in order to confirm the presence of the E−XMP* intermediate and determine if hydride transfer is reversible. IMPDH was incubated with IMP, thio-NAD, and NADH. Consumption of thio-NAD and NADH were monitored simultaneously at 395 nm and 340 nm, respectively (Table 3). No NADH consumption is observed in the absence of thio-NAD or when enzyme is incubated with XMP and NADH. However, NADH consumption is observed in the presence of IMP and thio-NAD. Thus NADH consumption must result from reduction of E−XMP* as outlined in Scheme 2, which demonstrates that hydride transfer is reversible. The rate of NADH consumption ($v_{NADH\rightarrow NAD}$) increases with NADH concentration (Figure 3A), while the rate of thio-NAD consumption ($v_{tNAD\rightarrow tNADH}$) is constant.

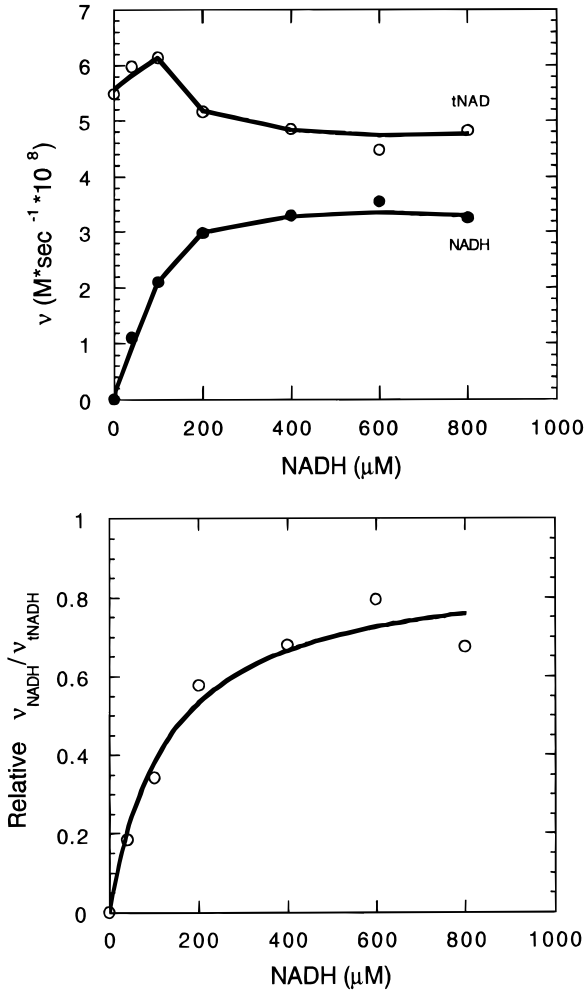


FIGURE 3: Hydride exchange experiment. Reactions contained 0.3 μM IMPDH, 250 μM IMP, 200 μM thio-NAD in 100 mM KCl, 50 mM Tris, pH 8.0, 1 mM dithiothreitol, and 2 mM EDTA at 25 °C. Absorbance was monitored simultaneously at 340 nm (NADH consumption) and 395 nm (thio-NADH production) using a Hewlett Packard 8453 diode array spectrophotometer. The concentration of thio-NADH was determined directly from the absorbance at 395 nm since NADH does not absorb at that wavelength (thio-NADH, $\epsilon_{395} = 11.3$ mM^{-1} cm^{-1}). The absorbance at 340 nm was corrected for the contribution of thio-NADH using the data of Table 3, i.e., $A_{340} = 6.22$ mM^{-1} cm^{-1} [NADH] + 0.43 mM^{-1} cm^{-1} [thio-NADH]. A. Rates of thio-NAD and NADH consumption versus [NADH]. B. Rate of NADH consumption relative to thio-NAD consumption versus [NADH].

Hydride transfer experiments were also used to examine the order of release of products. If thio-NADH release must

precede hydrolysis of E−XMP*, all of E−XMP* will be trapped at infinite NADH (Scheme 2). Thus, at infinite NADH, $v_{tNAD→tNADH}$ will equal $v_{NADH→NAD}$. However, if E−XMP* hydrolysis can precede thio-NADH release, $v_{tNAD→tNADH}$ will always be greater than $v_{NADH′NAD}$. Therefore the ratio of $v_{NADH→NAD}$ to $v_{tNAD→tNADH}$ was fit to the equation for a rectangular hyperbola (Figure 3B). At infinite NADH, the ratio is 0.9 ± 0.1, which indicates that $v_{tNAD→tNADH}$ will equal $v_{NADH→NAD}$. Therefore, product release is ordered with NADH release preceding hydrolysis of E−XMP*.

This conclusion is consistent with the observation that high concentrations of NAD trap E−XMP* (see above and Wu et al., 1995). Mycophenolic acid also inhibits by trapping E−XMP* (Hedstrom & Wang, 1990; Link & Straub, 1996; Fleming et al., 1996; Sintchak et al., 1996). Ordered release of products is further supported by the observation that 1 equiv of NADH is produced at saturating concentrations of mycophenolic acid (Fleming et al., 1996). If product release were random, more than 1 equiv of NADH would be produced before mycophenolic acid traps E−XMP*.

The hydrolysis of E−XMP* may be rate-determining in the thio-NAD reaction; in this case, a second pathway for the decomposition of E−XMP* would increase $v_{tNAD→tNADH}$. No such increase is observed in the experiment of Figure 3A. However, the thio-NAD concentration is not saturating in this experiment; under these conditions, formation of E−XMP* would be rate-limiting, and a second pathway for decomposition of E−XMP* would not increase $v_{tNAD→tNADH}$. An increase in $v_{tNAD→tNADH}$ is observed in the presence of NADH at saturating thio-NAD concentrations (data not shown). Unfortunately, substrate inhibition is significant at these thio-NAD concentrations, which complicates interpretation of this experiment: i.e., the increase in $v_{tNAD→tNADH}$ in the presence of NADH may simply result from NADH out-competing thio-NAD for the E−XMP* intermediate. In order to avoid complications arising from substrate inhbition, a hydride exchange experiment was performed using NAD and thio-NADH. Unfortunately, thio-NADH inhibits the IMPDH reaction and no enzyme-dependent consumption of thio-NADH was observed (data not shown). The failure to observe consumption of thio-NADH may result from the unfavorable thermodynamics of the hydride exchange reaction ($E^{0′} = -0.32$ for NAD versus -0.285 for thio-NAD (Hermes et al., 1984)). In addition, the increased values of the $^{D}V/K_m$ isotope effects in the thio-NAD reaction relative to the NAD reaction indicates that the kinetic barrier for hydride transfer is greater for thio-NAD than for NADH.

CONCLUSIONS

These experiments demonstrate that the IMPDH reaction proceeds via random addition of substrates and ordered release of products as shown in Scheme 3. Hydride transfer is fast and reversible; either E−XMP* hydrolysis or XMP release is rate-limiting in the overall reaction.

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