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HUMAN IMP DEHYDROGENASE

KINETICS AND REGULATORY PROPERTIES

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SUMMARY

Human IMP dehydrogenase has been partially purified from placenta and characterized. The enzyme activity is located in the cytosol. The Michaelis constants for IMP and NAD are 14 and 46 μM, respectively. In addition, the enzyme requires a monovalent cation for maximal activity and the apparent K_m for K^+ is 17 mM. The purine ribonucleotides XMP, GMP, and AMP inhibit the enzyme in a manner which is competitive with respect to IMP. The K_i values are 30 μM, 100 μM, and 530 μM, respectively. The enzyme is also sensitive to inhibition to a lesser degree by pyrimidine ribonucleotides. The pharmacologic agents, 6-mercaptopurine ribonucleotide, allopurinol ribonucleotide and mycophenolic acid are also inhibitors of human IMP dehydrogenase. A kinetic analysis indicates an ordered sequential reaction mechanism in which IMP binds first and XMP is released last.

INTRODUCTION

IMP dehydrogenase (IMP:NAD^+ oxidoreductase, EC 1.2.1.14) catalyzes the NAD^+-dependent conversion of IMP to XMP. This reaction is the first in a branched pathway leading to the synthesis of GMP and consequently it is a potential site for the regulation of this pathway [1]. Data available from the study of this enzyme in bacterial [2–9], fungal [10], and neoplastic mammalian cells [11, 12] are conflicting with regard to its regulation. Some studies have suggested that it is a complex allosteric protein with subunits that interact with both negative and positive effector molecules (purine ribonucleotides) [6, 7, 9, 10]. Other studies have failed to demonstrate an allosteric nature of this enzyme [4, 5, 8, 12]. Because of the importance of IMP dehydrogenase in the regulation of GMP biosynthesis, we have undertaken a study of the kinetic mechanism and regulation of the human enzyme. In this paper we report the results of these studies.

MATERIALS AND METHODS

[8-^{14}C]Hypoxanthine (2.48 Ci/mole) was purchased from New England Nuclear

Corporation and [8-^{14}C]IMP was enzymatically synthesized using a partially purified preparation of human hypoxanthine–guanine phosphoribosyltransferase as previously described [13]. *P*-Rib-*P*-*P* (sodium salt) and NAD^+ were purchased from Sigma Chemical Company. Mycophenolic acid and allopurinol ribonucleotide were gifts from Eli Lilly and Company and Burroughs–Wellcome Company, Inc., respectively. All other reagents were of the highest grades commercially available and the concentration of all compounds used was based on data provided by the manufacturer.

The kinetic constants, K_m and K_i, were obtained from graphical solutions of the abscissa intercept of the primary intercept and slope replots, respectively [15–17, 20].

Enzyme assays

IMP dehydrogenase was routinely assayed at 37 °C in 100 μl of 50 mM potassium phosphate buffer, pH 7.4, containing 0.043 mM [8-^{14}C]IMP, 0.3 mM NAD, 100 mM KCl and 1 mM disodium EDTA. The reaction was stopped by the addition of 100 μl of cold 95% ethanol and the protein removed by centrifugation at 1200 $\times$ *g* for 15 min. An aliquot (75 μl) of the supernatant fluid was spotted on Whatman DE-81 paper with 0.1 μmole of carrier XMP and developed in a descending system for 2.5 h in 0.2 M ammonium formate buffer, pH 5.0. The XMP spot was identified with an ultraviolet light source, cut out and counted at 63% efficiency in a Packard Tri-carb liquid scintillation counter. As previously reported this system provides adequate separation of XMP and IMP and the authenticity of XMP has been varified in two other systems [13]. XMP production was linear with respect to incubation time and protein concentration with all enzyme preparations except for the crude placental extract.

The partially purified enzyme preparation used for the kinetic studies provided linear production of XMP with respect to incubation time and protein concentration at all concentrations of substrates and inhibitors employed. Using this partially purified enzyme preparation the reaction catalyzed by human IMP dehydrogenase demonstrated the following stoichiometry: 0.93 mole of IMP consumed and 1.07 moles of NADH formed for each 1.00 mole of XMP produced. The standard radiochemical assay was used for measuring the relative conversion of IMP to XMP while NADH and XMP were quantitated in a Zeiss spectrophotometer by observing the change in absorbance at 340 and 290 nm [3], respectively.

Nucleotidase activity was assayed under the same conditions as described above for IMP dehydrogenase. At the conclusion of the incubation period, a 20-μl aliquot of the reaction mixture was spotted on Whatman 3 MM chromatography paper with carrier IMP, inosine and hypoxanthine. The purine compounds were then separated by high-voltage electrophoresis at 5000 V for 15 min in 50 mM sodium borate buffer, pH 9.0. IMP, inosine, and hypoxanthine spots were located with ultraviolet light, cut out and counted as described above.

Protein was determined by the method of Lowry et al. [14] with bovine serum albumin as standard.

Enzyme purification

Step 1. Human placenta was obtained immediately following delivery. The cotyledons were dissected from the fetal membranes and homogenized in 1 vol. of

50 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol. This and all subsequent steps were performed at 4 °C. The homogenate was centrifuged at 15 000 × *g* for 15 min.

Step 2. The supernatant fluid was applied to a Whatman DE 52 column (1.5 cm × 20 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM dithiothreitol (Buffer A). The column was washed with 500 ml of Buffer A. A linear gradient of 0–500 mM KCl in 500 ml of Buffer A was applied to the column. The enzyme activity was eluted at a Cl^- concentration of approx. 250 mM and those fractions containing IMP dehydrogenase were pooled and concentrated on a Diaflo ultrafiltration cell fitted with a UM 10 ultrafiltration membrane (Amicon Corporation). This enzyme concentrate was then dialyzed against 1000 vol. of 50 mM potassium phosphate buffer, pH 7.4, for 2 h.

Step 3. The dialyzed concentrate of IMP dehydrogenase was applied to another Whatman DE 52 column and Step 2 of the purification was repeated in its entirety. Following this the dialyzed concentrate of IMP dehydrogenase was stored at −70 °C.

Subcellular fractionation

Human placenta obtained immediately after delivery was homogenized at the slowest speed in a blender for 15 s in 2 vol. of 50 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose. The homogenate was centrifuged at 1000 × *g* for 15 min. Mitochondria were prepared by a subsequent centrifugation of the supernatant fluid at 11 000 × *g* for 15 min. The soluble supernatant fraction was obtained by another centrifugation at 100 000 × *g* for 60 min. The residue of each centrifugation was resuspended in 50 mM potassium phosphate buffer, pH 7.4, and the subcellular fractions were disrupted by freeze–thawing twice.

RESULTS

Enzyme purification

The purification procedure for human IMP dehydrogenase described in this paper results in an enzyme preparation that is stable for more than 3 months when stored at −70 °C and it contains no detectable nucleotidase activity (less than 1.0 pmole/h). However, the extent of purification and recovery of enzyme activity cannot be quantitated reliably because the large amount of nucleotidase activity in the crude placental homogenate prevents an accurate measure of enzyme activity. The purification procedure did result in a 60-fold reduction in total protein content of the enzyme preparation. 1 mg of this protein preparation converted 16 nmoles of IMP to XMP in 1 h, which is roughly 10 000 times the specific activity of IMP dehydrogenase found in normal human erythrocytes [13].

Subcellular fractionation

Because of the inability to assay IMP dehydrogenase in the crude homogenate accurately, it is not possible to calculate recovery of enzyme activity in any subcellular fraction. However, negligible amounts of IMP dehydrogenase activity were detected in the 1000 × *g*, 11 000 × *g*, and 100 000 × *g* pellets, while the amount of enzyme activity in the 100 000 × *g* supernatant fluid was the same as that observed in the starting preparation.

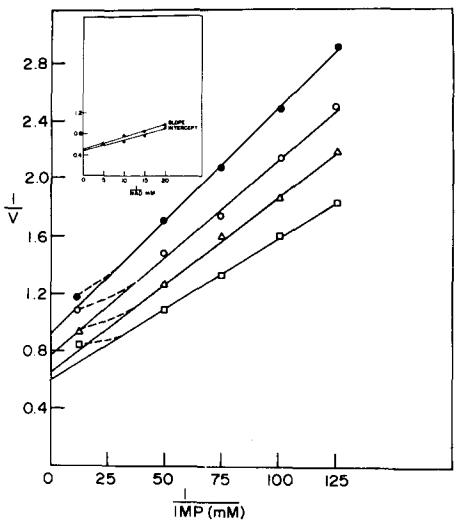

Fig. 1. Lineweaver–Burk plot with IMP as the variable substrate. The assays were performed in 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl and 1 mM EDTA. The concentrations of IMP are indicated on the abscissa; NAD was used in the following concentrations: 0.20 mM (□—□), 0.10 mM (△—△), 0.067 mM (○—○), and 0.050 mM (●—●). A replot of the slopes and intercepts is shown in the inset.

Initial velocity kinetics

Fig. 1 is a Lineweaver–Burk plot in which enzyme activity was determined at variable concentrations of IMP. Each line represents a set of experiments performed at different fixed concentrations of NAD. The inset graph is a replot of the slopes and intercepts. From this analysis the K_m for NAD was determined to be 46 μM (Table I). As demonstrated in this figure the Lineweaver–Burk plots were linear except at high concentrations of substrate where substrate inhibition was noted. The human enzyme exhibited no evidence of sigmoidal kinetics. In addition the non-parellel lines suggest that the reaction mechanism is a sequential one [15–17].

Fig. 2 is similar to Fig. 1 except NAD was the variable substrate and each line represents different fixed concentrations of IMP. From this set of experiments the K_m for IMP was determined to be 14 μM (Table I). As with IMP, the reciprocal plots for NAD were always linear except for substrate inhibition.

Cation requirement

The human enzyme like that from other species demonstrates a requirement

TABLE I

KINETIC CONSTANTS FOR HUMAN IMP DEHYDROGENASE

Compound	K_m (μM)	K_i (μM)
IMP	14	
NAD	46	
XMP		30
GMP		100
AMP		530

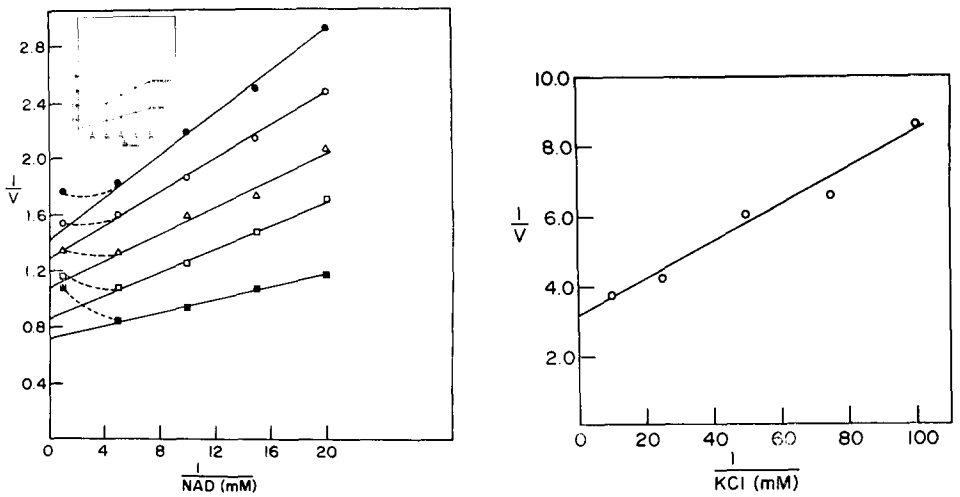

Fig. 2. Lineweaver–Burk plot with NAD as the variable substrate. Assay conditions were the same as in the legend of Fig. 1. The concentration of NAD is indicated on the abscissa; IMP was used in the following concentrations: 0.083 mM (■—■), 0.02 mM (□—□), 0.013 mM (△—△), 0.0098 mM (○—○), and 0.0079 mM (●—●). The replot of the slopes and intercepts is shown in the inset.

Fig. 3. Apparent K_m for K^+. The standard enzyme preparation was passed through a Sephadex G-25 column equilibrated with 50 mM Tris–HCl buffer, pH 7.4, just prior to use. The standard assay was used with IMP at a concentration of 0.043 mM and NAD at 0.30 mM.

for a metal cation for maximal catalytic activity [2, 8, 12]. Following passage through a Sephadex G-25 column equilibrated with 50 mM Tris–HCl buffer, pH 7.4, the enzyme assayed in the absence of any added K^+ had only 10% of the activity observed with a saturating concentration of this ion. The apparent K_m for K^+ was determined to be 17 mM (Fig. 3). K^+ could be at least partially replaced by NH_4^+ in this reaction. The velocity with 100 mM NH_4Cl was 40% of that observed with 100 mM KCl.

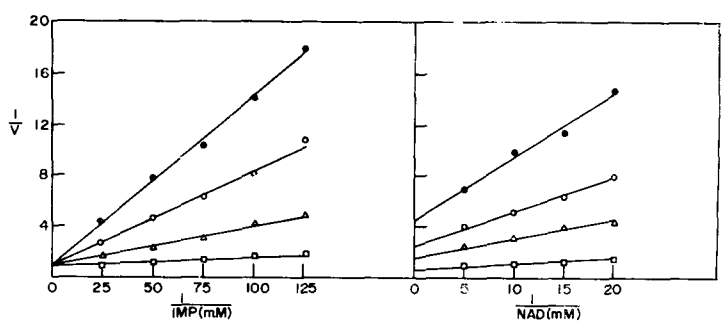

Fig. 4. Inhibition of human IMP dehydrogenase by XMP. The left-hand panel depicts the inhibition pattern produced by XMP when IMP was the variable substrate. NAD concentration was 0.2 mM in all assays and XMP was used in the following concentrations: control (□—□), 0.1 mM (▲—▲), 0.25 mM (○—○), and 0.5 mM (●—●). The right-hand panel depicts the inhibition pattern produced by XMP when NAD was the variable substrate. IMP concentration was 0.042 mM in all assays and XMP was used in the following concentrations: control (□—□), 0.25 mM (△—△), 0.5 mM (○—○), and 1.0 mM (●—●).

Product-inhibition patterns

Since the data presented above suggested a sequential reaction mechanism, product-inhibition studies were performed to determine whether this was an ordered or a random sequential mechanism. Studies performed with the product XMP are presented in Fig. 4. When IMP was the variable substrate, the inhibition produced by XMP was competitive. With NAD as the variable substrate, the pattern of inhibition was non-competitive. Studies performed with the product NADH are presented in Fig. 5. No competitive inhibition was observed with either NAD or IMP as the variable substrate.

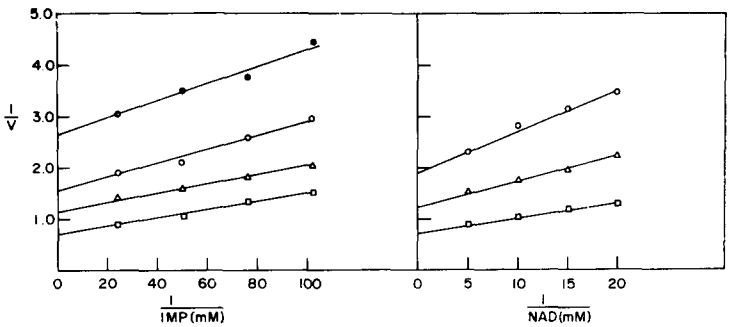

Fig. 5. Inhibition of human IMP dehydrogenase by NADH. The left-hand panel depicts the inhibition pattern produced by NADH when IMP was the variable substrate. NAD concentration was 0.2 mM in all assays and NADH was used in the following concentrations: control (□--□), 0.1 mM (△—△), 0.2 mM (○—○), and 0.4 mM (●—●). The right-hand panel depicts the inhibition pattern produced by NADH when NAD was the variable substrate. IMP concentration was 0.042 mM in all assays and NADH was used in the following concentrations: control (□—□), 0.1 mM (△—△), and 0.5 mM (○—○).

Nucleotide inhibition

The inhibition produced by a number of different compounds is listed in Table II. Human IMP dehydrogenase demonstrated the following order of sensitivity to inhibition by purine ribonucleotides: XMP > GMP > AMP. Allopurinol ribonucleotide and 6-mercaptopurine ribonucleotide were also inhibitors of the enzyme. Although the enzyme was inhibited by pyrimidine ribonucleotides, the degree of inhibition was less marked than that observed with equimolar concentrations of purine ribonucleotides. The sensitivity to both purine and pyrimidine ribonucleotide inhibition demonstrated the following order: mono > di > triphosphate. Although purine nucleosides and bases had no effect on enzyme catalytic activity, the most striking inhibition of human IMP dehydrogenase was produced by mycophenolic acid, an experimental drug with anti-neoplastic potential [18, 19].

Studies on the inhibition of human IMP dehydrogenase by the products of purine biosynthesis, GMP and AMP, are presented in Fig. 6. Both GMP and AMP inhibited the human enzyme in a strictly competitive fashion with respect to IMP. The Lineweaver–Burk plots were linear at all concentrations of nucleotide studied and the Hill coefficient was not greater than 1.1 for any concentration of GMP studied. The K_i values for XMP, GMP and AMP were determined to be 30 μM, 100 μM, and 530 μM, respectively (Table I). The inhibition produced by the combination of GMP and AMP was strictly additive and no evidence of synergistic inhi-

TABLE II

INHIBITION OF HUMAN IMP DEHYDROGENASE BY PURINE AND PYRIMIDINE COMPOUNDS

Compounds*	Percentage residual activity**
None	100
XMP	5
Guanine	96
Guanosine	108
GMP	9
GDP	29
GTP	28
Adenine	102
Adenosine	95
AMP	22
ADP	66
ATP	73
CMP	52
CDP	97
CTP	92
6-Mercaptopurine	102
6-Mercaptopurine ribonucleotide	6
Allopurinol ribonucleotide	50
Mycophenolic acid	0

* All compounds were included in the enzyme assay at a final concentration of 5 mM.
** The standard assay was used with IMP at a concentration of 0.043 mM and NAD at 0.30 mM.

bition was observed. *P*-Rib-*P*-*P* had no effect on enzyme activity either in the presence or absence of GMP. However, we did observe an apparent "stimulation" of enzyme catalytic activity by numerous purine and pyrimidine nucleotides and nucleosides in less pure enzyme preparations containing significant amounts of nucleotidase activity (through Step 2 of the purification). When the enzyme was further purified (comple-

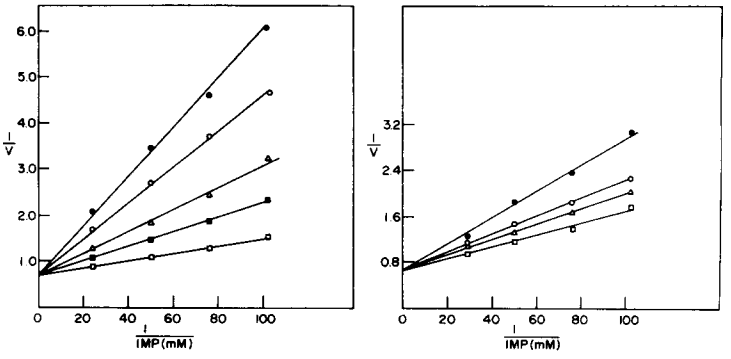

Fig. 6. Inhibition of human IMP dehydrogenase by GMP and AMP. The left-hand panel depicts the pattern of inhibition produced by GMP when IMP was the variable substrate. GMP was used in the following concentrations: control (□—□), 0.1 mM (■—■), 0.2 mM (△—△), 0.4 mM (○—○), and 0.6 mM (●—●). The right-hand panel depicts the pattern of inhibition produced by AMP when IMP was the variable substrate. AMP was used in the following concentrations: control (□—□), 0.1 mM (△—△), 0.25 mM (○—○), and 0.50 mM (●—●).

tion of Step 3) and nucleotidase activity was no longer detectable, the "stimulation" produced by these compounds was no longer evident (Table II).

DISCUSSION

The results of the initial velocity and product-inhibition studies of human IMP dehydrogenase are consistent with an ordered sequential reaction mechanism in which IMP binds to the enzyme first followed by the binding of NAD [14, 16, 17, 20]; the first product to be released is NADH and the last is XMP (Fig. 7). This mechanism has been proposed for IMP dehydrogenase isolated from *Aerobacter aerogenes* [4, 5], *Escherichia coli* [8], and sarcoma 180 ascites tumor cells [12], as well as a number of different pyridine nucleotide dehydrogenase enzymes [20].

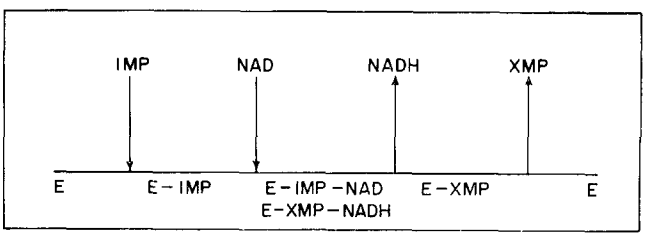

Fig. 7. Proposed kinetic mechanism for human IMP dehydrogenase.

The K_m for IMP observed with the human enzyme (14 μM) is similar to that observed for the IMP dehydrogenase from sarcoma 180 cells [12], Ehrlich ascites tumor cells [11], *A. aerogenes* [4, 5] and *E. coli* [8]. The K_m for NAD noted with the human IMP dehydrogenase (46 μM) is also in close agreement with that observed with the enzyme from sarcoma 180 cells [12] and *E. coli* [8], but somewhat lower than that observed for IMP dehydrogenase from *A. aerogenes* [4, 5]. In addition, the human enzyme was similar to that from other mammalian sources [12] and bacteria [2,8] in that it required a monovalent cation for maximal catalytic activity. As demonstrated with other forms of the enzyme [8, 12], K^+ appeared to be more effective than NH_4^+ in producing maximal activity of human IMP dehydrogenase.

The K_i values for the purine ribonucleotides appear to be somewhat lower for the human enzyme than those reported for the *E. coli* enzyme [8]. Human IMP dehydrogenase demonstrated the following order of sensitivity to inhibition by purine ribonucleotides (XMP > GMP > AMP), whereas the order of sensitivity for the *E. coli* enzyme was GMP > XMP > AMP [8]. However, the human and *E. coli* enzymes were similar in that sensitivity to ribonucleotide inhibition was observed in the order: mono > di > triphosphate. There was no evidence that human IMP dehydrogenase was inhibited by purine nucleosides or bases.

Human IMP dehydrogenase exhibited Michaelis–Menten kinetics under all conditions examined in the present study and no evidence was found to suggest the human enzyme was an allosteric protein. These results are in accord with previous studies of IMP dehydrogenase isolated from sarcoma 180 ascites tumor cells [12], *A. aerogenes* [4, 5] and *E. coli* [8]. However, these results differ from those obtained

with IMP dehydrogenase isolated from *Salmonella typhimurium* [6], *Bacillus subtilis* [7], *E. coli* [9], and *Schizosaccharomyces pombe* [10] which suggest the enzyme may be an allosteric protein. Negative cooperativity with GMP has been observed for IMP dehydrogenase from some bacteria [6, 7, 9] and yeast [10], while the human enzyme demonstrated only competitive binding of GMP and IMP. There was no evidence of a change from hyperbolic to sigmoidal kinetics in the presence of any purine ribonucleotide. Human IMP dehydrogenase also differed from the bacterial enzyme in that it did not demonstrate positive cooperativity with AMP [9].

Before initiation of the present study it was not possible to predict the mechanism by which human IMP dehydrogenase was regulated, since the data available from the study of this enzyme in other species had been conflicting with regard to its regulation [2–12]. In this paper data have been presented that indicate human IMP dehydrogenase is regulated by a feedback control in which purine ribonucleotides (most likely monophosphates) produce inhibition through competitive binding with the substrate IMP. There is no evidence for cooperative interaction in this binding and the human enzyme does not appear to be an allosteric protein. In addition these studies provide another potential step at which 6-mercaptopurine and allopurinol may alter purine metabolism in man. Finally, they support previous work in other species which has suggested that the anti-neoplastic action of mycophenolic acid may be the result of inhibition of IMP dehydrogenase [18, 19].

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