Biochemistry

 Volume 29, Number 4 January 30, 1990

Articles

Mycophenolic Acid and Thiazole Adenine Dinucleotide Inhibition of *Tritrichomonas foetus* Inosine 5′-Monophosphate Dehydrogenase: Implications on Enzyme Mechanism†

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Received June 26, 1989; Revised Manuscript Received September 14, 1989

ABSTRACT: Inosine 5′-monophosphate dehydrogenase (IMPDH) catalyzes the oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) with the conversion of NAD to NADH. An ordered sequential mechanism where IMP is the first substrate bound and XMP is the last product released was proposed for *Tritrichomonas foetus* IMPDH on the basis of product inhibition studies. Thiazole adenine dinucleotide (TAD) is an uncompetitive inhibitor versus IMP and a noncompetitive inhibitor versus NAD, which suggests that TAD binds to both E–IMP and E–XMP. Mycophenolic acid is also an uncompetitive inhibitor versus IMP and noncompetitive versus NAD. Multiple-inhibitor experiments show that TAD and mycophenolic acid are mutually exclusive with each other and with NADH. Therefore, mycophenolic acid most probably binds to the dinucleotide site of *T. foetus* IMPDH. The mycophenolic acid binding site was further localized to the nicotinamide subsite within the dinucleotide site: mycophenolic acid was mutually exclusive with tiazofurin, but could form ternary enzyme complexes with ADP or adenosine diphosphate ribose. NAD inhibits the IMPDH reaction at concentrations >3 mM. NAD substrate inhibition is uncompetitive versus IMP, which suggests that NAD inhibits by binding to E–XMP. TAD is mutually exclusive with both NAD and NADH in multiple-inhibitor experiments, which suggests that there is one dinucleotide binding site. The ordered mechanism predicts that multiple-inhibitor experiments with XMP and TAD, mycophenolic acid, or NAD should have an interaction constant (α) between 0 and 1. However, α was greater than 1 in all cases. These results indicate that TAD, mycophenolic acid, and NAD do not inhibit simply by binding to E–XMP and suggest that the mechanism must include an isomerization step either between IMP binding and NAD binding or between NADH release and XMP release.

Inosine 5′-monophosphate dehydrogenase (IMPDH)[1] (EC 1.1.1.205) catalyzes the oxidation of IMP to XMP with the conversion of NAD to NADH. The reaction is apparently irreversible. The conversion of IMP to XMP is the rate-determining step of de novo guanine nucleotide biosynthesis in mammalian systems. IMPDH is a potential target for antitumor chemotherapy (Jackson et al., 1975; Robins, 1982; Weber, 1983), as well as the target for antiviral agents, notably ribavirin and tiazofurin (Malinoski & Stollar, 1981; Streeter et al., 1973). Recently, IMPDH was recognized as a potential target for the development of antiparasitic chemotherapy for *Plasmodium falciparum* (Webster & Whaun, 1982), *Eimeria tenella* (Hupe et al., 1986), and *Tritrichomonas foetus* (Verham et al., 1987).

We have previously reported purification and kinetic studies on *T. foetus* IMPDH (Verham et al., 1987). The *T. foetus*

†This work was supported by NIH Postdoctoral Fellowship 1F32-GM10504 (L.H.) and NIH Grant AI 19391.

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[1] Abbreviations: NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; TAD, thiazole adenine dinucleotide; tiazofurin, 2-β-D-ribofuranosylthiazole-4-carboxamide; ADP, adenosine diphosphate; ADPR, adenosine diphosphate ribose; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; IMP, inosine monophosphate; IMPDH, inosine monophosphate dehydrogenase.

0006-2960/90/0429-849$02.50/0

Table I: Inhibitors of *T. foetus* IMPDH[a]

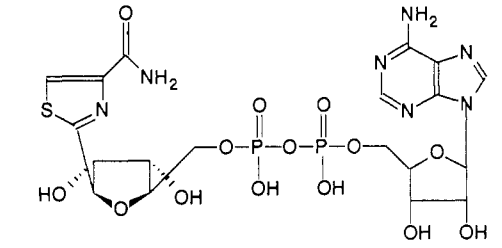
inhibitor	vs IMP			vs NAD		
	pattern type	K_{is}	K_{ii}	pattern type	K_{is}	K_{ii}
XMP	C	11 ± 3 μM		NC	660 ± 60 μM	610 ± 50 μM
NADH[b]	NC	260 ± 100 μM	400 ± 40 μM	NC	320 ± 70 μM	240 ± 20 μM
ribavirin5P	C	0.12 ± 0.01 μM		NC	15 ± 5 μM	4.5 ± 0.3 μM
TAD	UC		1.49 ± 0.09 μM	NC	2.6 ± 0.9 μM	1.6 ± 0.2 μM
tiazofurin	UC		72 ± 5 mM	NC	50 ± 10 mM	69 ± 9 mM
ADPR	UC		35 ± 4 mM	NC	26 ± 3 mM	120 ± 20 mM
ADP[c]	NC	9 ± 3 mM	64 ± 9 mM	C	31 ± 2 mM	
MPA	UC		14 ± 1 μM	NC	20 ± 5 μM	14 ± 2 μM
NAD	UC		10 ± 1 mM			

[a] Experimental conditions: 50 mM Tris-HCl, pH 8.1, 1.0 mM dithiothreitol, 100 mM KCl, 3.0 mM EDTA. Fixed concentrations of IMP and NAD were 200 μM and 1.0 mM, respectively, unless otherwise noted. MPA, mycophenolic acid; C, competitive inhibition; NC, noncompetitive inhibition; UC, uncompetitive inhibition; K_{is} and K_{ii}, slope and intercept inhibition constants, respectively. [b] 100 μM IMP, 2.0 mM NAD. [c] 200 μM IMP, 200 μM NAD.

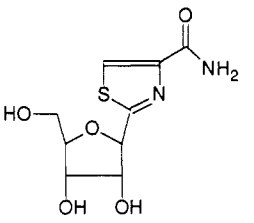
enzyme has two properties that distinguish it from mammalian IMPDH: it does not require K^+ for activity and mycophenolic acid is a less potent inhibitor (K_i = 14 μM versus K_i = 0.03 μM for mammalian IMPDH) (Franklin & Cook, 1969). We proposed an ordered sequential mechanism of substrate binding and product release for *T. foetus* IMPDH on the basis of initial velocity and product inhibition studies (Verham et al., 1987). In the proposed mechanism, IMP is the first substrate bound and XMP is the last product released. This is consistent with the observations that XMP is a competitive inhibitor of IMP and noncompetitive with respect to NAD, while NADH is a noncompetitive inhibitor of both substrates. Similar ordered mechanisms have been proposed for IMPDH isolated from many other sources (Anderson & Satorelli, 1986; Brox & Hampton, 1968; Holmes et al., 1974; Jackson et al., 1977).

In this paper we have characterized the interaction of TAD and mycophenolic acid with *T. foetus* IMPDH (for structures see Chart I). TAD, the active metabolite of tiazofurin (Cooney et al., 1983), is a potent inhibitor of mammalian IMPDH (Gebeyehu et al., 1985; Yamada et al., 1988). Several research groups have suggested that TAD is an NADH analogue rather than an NAD analogue (Gebeyehu et al., 1985; Yamada et al., 1988). This seems reasonable because TAD does not have a positive charge corresponding to the positive charge on the nicotinamide ring of NAD (see Chart I). Mycophenolic acid is also a specific inhibitor of IMPDH from many different sources (Franklin & Cook, 1968; Hupe et al., 1986; Verham et al., 1987). It has antitumor and immunosuppressive activity and is used clinically to treat psoriasis (Carter et al., 1969; Ohsugi et al., 1976; Sweeney, 1977). Mycophenolic acid is an especially interesting compound because it has no obvious structural resemblance to substrates or products of the IMPDH reaction (see Chart I). Even small changes in the structure of mycophenolic acid (e.g., methylation, reduction of the double bond, etc.) eliminate drug activity (Beisler & Hillery, 1975; Jones & Mills, 1971; Sweeney, 1977). We previously reported that mycophenolic acid is an uncompetitive inhibitor of *T. foetus* IMPDH (Verham et al., 1987). Mycophenolic acid is also an uncompetitive inhibitor of the *E. tenella* IMPDH (Hupe et al., 1986). Uncompetitive inhibitors are especially interesting compounds for drug design because the inhibition will not be relieved by the accumulation of substrates. Therefore, it is important to understand how mycophenolic acid inhibits IMPDH. We have used multiple-inhibitor experiments to show that both TAD and mycophenolic acid bind to the dinucleotide site of *T. foetus* IMPDH. However, mycophenolic acid and TAD inhibition does not result from these compounds simply binding to E–XMP as previously proposed (Hupe et al., 1986; Verham et al., 1987). This suggests that the kinetic mechanism contains isomerization steps in addition to substrate binding and product release.

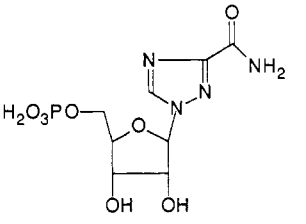
Chart I: IMPDH Inhibitors



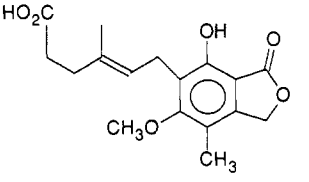
TAD



Tiazofurin



Ribavirin 5-phosphate



Mycophenolic acid

Materials and Methods

Materials. IMP, XMP, NAD, NADH, ADP, ADPR, and mycophenolic acid were purchased from Sigma. NADH was further purified on a Pharmacia Mono Q column (Orr & Blanchard, 1984). TAD, tiazofurin, and ribavirin 5′-phosphate were the generous gift of Dr. Roland K. Robins, Nucleic Acid Research Institute, Costa Mesa, CA.

Purification of IMPDH. IMPDH was purified from *T. foetus* by a modification of the published procedure (Verham et al., 1987). *T. foetus* was harvested in late log phase (8×10^6 cells/mL) by centrifugation at 5000 rpm in a Sorvall GSA rotor. The cells were washed 3 times in 0.1 M Tris-HCl, pH 7.2, containing 1.0 mM dithiothreitol (buffer A). The cells were resuspended in an equal volume of buffer A containing 1.0 mM *o*-phenanthroline and 1.0 mM benzamidine and frozen

at −70 °C until needed. Frozen cells (40 mL) were thawed, sonicated, and centrifuged at 78000*g* for 135 min. The supernatant [specific activity (SA) = 1 nmol/(min·mg)] was chromatographed on a Bio-Gel A 5-m column (500mL) equilibrated in buffer A. The IMPDH-containing fractions were pooled [SA = 14 nmol/(min·mg), 70% recovery] and applied to an IMP affinity column (Ikegami et al., 1987). The column was washed with buffer A and eluted with buffer A containing 10% glycerol and 0.5 mM IMP. The enzyme was concentrated to 0.4 mg/mL and stored at −70 °C. Purified IMPDH had a specific activity of 700 nmol/(min·mg), which represents a 700-fold purification with a 60% overall yield. The enzyme preparation was free of NADH oxidase activity.

Enzyme Assays. IMPDH was assayed spectrophotometrically on a Beckman DU7 UV–visible spectrophotometer. Assay mixture (0.5 mL) contained 50 mM Tris-HCl, pH 8.1, 100 mM KCl, 3.0 mM EDTA, 1.0 mM dithiothreitol, 1.0 mM NAD, and 200 μM IMP. The reaction was initiated by the addition of 1–2 μg of purified IMPDH to the assay mixture incubated at 37 °C. The reaction was monitored at 290 nm during enzyme purification (the background rate was A_{290}/min in the absence of IMP) and at 340 nm after purification (the background rate was the A_{340}/min in the absence of enzyme).

Data Analysis. Initial rate data were fitted to eq 1–4 by using Hewlett-Packard kinetics software with Marquadt analysis. The best fits were determined by the relative fit error

competitive inhibition

$$v = VA/[K_a(1 + I/K_{is}) + A] \quad (1)$$

noncompetitive inhibition

$$v = VA/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (2)$$

uncompetitive inhibition

$$v = VA/[K_a + A(1 + I/K_{ii})] \quad (3)$$

multiple inhibition

$$v = v_0/[1 + I/K_i + J/K_j + IJ/\alpha K_i K_j] \quad (4)$$

and errors in the constants. The nomenclature is that of Cleland (1963): v, velocity; V, maximum velocity; A, substrate concentration; K_a, apparent Michaelis constant for A; K_{ii} and K_{is}, intercept and slope inhibition constants, respectively; I and J, concentrations of inhibitors I and J; v_0, initial velocity in the absence of inhibitors I and J; K_i and K_j, apparent inhibition constants for inhibitors I and J, respectively; α, interaction constant between inhibitors I and J. Multiple-inhibitor experiments were fitted by substituting $1/J$ for A in the equations for single inhibitors (Schimerlik & Cleland, 1977). v_0 becomes V. The equations rearrange so that $K_j = 1/K_a$, $K_i = K_{ii}$, and $\alpha = K_{is}/K_{ii}$.

Results and Discussion

Dead-End Inhibitors of T. foetus IMPDH. We have studied several IMPDH inhibitors to test the ordered sequential mechanism of substrate binding proposed for *T. foetus* IMPDH. The results are summarized in Table I. All of the inhibition patterns are linear. The K_ms for IMP and NAD are 18 ± 3 and 340 ± 30 μM, respectively (Verham et al., 1987).

Ribavirin 5'-phosphate, a metabolite of ribavirin (Streeter et al., 1973), is a potent inhibitor of mammalian IMPDHs (Gebeyehu et al., 1985; Yamada et al., 1988). Ribavirin 5'-phosphate is a competitive inhibitor versus IMP for *T. foetus* IMPDH and noncompetitive versus NAD (Table I). Interestingly, the K_i for ribavirin 5'-phosphate binding to *T. foetus* IMPDH is 0.12 μM, which is 8–40-fold lower than that reported for mammalian enzymes (Gebeyehu et al., 1985; Yamada et al., 1988). No time dependence was observed for ribavirin 5'-phosphate inhibition of *T. foetus* IMPDH, in contrast to the time-dependent inhibition reported for the rat hepatoma 3924A IMPDH (Yamada et al., 1988).

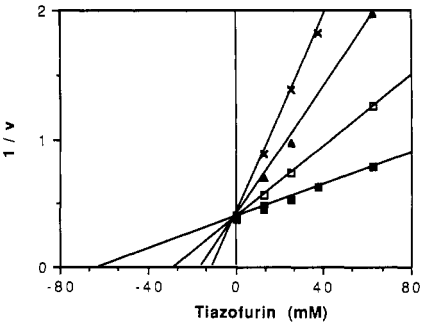


FIGURE 1: Multiple inhibition by tiazofurin and ADP. The solutions contained 0.1 M Tris-HCl, pH 8.1, 1.0 mM dithiothreitol, 1.0 M KCl, 3.0 mM EDTA, 1.0 mM NAD, and 200 μM IMP. The reaction was monitored at 340 nm at 37 °C. The rates are in arbitrary units. (■) No ADP; (□) 1.6 mM ADP; (▲) 4.0 mM ADP; (×) 8.0 mM ADP.

TAD is an uncompetitive inhibitor versus IMP (K_{ii} = 1.5 μM) and a noncompetitive inhibitor versus NAD (K_{is} = 2.6 μM, K_{ii} = 1.6 μM) with *T. foetus* IMPDH (Table I). Similar patterns were observed with mammalian IMPDHs (Gebeyehu et al., 1985; Yamada et al., 1988). Tiazofurin itself is a weak inhibitor of *T. foetus* IMPDH (Table I). It is an uncompetitive inhibitor versus IMP (K_{ii} = 72 mM) and noncompetitive inhibitor versus NAD (K_{is} = 50 mM, K_{ii} = 69 mM). A similar pattern was observed with ADPR: uncompetitive versus IMP (K_{ii} = 35 mM) and noncompetitive versus NAD (K_{is} = 26 mM, K_{ii} = 120 mM).

Several NAD analogues were tested as inhibitors of *T. foetus* IMPDH. No significant inhibition was observed with nicotinic acid, nicotinamide, 3-aminopyridine adenine dinucleotide, nicotinamide hypoxanthine dinucleotide, or thionicotinamide adenine dinucleotide. Only ADP is a competitive inhibitor versus NAD (K_{is} = 31 mM). ADP is a noncompetitive inhibitor of IMP (K_{is} = 9 mM, K_{ii} = 64 mM).

Dinucleotide Binding Site. The noncompetitive inhibition by TAD versus NAD suggests that TAD can bind to both E–IMP and E–XMP. Alternatively, TAD might be binding to a third allosteric site. We have used a series of multiple-inhibitor experiments to show that the binding of TAD and that of NADH are mutually exclusive. In a multiple-inhibitor experiment, α is defined as the interaction constant (see eq 4). If two inhibitors I and J bind mutually exclusively, $\alpha = \infty$, and a series of parallel lines results when $1/v$ is plotted against [I] at changing fixed [J] and constant fixed substrate concentrations. If the ternary complex E–I–J can form, α will be between 0 and ∞ (Yonetani & Theorell, 1964; Northrop & Cleland, 1974; Cleland, 1977; Schimerlik & Cleland, 1977; Grimshaw & Cleland, 1981). Table II shows that a multiple-inhibitor experiment between TAD and NADH results in an $\alpha = \infty$. This indicates that TAD binding and NADH binding are mutually exclusive and suggests that there is only one dinucleotide binding site.

Further multiple-inhibitor experiments indicate that tiazofurin binding is also mutually exclusive with both NADH and TAD (Table II). These results suggest that tiazofurin also binds to the NADH site, presumably to the nicotinamide portion. To verify that tiazofurin binds to the nicotinamide portion of the NADH site, multiple-inhibitor experiments were done with tiazofurin and ADP or ADPR. Figure 1 shows that tiazofurin and ADP interact synergistically with a pattern of

Table II: Multiple-Inhibitor Experiments[a]

inhibitor I	inhibitor J	α	K_i	K_j
TAD	NADH	∞	1.13 ± 0.05 μM	270 ± 20 μM
TAD	tiazofurin	∞	0.85 ± 0.03 μM	63 ± 3 mM
TAD	ADPR[b]	∞	0.84 ± 0.08 μM	60 ± 10 mM
TAD	ADP[b]	∞	1.6 ± 0.1 μM	55 ± 2 mM
TAD	XMP	7 ± 2	0.93 ± 0.03 μM	590 ± 20 μM
MPA	TAD	∞	12.5 ± 0.6 μM	0.91 ± 0.04 μM
MPA	NADH	∞	7.7 ± 0.6 μM	420 ± 30 μM
MPA	tiazofurin	∞	9.5 ± 0.4 μM	68 ± 4 mM
MPA	ADP[c]	2.5 ± 0.7	16 ± 1 μM	42 ± 2 mM
MPA	ADPR[c]	1.0 ± 0.2	18 ± 1 μM	43 ± 2 mM
MPA	XMP	8 ± 3	10.7 ± 0.4 μM	450 ± 10 μM
tiazofurin	NADH	∞	50 ± 5 mM	280 ± 20 μM
tiazofurin	ADP	<0.002	63 ± 5 mM	[e]
tiazofurin	ADPR	0.4 ± 0.1	83 ± 7 mM	24 ± 3 mM
NAD	TAD[d]	∞	6.2 ± 0.8 mM	0.6 ± 0.1 μM
NAD	XMP[d]	4 ± 1	6.3 ± 0.8 mM	700 ± 100 μM

[a] Conditions as defined in Table I. MPA, mycophenolic acid. [b] [NAD] = 200 μM. [c] [NAD] = 250 μM. [d] [NAD] ≥ 4.0 mM. [e] The fit for K_j is 700 ± 9000 mM. See Figure 1.

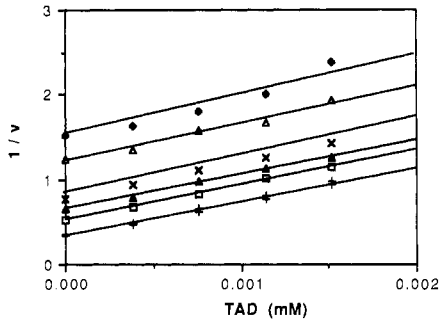


FIGURE 2: Multiple inhibition by TAD and mycophenolic acid. The conditions are as described in Figure 1. (+) No mycophenolic acid; (□) 5.0 μM mycophenolic acid; (▲) 10 μM mycophenolic acid; (×) 20 μM mycophenolic acid; (△) 30 μM mycophenolic acid; (◆) 40 μM mycophenolic acid.

lines intersecting on the $1/v$ axis (α < 0.002). ADPR and tiazofurin also interact synergistically (α = 0.4). This is somewhat surprising because the ribose moiety of tiazofurin would be expected to overlap with the ribose ring of ADPR in the binding site. There is apparently a great advantage to filling both the tiazofurin and ADP portions of the dinucleotide binding site, enough to overcome the expected steric overlap of the two ribose portions. The values of α for tiazofurin and ADPR are much greater than the α for tiazofurin and ADP (Table II), which probably reflects the interaction of the two ribose portions. ADPR and ADP were mutually exclusive with TAD, as expected (Table II).

Mycophenolic Acid Inhibition. We previously reported that mycophenolic acid is an uncompetitive inhibitor with respect to both IMP and NAD for the *T. foetus* IMPDH (Verham et al., 1987). Our more recent results with enzyme that is free of NADH oxidase contamination show that mycophenolic acid is an uncompetitive inhibitor with IMP (K_{ii} = 14 μM) but noncompetitive with NAD (K_{is} = 20 μM, K_{ii} = 14 μM). This suggests that mycophenolic acid may also bind to the dinucleotide site like TAD. A series of multiple-inhibitor experiments was used to test this hypothesis. Figure 2 shows that a multiple-inhibitor experiment with mycophenolic acid versus TAD results in a parallel line pattern. This indicates that TAD and mycophenolic acid are mutually exclusive and implies that they bind to the same site on the enzyme. Similar results were obtained for the combination of NADH and mycophenolic acid (Table II). Therefore, mycophenolic acid binds to the dinucleotide site of *T. foetus* IMPDH.

Tiazofurin, ADP, and ADPR were used to map the binding of mycophenolic acid in the dinucleotide site. Tiazofurin and mycophenolic acid were mutually exclusive, suggesting that mycophenolic acid binds in the tiazofurin, hence nicotinamide, portion of the NADH site (Table II). Mycophenolic acid, like tiazofurin, can form ternary enzyme complexes with ADP or ADPR (Table II), which also suggests that mycophenolic acid binds to the nicotinamide portion of the dinucleotide site. Unlike tiazofurin, the interaction of mycophenolic acid with ADP or ADPR is not synergistic.

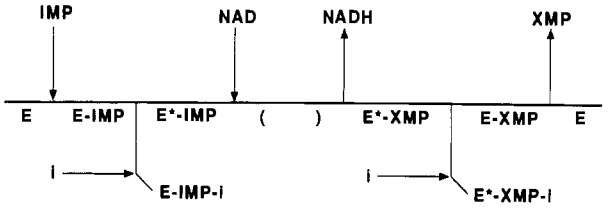
The binding of mycophenolic acid to the nicotinamide subsite of the NADH binding site is a particularly interesting result because there are many compounds (Cibacron blue, salicylate, anilinonaphthalenesulfonates, tetraiodofluorescein) that bind to the adenosine portion of NAD binding sites (Brand et al., 1967; Wassarman & Lentz, 1971; Einarsson et al., 1974; Beillman et al., 1979). These results suggest that more potent mycophenolic acid analogues might be designed by adding a moiety that could bind in the adenosine subsite.

NAD Substrate Inhibition. We previously reported that NAD is an inhibitor of *T. foetus* IMPDH at concentrations greater than 3 mM (Verham et al., 1987). Table I shows that NAD inhibition is uncompetitive with respect to IMP. Uncompetitive substrate inhibition is a characteristic of ordered sequential mechanisms (Cleland, 1979). This suggests that NAD inhibits by binding to E–XMP. Alternatively, NAD substrate inhibition could result from binding to a third allosteric site. A multiple-inhibitor experiment with TAD and NAD results in α = ∞ (Table II). This indicates that NAD and TAD are mutually exclusive and suggests that NAD, TAD, and NADH inhibit by binding to a single dinucleotide site.

Multiple-Inhibition Experiments with XMP. The above results suggest that TAD, mycophenolic acid, and NAD should all inhibit by binding to E–XMP. Therefore, α should be between 0 and 1 for multiple-inhibitor experiments with XMP and TAD, mycophenolic acid, or NAD (Northrop & Cleland, 1974; Schimerlik & Cleland, 1977; Cleland, 1977, 1979). However, Table II indicates that the value of α is greater than 1 for TAD versus XMP (α = 7), mycophenolic acid versus XMP (α = 8), and NAD versus XMP (α = 4). These results suggest that TAD inhibition, mycophenolic acid inhibition, and NAD inhibition do not result from simply binding to E–XMP and are therefore not consistent with a simple ordered sequential mechanism where NADH is the first product released.

Implications on the Mechanism of IMPDH. The studies reported above suggest that IMP is the first substrate to bind to *T. foetus* IMPDH as originally proposed. Three classes of

Scheme I

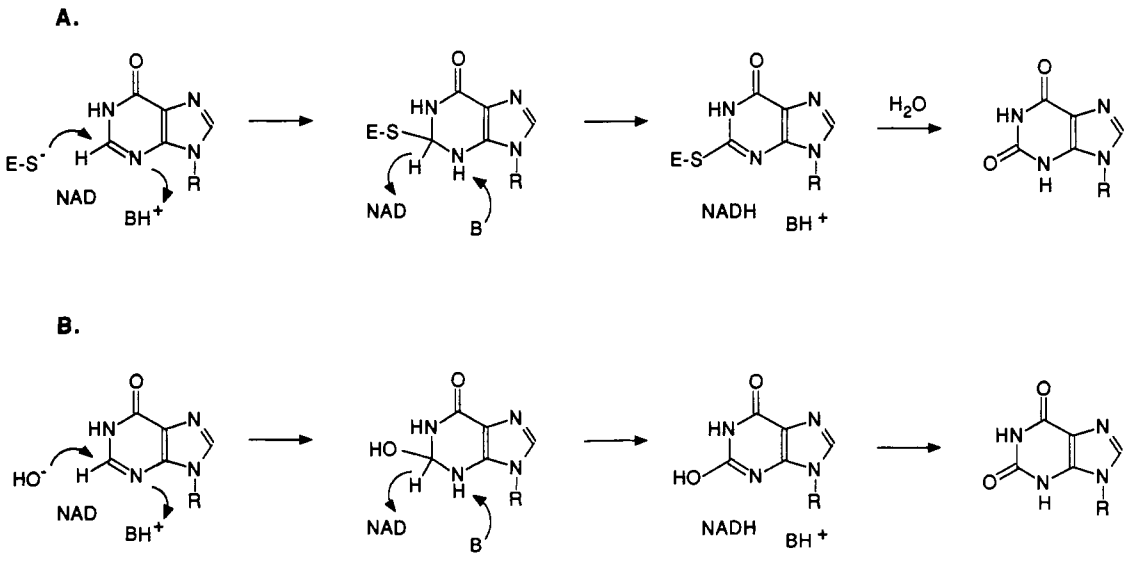


dead-end inhibitors were identified. All of the inhibition patterns were linear, which suggests that the inhibitors are binding to single sites. The first class is competitive versus IMP, noncompetitive versus NAD, and is represented by ribavirin 5′-phosphate. This behavior is consistent with an ordered sequential mechanism where IMP is the first substrate bound, but does not distinguish from random substrate binding. The second class is uncompetitive versus IMP, noncompetitive versus NAD. This class includes four different compounds—TAD, tiazofurin, ADPR, and mycophenolic acid—with K_is ranging from 1.49 μM to 72 mM. Uncompetitive inhibition requires that IMP bind before these inhibitors. NAD substrate inhibition is also uncompetitive versus IMP. Therefore, a great deal of evidence suggests that IMP binds first to *T. foetus* IMPDH, followed by NAD. The last class of inhibitors is represented by ADP. ADP is a competitive inhibitor versus NAD and noncompetitive versus IMP. This is the one observation that contradicts the proposed ordered sequential mechanism of substrate binding, which requires that a competitive inhibitor of NAD is uncompetitive versus IMP. It is possible that ADP is small enough to bind to free enzyme where NAD cannot. We therefore conclude that IMP is the first substrate bound in the *T. foetus* IMPDH reaction.

Several observations suggest that the simple ordered sequential mechanism must be modified to include a step or steps in addition to substrate binding. TAD, tiazofurin, ADPR, and mycophenolic acid are all noncompetitive inhibitors versus NAD, which suggests that they bind to both E–IMP and E–XMP. The value of α is greater than 1 in multiple-inhibitor experiments with XMP and TAD or mycophenolic acid. This suggests that TAD and mycophenolic acid prefer to bind to E–IMP. However, Table I shows that K_{ii} and K_{is} are equivalent for these inhibitors, which suggests that they bind equally to E–IMP and E–XMP. These data suggest that TAD and mycophenolic acid inhibit by combining with an additional enzyme form, not simply E–XMP. Further, uncompetitive NAD substrate inhibition suggests that NAD binds to E–XMP, yet α is greater than 1 for the multiple-inhibitor experiment between NAD and XMP. This indicates that NAD does not inhibit by combining directly with E–XMP. There are several possible explanations for this behavior. NAD might inhibit by binding to the central complexes as proposed for α-ketoglutarate substrate inhibition in the isocitrate dehydrogenase reaction (Northrop & Cleland, 1974). This seems unlikely because TAD is mutually exclusive with NAD and NADH, which suggests that there is only one dinucleotide binding site. If NAD bound to ternary complexes, one would also expect NAD to bind to ternary complexes containing TAD. A second possibility is that there is an additional step, E–IMP → E*–IMP, between IMP and NAD binding. NAD inhibition would then result from formation of a nonproductive E–IMP–NAD complex. This is intuitively a somewhat unsatisfying explanation, though it has been proposed to explain substrate inhibition by alanine in the alanine dehydrogenase reaction (Grimshaw & Cleland, 1981). In this case, TAD inhibition would result from combination with E–IMP, not E*–IMP, which predicts the observed inhibition patterns and multiple-inhibitor data. However, given the structural resemblance of TAD and NADH, it seems likely that TAD should also combine with product complexes. A third explanation is that there is an additional step, E*–XMP → E–XMP, between NADH and XMP release. NAD inhibition would now result from combination with E*–XMP, not E–XMP. TAD inhibition would result from binding to both E–IMP and E*–XMP. Unfortunately, we are unable to assay the reverse reaction and identify the position of the isomerization step(s).

Scheme I shows a mechanism with both possible isomerization steps, where i is NAD, TAD, or mycophenolic acid. The isomerization steps could be conformational changes or chemical steps (see below), with the equilibrium favoring E–IMP and E–XMP. Scheme I is also consistent with the probable chemical mechanism of the IMPDH reaction. A nucleophile must add to the 2-position of IMP before hydride transfer to NAD can occur. There are two likely candidates for this nucleophile: an enzyme cysteine thiol and OH^-. Involvement of a cysteine thiol would be analogous to the glyceraldehyde-3-phosphate dehydrogenase reaction (Harris & Waters, 1976). In this case E*–IMP might be a binary co-

Scheme II: Proposed IMPDH Mechanism



valent enzyme–IMP adduct as in Scheme IIA. Hydride transfer from this adduct to NAD would result in an enzyme–XMP covalent adduct (E*–XMP) that must hydrolyze to release XMP. This step would be expected to be essentially irreversible and would therefore fit the multiple-inhibitor data. There is evidence for a cysteine in the active site of *Aerobacter aerogenes* IMPDH (Brox & Hampton, 1968). The second possibility is that the nucleophile is OH^- by analogy to the mechanism proposed for the adenosine deaminase reaction (Kurz & Frieden, 1983; Weiss et al., 1987). E*–IMP would now be the hydroxide addition product, and E*–XMP might be a tautomer of XMP that collapses to XMP as shown in Scheme IIB. Interestingly, there is also a cysteine involved in the mechanism of adenosine deaminase (Kurz & Frieden, 1983; Weiss et al., 1987). We are currently testing these mechanisms.

ACKNOWLEDGMENTS

We thank Dr. Thomas Meek of Smith, Kline and French for many helpful discussions.

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