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Inhibition of Inosine Monophosphate Dehydrogenase (IMPDH) by 2-[2-(*Z*)-Fluorovinyl]inosine 5′-Monophosphate

Vasu Nair* and Ramesh C. Kamboj

Department of Pharmaceutical and Biomedical Sciences, The University of Georgia, Athens, GA 30602, USA

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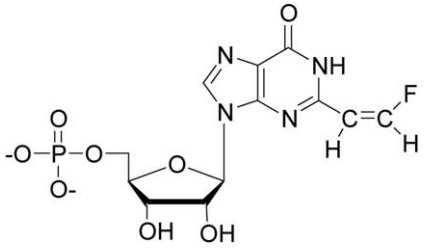
Abstract—Inosine 5′-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) isolated from *Escherichia coli* B3 cells was strongly inhibited by 2-[2-(*Z*)-fluorovinyl]inosine 5′-monophosphate (2-FVIMP). Inhibition of IMPDH appears to be irreversible with k_{inact} and K_i values of 0.0269 s^{-1} and 1.11 μM, respectively.

Inosine monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) catalyses the oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) with the concomitant reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH.[1–4] Its role at the metabolic branch point in the de novo purine nucleotide biosynthetic pathway makes it an interesting target in the discovery of drugs for antiviral and anticancer chemotherapy and in the area of immunosuppressive agents. Thus, inhibitors of IMPDH have been found to have anticancer, antiviral and immunosuppressive activity.[1–4] IMPDH is a sulfhydryl enzyme in which the active-site Cys-331 residue may act as a nucleophilic participant in interactions with inhibitors that are Michael acceptors. This is also consistent with the mechanism of substrate action of IMPDH which involves interaction of the enzyme and coenzyme (NAD^+) complex at the 2-position of IMP.[5] X-ray crystallographic data support the suggestion of a covalent adduct formation between Cys-331 of IMPDH and IMP during catalysis.[6] In an ongoing drug discovery program in our laboratory on antiviral agents, we designed and synthesized the novel compound, 2-[2-(*Z*)-fluorovinyl]inosine 5′-monophosphate (2-FVIMP, **1**), which is related to 2-vinylinosine and its monophosphate previously investigated in our laboratory and also by others.[7–10] This communication describes our studies on the inhibition of IMPDH by the interesting compound, 2-FVIMP.



Materials and Methods

Materials

IMP, NAD^+ and DTT were purchased from Sigma and glycerol, KCl, EDTA and $(NH_4)_2SO_4$ from Fisher. The other chemicals used in the study were of the highest purity available. The chromatographic medium, Green A-Sepharose was procured from Millipore (Bedford, MA, USA). *Escherichia coli B3* cells were grown at the Center for Biocatalysis and Bioprocessing, University of Iowa, Iowa City, USA. Concentration of enzyme samples at various levels of purification was carried out using an Amicon ultrafiltration cell equipped with YM-10 membrane. Compound **1** was synthesized from its nucleoside **3**, (Scheme 1) by standard phosphorylation.[11]

Purification of IMPDH from *E. coli B3*

All the purification steps were performed at 4 °C unless stated otherwise. IMPDH was purified from *E. coli B3* cells using a modification[7] of the procedure previously described by Hager et al.[12] Briefly, the bacterial cells (50

*Corresponding author. Tel.: +1-706-542-6293; fax: +1-706-583-8283; e-mail: vnair@rx.uga.edu

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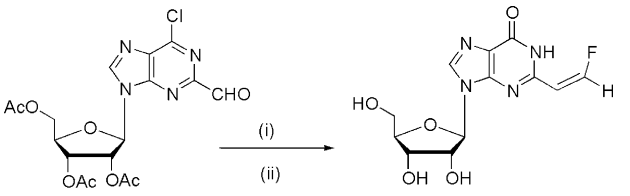

2 3

Scheme 1. Reagents and conditions: (i) (a) Bu_3P, $CFCl_3$, DCM, 0 °C – rt, 20 h, (b) 10% NaOH, rt, 24 h; (ii) NH_3/MeOH.

g) were harvested and then disrupted (French press) in 150 mL of 100 mM Tris–HCl buffer (pH 7.4) containing 150 mM KCl, 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF (Buffer A). The cell debris was removed by centrifugation at 10,000*g* for 15 min. The supernatant was treated with solid $(NH_4)_2SO_4$ (20% saturation) and centrifuged at 18,000*g* for 30 min. The supernatant so obtained was further saturated (70%) with solid $(NH_4)_2SO_4$ and centrifuged. The resulting pellet was dissolved in buffer A and dialyzed against it for 24 h at 4 °C with intermittent changes of buffer to remove $(NH_4)_2SO_4$. This enzyme solution was concentrated and then dialyzed against buffer B [20 mM Tris–HCl buffer (pH 7.4) containing 150 mM KCl, 1 mM EDTA and 1 mM DTT] for 24 h and was introduced onto a Green A-Sepharose column developed in buffer B. Unbound proteins were removed with buffer B. Bound enzyme was eluted from the column with a linear gradient of 0.15–1.0 M KCl in buffer B. The eluted fractions with IMPDH activity were pooled together, concentrated and dialyzed against 50 mM Tris–HCl buffer (pH 7.4) containing 150 mM KCl, 1 mM EDTA and 1 mM DTT). The specific activity of the enzyme was 78.7 μmoles/mg/min.

Enzyme assay

The standard assay solutions contain 100 mM Tris–HCl buffer pH 8.0, 1 mM dithiothreitol (DTT), 150 mM KCl and 1 mM EDTA. The appropriate amount of enzyme was added to this solution. Then the reaction was initiated by the addition of 50 μL substrate stock solution (20 mM IMP and 50 mM NAD^+) which yielded the final substrate concentration as 1 mM for IMP and 2.5 mM for NAD^+ in a total of 1.0 mL of assay solution. The IMPDH activity was measured by using a Cary 3 spectrophotometer by monitoring the formation of NADH at 340 nm for 2 min at 25 °C. One unit of enzyme activity is defined as the conversion of 1 μmol of NAD^+ to NADH per min per mg of enzyme protein at 25 °C.

Inhibition of IMPDH by 2-FVIMP

The purified IMPDH was desalted on Sephadex G-25 to remove DTT from the buffer just before the inhibition reaction. The inhibition reaction was initiated by the addition of various concentrations of 2-FVIMP to the enzyme solution. Aliquots were removed at the different time intervals and quenched by dilution into assay mixture [100 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 150 mM KCl without DTT]. The remaining activity was measured with final substrate and coenzyme concentrations of 1 mM IMP and 2.5 mM NAD^+, by monitoring the increase in absorbance at 340 nm at 25 °C. Less than 10% of the substrate was consumed during data collection. The data were fitted into the following eq 1:

$$\ln(V_t/V_o) = -k_{obs}\ t \quad (1)$$

where V_t is the activity at time t and V_o is the activity at time $t=0$. The k_{obs} values obtained for inactivation of IMPDH were then fitted into eq 2:

$$k_{obs} = k_{inact}[I]/(K_i + [I]) \quad (2)$$

where k_{inact} is the inactivation rate constant, K_i is the apparent dissociation constant and [I] is the inhibitor (2-FVIMP) concentration.

Inactivation, denaturation and renaturation of IMPDH by 2-FVIMP

IMPDH (100 μL) was incubated with 2-FVIMP (2.0 μM) for 1 h at 25 °C in the assay buffer (2.0 mL) having no DTT. Then urea was added to a final concentration of 8 M and the solution was kept at 25 °C for 1 h. The denatured enzyme was diluted 6-fold with 100 mM Tris–HCl buffer (pH 8.0) containing 150 mM KCl, 1 mM EDTA and 8 M urea and concentrated to its original volume. This dilution-concentration process was repeated four times and the final concentrate was dialyzed overnight against 100 mM Tris–HCl buffer (pH 8.0) containing 150 mM KCl and 1 mM EDTA. A control sample of IMPDH (not incubated with inhibitor) was also treated likewise using the same procedure. The enzymatic activity for both control and inhibitor treated enzyme were then measured.

Results and Discussion

Incubation of IMPDH with 2-FVIMP exhibited a time-dependent decrease in V_t/V_o as shown in Figure 1 which

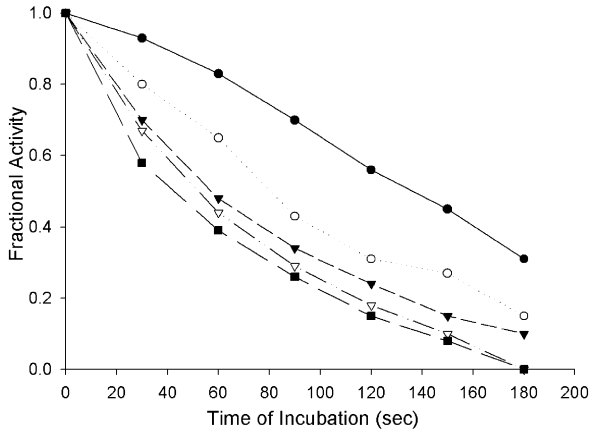

Figure 1. Inhibition of *E. coli* IMPDH by 2-FVIMP with respect to time (●, 0.25 μM; ○, 0.50 μM; ◀, 0.75 μM; △, 1.0 μM; ■, 1.5 μM).

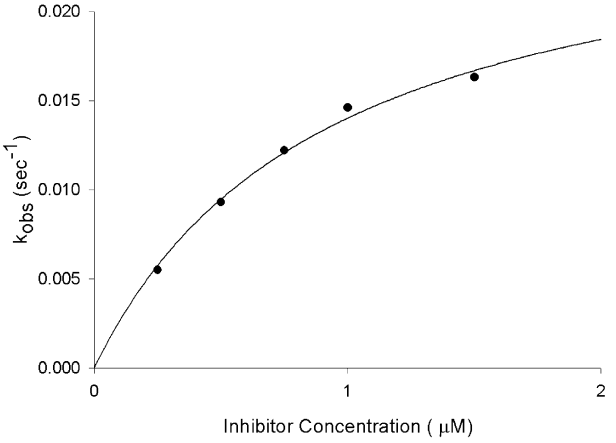

Figure 2. Relationship of k_{obs} values with the inhibitor (2-FVIMP) conc.

indicates that 2-FVIMP inactivates the enzyme. The activity of inhibited/inactivated enzyme could not be restored by dialysis. Urea denaturation and renaturation of the 2-FVIMP-inactivated IMPDH also did not restore enzyme activity. In a control experiment containing untreated enzyme there was 70% recovery of activity after urea treatment. These results imply that a covalent bond is formed between 2-FVIMP and IMPDH and, during the process, the enzyme is irreversibly inactivated.

For determining the mechanism of inhibition, the k_{obs} values obtained by using eq 1 were plotted against inhibitor concentration. The k_{obs} values displayed a hyperbolic relationship with 2-FVIMP concentration (Fig. 2). Therefore, 2-FVIMP reacts with IMPDH through a two-step mechanism as follows:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.I \xrightarrow{k_{inact}} E - I$$

where $K_i = k_1/k_{-1}$ is the dissociation constant, k_{inact} is rate constant of inactivation, E is IMPDH, E.I is the reversibly bound enzyme–inhibitor complex and E-I is the irreversibly inactivated enzyme. Thus, the mechanism of inactivation of IMPDH by 2-FVIMP involves the initial reversible formation of an E.I complex followed by the inactivation step. The values of k_{inact} and K_i were determined using eq 2 by plotting the reciprocal of k_{obs} versus the reciprocal of inhibitor concentration. The values of k_{inact} and K_i were 0.0269 s^{-1} and 1.11 μM, respectively, whereas the well-known IMPDH inhibitor, 6-chloropurine ribonucleoside monophosphate in these studies gave values of 0.076 min^{-1} and 62.0 μM. The type of inactivation of IMPDH shown by 2-FVIMP is related to that exhibited by ethynylimidazole carboxamide riboside monophosphate (EICARMP).[13] We conclude that 2-FVIMP is a potent inhibitor of IMPDH. The inactivation is time-dependent and follows a two-step mechanism. Antiviral screening of **3** against the vaccinia virus (HFF cell line) showed moderate activity (T.I. <4). The mechanism of this antiviral activity is likely associated with the ability of the cellularly produced monophosphate, **1**, to be an inhibitor of IMPDH.

Acknowledgements

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Data for **3**: mp 216–217 °C (decomp); UV (MeOH) $_{max}$ 299 (ε 6871), 253 (ε 7863).; ^{19}F NMR (CD_3OD): δ −105.23 (dd, $J = 39.4$, 76.2 Hz, 1F); ^{13}C NMR (CD_3OD) δ 63.1, 72.1, 75.8, 87.4, 90.6, 106.0 (d, $J_{F,C} = 4.3$ Hz), 124.5, 141.6, 149.9, 152.2, 156.2 (d, $J_{F,C} = 283.4$ Hz), 157.8; HRMS (FAB) calcd for $C_{12}H_{13}FN_4O_5$ $M + H^+$ 313.0950, found 313.0945.
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