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Action of the Active Metabolites of Tiazofurin and Ribavirin on Purified IMP Dehydrogenase†

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Received August 31, 1987; Revised Manuscript Received November 18, 1987

ABSTRACT: The inhibitory mechanisms of ribavirin 5′-monophosphate (RMP) and thiazole-4-carboxamide adenine dinucleotide (TAD), the active forms of the antimetabolites ribavirin and tiazofurin, were investigated in IMP dehydrogenase purified to homogeneity from rat hepatoma 3924A. The hepatoma IMP dehydrogenase has a tetrameric structure with a subunit molecular weight of 60000. For the substrates IMP and NAD^+, K_m's were 23 and 65 μM, respectively. Product-inhibition patterns showed an ordered Bi-Bi mechanism for the enzyme reaction where IMP binds to the enzyme first, followed by NAD^+; NADH dissociates from the ternary complex first and then XMP is released. XMP interacts with the free enzyme and competes for the ligand site with IMP, while NADH binds to the enzyme·XMP complex. RMP exerted the same inhibitory mechanisms as XMP, and the inhibition by TAD was similar to that by NADH. However, the K_i values for RMP (0.8 μM) and TAD (0.13 μM) were orders of magnitude lower than those of XMP (136 μM) and NADH (210 μM). Thus, the drugs interact with IMP dehydrogenase with higher affinities than the natural substrates and products, RMP with the IMP–XMP site and TAD with the NADH site. Preincubation of the purified enzyme with RMP enhanced its inhibitory effect in a time-dependent manner. The enzyme was protected from this inactivation by IMP or XMP. These results provide a biochemical basis for combination chemotherapy with tiazofurin and ribavirin targeted against the two different ligand sites of IMP dehydrogenase.

IMP dehydrogenase (EC 1.1.1.205), the rate-limiting enzyme of de novo GTP biosynthesis, is a promising target in cancer chemotherapy (Weber et al., 1976; Weber, 1983; Robins, 1982; Tricot et al., 1987). The antimetabolites such as tiazofurin and SM-108,[1] targeted against IMP dehydrogenase, have a broad spectrum of action against tumors including Lewis lung carcinoma, which is refractory to most drugs (Yoshida et al., 1980; Robins et al., 1982). The oncolytic action of these drugs has been linked with the depletion of GTP and dGTP pools (Weber et al., 1984; Lui et al., 1984; Fukui et al., 1986).

Because of the strategic importance of the guanylate pathway and its rate-limiting enzyme, IMP dehydrogenase, it is vital to clarify the inhibitory mechanisms and the sites of drug actions. Combinations of the drugs against IMP dehydrogenase that act at different ligand sites might potentiate the inhibitory effect on guanylate synthesis. The resistance to an inhibitor might be overcome by a suitable choice of drugs with different metabolism and mechanisms. We recently purified IMP dehydrogenase to homogeneity from rapidly growing hepatoma 3924A (Ikegami et al., 1987). The present study determined the kinetic properties of purified IMP dehydrogenase with the natural ligands (IMP, XMP, NAD, and NADH) and elucidated the inhibitory mechanisms of the active metabolite of ribavirin (RMP) and tiazofurin (TAD).

Materials and Methods

Materials. [8-^{14}C]IMP was purchased from Amersham. Epoxy-activated Sepharose 6B was from Sigma and hydroxylapatite (Bio-Gel HTP) was from Bio-Rad. Sephacryl S-400 gel and an electrophoresis calibration kit were from Pharmacia. TAD and RMP were kindly supplied by Dr. R. K. Robins, Nucleic Acid Research Institute, Costa Mesa, CA. All other chemicals were of the highest purity available.

Enzyme Assay. In the enzyme purification IMP dehydrogenase activity was determined by a radiochemical assay and spectrophotometric assays (Ikegami et al., 1985, 1987). In the kinetic studies with the purified enzyme, however, adenosine 5′-α,β-methylenediphosphate and allopurinol were omitted from the reaction mixture.

Enzyme Purification. IMP dehydrogenase was purified from transplantable rat hepatoma 3924A as described by Ikegami et al. (1987) except that Sephacryl S-400 gel filtration was added to the purification steps prior to the IMP Sepharose affinity column. The gel (2.5 cm × 60 cm) was preequilibrated with 40 mM Tris-HCl, pH 7.4, containing 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol, and 0.15 M KCl. Fractions with enzyme activity were collected and applied on an IMP Sepharose column (0.8 cm^2 × 18 cm) equilibrated with the same

†This investigation was supported by Outstanding Investigator Grant CA-42510 and Program Project Grant CA-13526 awarded by the U.S. Public Health Service, National Cancer Institute, Department of Health and Human Services.
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[1] Abbreviations: SM-108, 4-carbamoylimidazolium 5-oleate; RMP, ribavirin 5′-monophosphate; TAD, thiazole-4-carboxamide adenine dinucleotide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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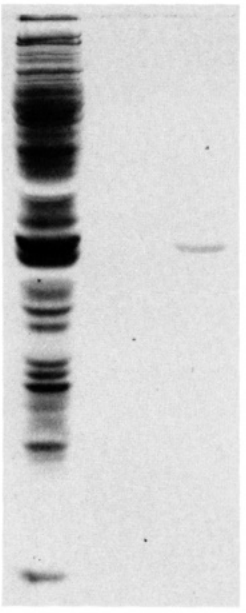

FIGURE 1: SDS–polyacrylamide gel electrophoresis of enzyme preparation before and after IMP Sepharose affinity column. (Left lane) Result of Sephacryl S-400 preparation (step 4). (Right lane) Electrophoretic pattern of the final purified IMP dehydrogenase preparation (step 5; about 0.5 μg).

buffer. After washing with 200 mL of the buffer, the enzyme was eluted with the buffer containing 0.5 mM IMP. The fractions with high enzyme activity were pooled and dialyzed for 4 h against 40 mM Tris-HCl, pH 7.4, containing 1 mM DTT, 1 mM EDTA, and 10% (v/v) glycerol to remove IMP.

Analytical Methods. SDS–polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). The electrophoresis was performed in a 10% polyacrylamide slab gel. Gel was fixed in a solution of 50% methanol and 10% acetic acid, and the polypeptide bands were detected by an ultrasensitive silver stain as reported by Oakley et al. (1980). Pharmacia's electrophoresis calibration kit was used for protein standards. The kit gives six calibration points covering a molecular weight range of 14 400–94 000. Protein concentration was determined by a Bio-Rad protein kit using crystalline bovine serum albumin as a standard.

Results and Discussion

Enzyme Purification, Stability, and Molecular Weight. IMP dehydrogenase has been purified to homogeneity from rat hepatoma 3924A. When the enzyme was purified according to an earlier method (Ikegami et al., 1987), we occasionally detected a few minor polypeptide bands besides the major band on SDS gel electrophoresis by an ultrasensitive silver staining. With the addition of the step of Sephacryl S-400 gel filtration, we have always obtained a homogeneous enzyme preparation that migrated as a single electrophoretic band in the presence of SDS (Figure 1). The specific activity of the final preparation was 103 μmol h^{-1} (mg of protein)$^{-1}$ and represented a 4980-fold purification over the original supernatant and a recovery of 30% of the initial activity. The enzyme activity of the final preparation was very unstable. During a 4-h dialysis to remove IMP from the preparation, the activity decreased to 8%, and after the preparation was stored at −70 °C for 24 h, the activity further dropped to 1% of the original preparation. To stabilize the purified enzyme for kinetic studies, 10 mg/mL bovine serum albumin was added to the preparation before the dialysis. With this method 95% of the activity was retained after the dialysis and 85% remained after 3 weeks at −70 °C. Stabilization with 1 mg/mL bovine serum albumin was less effective, and 10 mM DTT or 1.5 M KCl failed to stabilize the purified enzyme. The albumin could be completely removed from the stored sample in one step by an IMP Sepharose affinity column.

When either the 100000*g* supernatant or the partially purified enzyme after hydroxylapatite column chromatography was applied to a column of Sephacryl S-400, the activity was eluted from the column as a single peak, and a molecular weight of 245 000 was estimated by using thyroglobulin (669 000), catalase (232 000), bovine serum albumin, (67 000), and chymotrypsinogen (25 000) as standard proteins. The molecular weight of the subunit was about 60 000 by using 10% polyacrylamide gel electrophoresis in the presence of SDS. These data suggest that the native IMP dehydrogenase of hepatoma 3924A consists of four subunits with a molecular weight of about 60 000 each. This observation was different from that on the enzyme from Yoshida sarcoma ascites in which the estimated molecular weight of the native enzyme was 129 000 and the enzyme appeared to be composed of two identical subunits (Okada et al., 1983).

Kinetic Properties of Purified IMP Dehydrogenase. The double-reciprocal plots for dependence of velocity upon substrate concentration yielded K_m values for IMP and NAD^+ of 23 and 65 μM, respectively (data not shown). Excess NAD^+ caused substrate inhibition in the purified enzyme as described previously (Anderson & Sartorelli, 1968; Holmes et al., 1974; Jackson et al., 1977; Okada, 1983; Hupe et al., 1986). When NAD^+ concentration was increased to 1.0 mM, the activity of IMP dehydrogenase decreased to 80% of the maximum rate observed at 0.25 mM NAD^+. An apparent K_m value for K^+ of 7.8 mM was obtained from the double-reciprocal plots of various KCl concentrations in the presence of 250 μM IMP and 250 μM NAD^+ (data not shown).

The double-reciprocal plots for the inhibition by XMP indicated a competitive pattern versus IMP (data not shown). From a linear replot of slopes versus [XMP], a K_i of 136 μM was obtained. Inhibition by XMP at a fixed IMP concentration (250 μM) demonstrated a noncompetitive pattern versus NAD^+. The linear replots of slope and intercept gave $K_{i(slope)}$ of 320 μM and $K_{ii(intercept)}$ of 390 μM, respectively. In contrast, NADH exerted an uncompetitive inhibition with IMP (data not shown), yielding little slope effect but a large V_{max} effect. From a linear replot of intercept ($1/V_{max}$) versus [NADH], a K_i of 210 μM was obtained. A mixed type inhibition by NADH was observed with respect to NAD^+ (data not shown). NADH exerted both V_{max} and slope effects, but the ratio of slope/intercept ($K_{m,app}$) decreased, since the V_{max} effect was larger than the slope one. The replots of slope and intercept yielded $K_{i(slope)}$ of 290 μM and $K_{ii(intercept)}$ of 210 μM.

Inhibition by RMP and TAD. RMP and TAD, the chemotherapeutically active metabolites of ribavirin and tiazofurin, are potent inhibitors of IMP dehydrogenase (Streeter et al., 1973; Kuttan et al., 1982; Gebeyehu et al., 1986). The pattern obtained in double-reciprocal plots for these inhibitors is shown in Figures 2 and 3. RMP exerted competitive inhibition with IMP and noncompetitive inhibition with NAD^+ (Figure 2). The K_i value of 0.8 μM with IMP was obtained from a linear replot of slope versus [RMP], and $K_{i(slope)}$ of 3.6 μM and $K_{ii(intercept)}$ of 6.1 μM with NAD^+ were obtained from the replots of slope and intercept, respectively.

As shown in Figure 3, the inhibition by TAD was uncompetitive with IMP and a mixed type with NAD^+. TAD exerted only V_{max} effect with respect to IMP, and the replot of intercept yielded a K_i of 0.13 μM. TAD showed substantial effect on

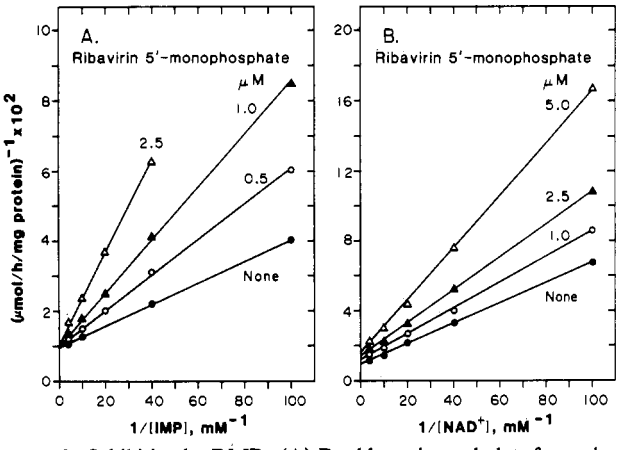

FIGURE 2: Inhibition by RMP. (A) Double-reciprocal plots for various IMP concentrations at a constant NAD^+ concentration (250 μM) and at RMP concentrations as indicated. (B) Double-reciprocal plots are shown for various NAD^+ concentrations at a constant IMP concentration (250 μM) and at various RMP concentrations.

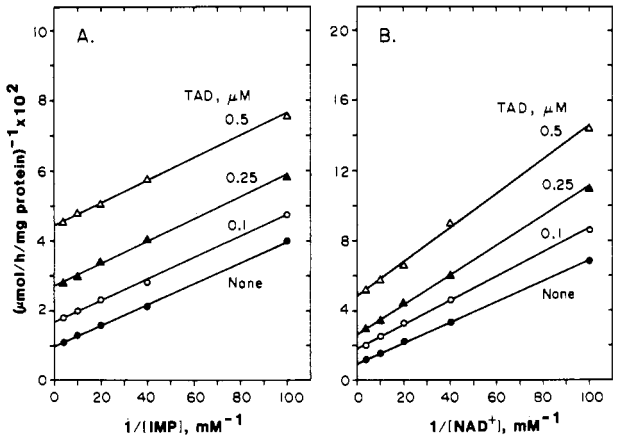

FIGURE 3: Inhibition by TAD. (A) Double-reciprocal plots are given for various IMP concentrations at a constant NAD^+ level (250 μM) and at various TAD concentrations. (B) Double-reciprocal plots are shown for various NAD^+ concentrations at a constant IMP level (250 μM) and at different TAD concentrations.

Table I: Inhibition of IMP Dehydrogenase by Products and Antimetabolites

compounds	substrates	K_i (μM)	types of inhibition
products			
XMP	IMP	136	competitive
XMP	NAD^+	320,[a] 390[b]	noncompetitive
NADH	IMP	210	uncompetitive
NADH	NAD^+	287,[a] 212[b]	mixed type
antimetabolites			
RMP	IMP	0.84	competitive
RMP	NAD^+	3.6,[a] 6.1[b]	noncompetitive
TAD	IMP	0.13	uncompetitive
TAD	NAD^+	0.73,[a] 0.13[b]	mixed type

[a] The values of $K_{i(slope)}$ are given. [b] The values of $K_{ii(intercept)}$ are given.

the V_{max} with a small slope effect with respect to NAD^+, and the replots of slope and intercept gave $K_{i(slope)}$ of 0.7 μM and $K_{ii(intercept)}$ of 0.13 μM, respectively.

The K_i values and the inhibition types of the reaction products and antimetabolites are summarized in Table I. The results indicate that RMP exerted the same inhibitory mechanism as XMP, and the inhibition by TAD was similar to that by NADH. However, the K_i values of RMP were 2 orders of magnitude lower than those of XMP, and the K_i values of TAD were 3 orders of magnitude lower than those of NADH. Thus, these drugs have different modes of action and interact at different ligand sites of the IMP dehydrogenase

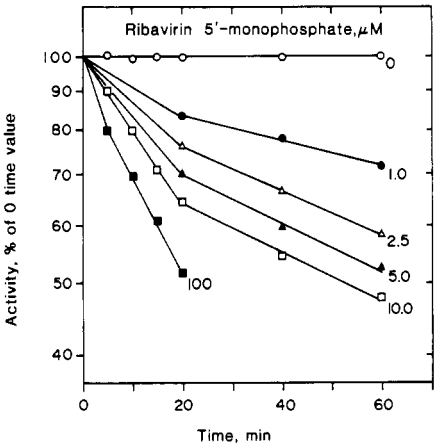

FIGURE 4: Effects of preincubation of purified IMP dehydrogenase with RMP. The purified enzyme was preincubated at 37 °C with various concentrations of RMP prior to the assay.

Table II: Effect of Preincubation of IMP Dehydrogenase with Ligands

ligands	concn (μM)	activity (% of control): not preincubated[a]	activity (% of control): preincubated[b]	activity recovered after preincubation
none		100[c]	100	100
RMP	2.5	89	62	70
TAD	0.5	73	73	100
XMP	1250	80	80	100
NADH	1250	63	62	98
IMP	1250	100	100	100
RMP	2.5	89	91	102
+IMP	1250			
RMP	2.5	76	60	79
+XMP	1250			
RMP	2.5	53	37	70
+NADH	1250			
RMP	2.5	65	41	63
+TAD	0.5			

[a] Activities of purified IMP dehydrogenase were determined in the absence or the presence of various ligands. [b] The purified enzyme was preincubated with ligands for 1 h at 37 °C prior to the assay. The assay mixture contained the same concentrations of the ligands, so that the concentrations of the ligands did not change throughout the preincubation of the assay. [c] The activity was expressed as percent of control.

with higher affinity than the natural substrates and products: RMP with the IMP–XMP site and TAD with the NADH site.

Increase of the Inhibition after Incubation of the Enzyme with RMP. The inhibitory effect was enhanced by preincubation of the purified IMP dehydrogenase with 1–100 μM RMP at 37 °C in a time-dependent manner (Figure 4). The enhancing action was also temperature dependent. When the enzyme was preincubated with 10 μM RMP for 20 min, the loss of activity at 0 °C (12%) was significantly less than that at 37 °C (36%). Although XMP has the same inhibitory mechanism as RMP, preincubation of the enzyme with XMP did not increase the inhibition (Table II). Incubation in the presence of TAD (0.5 μM), XMP (1250 μM), NADH (1250 μM), or IMP (1250 μM) did not exert any time-dependent change in the enzyme activity, whereas IMP and XMP protected against the time-dependent action induced by RMP (Table II). The results support the kinetic analysis that RMP shares the same binding site with IMP and XMP. In the presence of TAD, RMP further enhanced its inhibitory effect (Table II).

The reaction mechanism of IMP dehydrogenase and the mode of action of RMP and TAD are summarized in Figure

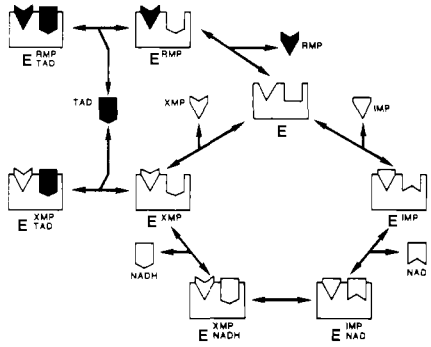

FIGURE 5: Reaction mechanisms of IMP dehydrogenase and the proposed mode of action of RMP and TAD.

5. The initial rate kinetics and the results of product inhibition studies show a pattern of ordered Bi-Bi mechanism (Cleland, 1963) in which IMP binds to free enzyme, followed by NAD^+. After rearrangement of the ternary complex, NADH is released, and finally XMP is liberated from the enzyme as proposed in previous studies with partially purified enzyme (Holmes et al., 1974; Jackson et al., 1977; Hupe et al., 1986). TAD has the same inhibitory mechanism as NADH and is thought to interact with E·XMP and produce E·XMP·TAD complex. By contrast, RMP competes in binding to the free enzyme with IMP and XMP and also enhances the inhibitory action in a time-dependent manner. The enzyme is protected from this effect in the presence of IMP or XMP by making an E·IMP (E·XMP) complex.

In physiological conditions in tissues the equilibrium between E, E·IMP, and E·XMP would be affected by concentrations of IMP and XMP. The content of IMP in rat liver and hepatomas is 19–63 nmol/g (Jackson et al., 1980), which is in the same order of magnitude as the K_m for IMP, 23 μM. For the forward reaction, concentrations of free NAD^+ ranging from 400 to 500 μM in rat liver cytosol (Tischler et al., 1977) would be saturating. By contrast, the XMP content in liver and hepatomas (3–10 nmol/g) (Jackson et al., 1980) is orders of magnitude lower than the K_i of 136 μM, probably because of its rapid removal by GMP synthase, which has high activity (Weber, 1983). The treatment by ribavirin would change this equilibrium by producing E·RMP complex. Although IMP competes with RMP for the same ligand site and protects against its time-dependent increase in inhibition, the combination of TAD with RMP might further shift this equilibrium to reduce E·IMP complex by producing E·RMP·TAD complex.

Synergistic antiviral effects are exerted by a combination of ribavirin with tiazofurin or its selenium analogue, selenazofurin, against some viruses (Higgins et al., 1984). In our preliminary studies the combination of ribavirin and tiazofurin synergistically killed hepatoma 3924A cells in culture (Yamada et al., 1987). Total cell kill by a single drug is rare in cancer chemotherapy because of the heterogeneity of tumor cells. Combination chemotherapy is usually mandatory to reduce the emergence of resistant tumor cells. The present studies show two distinct ligand sites of IMP dehydrogenase that provide promising drug targets. The results offer a biochemical basis for combination chemotherapy directed to the two sites on IMP dehydrogenase. The combination would potentiate the inhibitory action on the guanylate pathway and should reduce the emergence of resistant cells.

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