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REGULATION OF THE BIOSYNTHESIS OF PURINE NUCLEOTIDES IN *SCHIZOSACCHAROMYCES POMBE*

II KINETIC STUDIES OF IMP DEHYDROGENASE

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SUMMARY

1 IMP dehydrogenase was prepared from *Schizosaccharomyces pombe* Enzyme kinetics showed properties typical of allosteric enzymes

2 In the absence of any effector, the substrate NAD exerted homotropic effects No heterotropic interaction between NAD and substrate IMP was detected

3 IMP showed neither homotropic nor heterotropic effects

4 GMP exerted an allosteric effect upon the kinetic of fixation of NAD and showed an homotropic effect for its own fixation

INTRODUCTION

IMP dehydrogenase (IMP NAD oxidoreductase, EC 1 2 1 14) is the first enzyme in the metabolic pathway of the transformation of IMP into GMP This enzyme is expected to be submitted to some feedback regulation by GMP and thus to show allosteric properties The kinetic studies of BROX AND HAMPTON[1] and of ANDERSON AND SARTORELLI[2] have elucidated the reaction mechanism catalyzed by this enzyme, isolated from *Aerobacter aerogenes* and sarcosomal cells These authors proposed an ordered addition mechanism with IMP as first ligand, however they did not mention any allosteric effect On the other hand, BUZZEE AND LEVIN[3] present evidence suggesting that in *Salmonella typhimurium* separate binding sites for GMP and IMP exist Allosteric kinetics of the enzyme in the presence of GMP were briefly mentioned This paper further elucidates the allosteric kinetic properties of an IMP dehydrogenase prepared from *Schizosaccharomyces pombe*. Clear non-Michaelian kinetics could be found in the absence of any effector

MATERIALS AND METHODS

Enzyme preparation

A high level of IMP dehydrogenase was found in gu_2 mutants which are auxo-

trophic for guanine and presumably lack GMP synthetase (EC 6 3 4 1) The cells are grown aerobically at 30° for 48 h in a liquid medium containing yeast extract supplemented with guanine 20 mg/l They are harvested at the end of the log phase The pellet is resuspended in 1 M Tris–HCl (pH 8 4), 0 1 M KCl and 0 001 M glutathione The cells are disrupted by shaking with glass beads in a refrigerated Braun shaker and the resulting homogenate is centrifuged 30 min at 71 000 × g Nucleic acids are removed by precipitation with 10% (v/v) of 1 M $MnCl_2$ and then with 0 06% (w/v) protamine sulfate. The supernatant is further fractionated by precipitation between 35% to 55% of saturation with $(NH_4)_2SO_4$ The precipitated enzyme is redissolved in a minimum volume of buffer and dialyzed for 2 h against 0 5 M Tris–HCl (pH 8 4), 0 1 M KCl, $2\,5 \cdot 10^{-4}$ M glutathione After this partial purification, the specific activity rises from 0 002 to 0 008 unit/mg protein Glycerol is added to the dialyzed enzyme to a final concentration of 20–25% w/v, this solution can be stored for a few weeks at −20° without appreciable loss in activity Enzyme preparations once thawed are stable for a day but loose part of their activity when frozen and thawed again Each set of curves presented below have been obtained within a day with enzyme solutions which had never been previously thawed

Enzyme assay

The spectrophotometric assay described by MAGASANIK[4] has been slightly modified The reaction mixture contains in a total volume of 2 ml 0 1 M Tris–HCl, 0 1 M KCl, $5 \cdot 10^{-3}$ M glutathione, varying levels of NAD, water and enzyme. The enzyme is incubated for 6 min at 24° and the reaction is started by adding prewarmed IMP The reference cell contains no IMP. The activity is measured by the increase in absorbance obtained in 10 min at 290 mμ The increase in absorbance is linear during these 10 min, and the activity is a linear function of the enzyme concentration The values of v_{max} were computed from double reciprocal plots of the reaction velocity *versus* substrate concentrations

RESULTS

Properties of the enzyme

The enzyme shows a broad pH optimum between pH 8 2 and 9 5 However the pH of the reaction strongly influences the kinetic properties as well as the stability of the enzyme, in the absence of glycerol the enzyme is unstable and the inactivation

TABLE I

EFFECT OF NUCLEOTIDES UPON THE ACTIVITY OF IMP DEHYDROGENASE

Assay conditions are described in the text, pH of the reaction was 9 4, NAD concn , 0 5 mM, IMP concn , 0 6 mM

	Nucleotide added to the assay (mM)								
	AMP 0 8	*ADP 0 8*	*ATP 0 8*	*UMP 0 6*	*CMP 0 6*	*TMP 0 6*	*GMP 0 6*	*GDP 0 6*	*GTP 0 6*
Inhibition	0	0	0	25%	25%	25%	55%	45%	45%
Activation	0	0	30%	0	0	0	0	0	0

is faster in buffers of weak ionic strength Dialysis against 0 01 M Tris–HCl (pH 8 4), 0.01 M KCl and 2 5 10^{-4} M glutathione rapidly inactivates the enzyme However addition of either dialyzate or Tris–HCl buffer (*cf* ref 5) to the reaction mixture results in reactivation K^+ is required for enzyme activity, the K_m for K^+ has been found to be $2 \cdot 10^{-2}$ M at pH 8 4 The enzyme activity is affected by several nucleotides Table I shows the percentages of inhibition or of activation exerted by these nucleotides

Saturation of IMP dehydrogenase by its substrates

All kinetic results reported here were obtained at pH 9 4 and 24° In these conditions the allosteric effects were most visible The empirical Hill equation may be written as

$$\log \frac{v}{v_{max} - v} = n \log [S] - \log K$$

where v is the conversion rate of substrate to product, n the number of interacting sites, $[S]$ the concentration of substrate and K the product of the n dissociation constants for the separate binding steps CHANGEUX[6] has referred to n as an interaction coefficient depending on the number of interacting substrate binding sites and on the strength of interaction, when interactions are very weak, n equals 1 n was

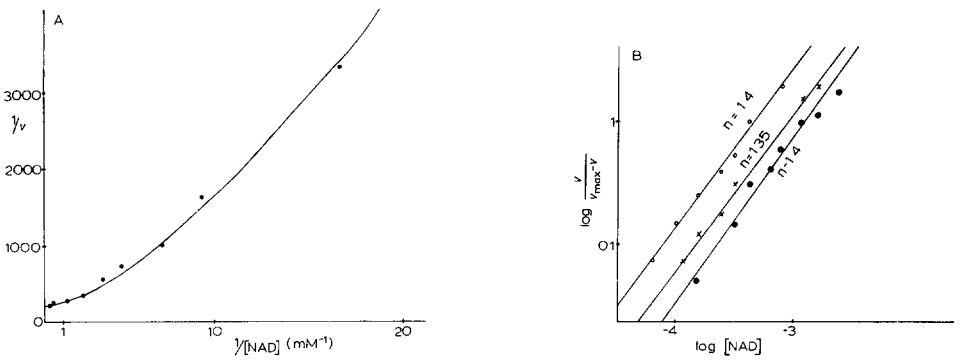

Fig 1 Saturation of IMP dehydrogenase by NAD (A) Double-reciprocal plot of reaction rate, measured as absorbance increase/min *vs* [NAD] Assay conditions are described in the text pH of the reaction was 9 4, IMP concn , 3 mM (B) Plot of log $v/(v_{max} - v)$ against log [NAD] Assay conditions are those of (A), IMP concn ○, 3 mM, ×, 1 mM, ●, 0 15 mM

calculated as the slope of the straight line which is obtained by plotting log $v/(v_{max} - v)$ as function of log $[S]$, for each of the substrates at three different constant concentrations of the other one In Fig 1B, the reaction velocities at varying NAD levels are plotted as described above The slope of the straight line obtained is approx 1 4 Different constant IMP concentrations displace the curve but do not significantly alter its slope On the other hand, it can be seen in Fig 2B that when IMP is the variable substrate, n has a constant value of 1 which remains constant for the levels of NAD tested

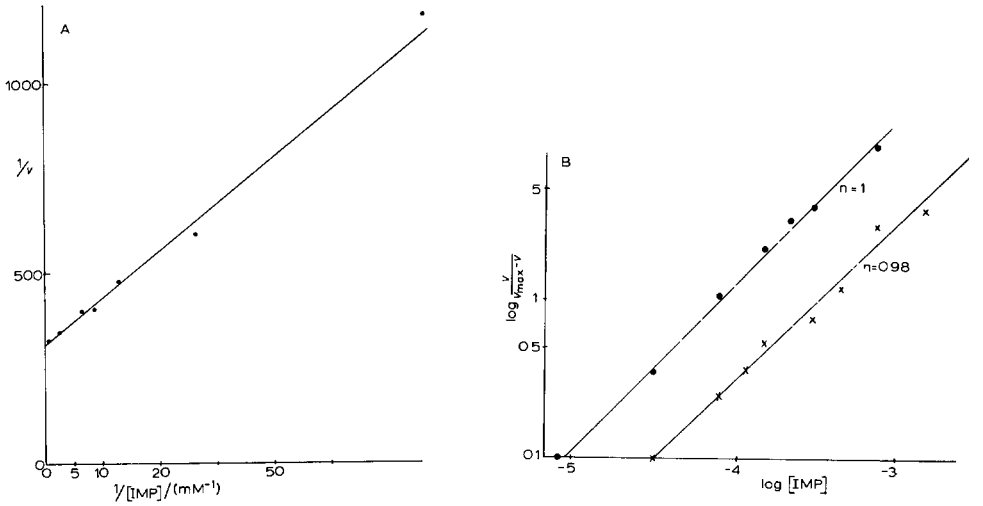

Fig 2 Saturation of IMP dehydrogenase by IMP (A) Double-reciprocal plot of reaction rate, measured as absorbance increase/min *vs* [IMP] Assay conditions are described in the text, pH of the reaction was 9 4, NAD concn , 1 6 mM (B) Plot of log $v/(v_{max} - v)$ against log [IMP] Assay conditions are those of Fig 2A NAD concn ●, 1 6 mM, ×, 0 4 mM

Inhibition by GMP

Addition of GMP to the reaction mixture is inhibitory Plots of enzyme activity against GMP concentration (Fig 3A) give sigmoidal curves, suggesting that more than one GMP molecule per enzyme molecule participate in the formation of the inactive inhibitor–enzyme complex According to TAKETA AND POGELL[7], if one assume the over-all reaction

$$E + n \text{ GMP} \rightleftarrows E \text{ GMP } n,$$

then

$$\log \frac{v_0 - v}{v} = \log K + n \log [\text{GMP}]$$

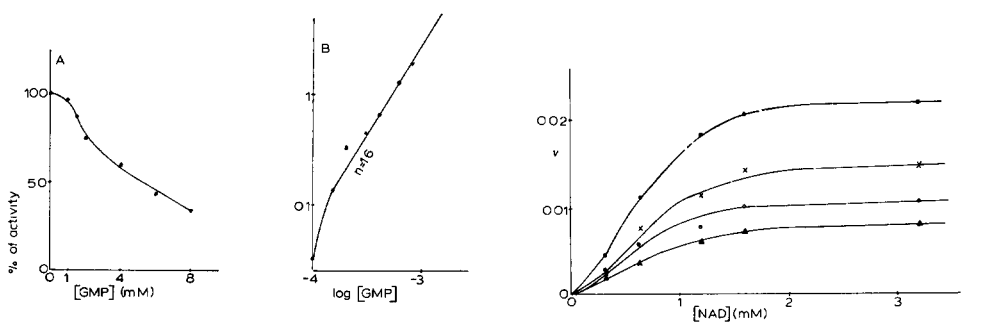

Fig 3 Inhibition of IMP dehydrogenase activity by GMP (A) Plot of the percentage of activity *vs* [GMP] pH of the reaction was 9 4 IMP concn , 0 15 mM, NAD concn , 1 6 mM (B) Plot of log $(v_0 - v)/v$ against log [GMP] constructed after (A)

Fig 4 Saturation of IMP dehydrogenase by NAD at different constant GMP concn Plots of the activity v measured as absorbance increase/min *vs* [NAD] Assay conditions are the same as for Fig 1 except pH which is 8 4 GMP concn ●, 0 mM, ×, 0 2 mM, ▲, 0 6 mM, ○, 0 4 mM

where E is the enzyme concentration, v_0 the reaction rate in the absence of GMP and n the apparent number of GMP molecules reacting per enzyme molecule Fig 3B shows that n approximates 1 6 at GMP concentrations higher than 3 mM Fig. 4 shows that GMP mainly inhibits the v_{max} of the reaction and is not competitive with NAD Similar results are obtained by varying the IMP concentration These last results have been obtained at pH 8 4 where the curve is less sigmoidal than at pH 9 4 However similar results are obtained at pH 9 4, the interaction coefficient of NAD passes from $n = 1\ 4$ in the absence of GMP to $n = 1\ 7$ in the presence of $4 \cdot 10^{-4}$ M GMP

DISCUSSION

Our results show that by varying the NAD or GMP concentration, the enzyme kinetics do not obey simple mass action laws Apparent homotropic effects were obtained only for NAD and GMP and not for IMP No heterotropic effects either from IMP or NAD on the fixation of NAD or IMP have been detected The sigmoidal curves with interacting coefficients greater than one, obtained when the reaction velocity is plotted *versus* substrate or effector concentrations, suggest that IMP dehydrogenase is a multisite enzyme which manifests some of the characteristic kinetic properties predicted by the allosteric theory of MONOD *et al* [8]

The kinetic properties of IMP dehydrogenase have previously been studied using the classical procedure of CLELAND[9,10] These results obtained in other conditions are consistent with a classical ordered addition mechanism, IMP being the first ligand Although no allosteric kinetic was reported, NICHOLS *et al* [11] have postulated the existence of a configuration modification in the enzyme mediated by the phosphate moiety of IMP, BROX AND HAMPTON[1] have suggested that such a modification might be a prerequisite for the fixation of NAD BUZZEE AND LEVIN[3] were first to report allosteric kinetics of the enzyme in the presence of GMP, while the present paper reports allosteric kinetics in the absence of any effector All these observations are not necessarily contradictory While studies carried out in conditions where the enzyme shows classical kinetics elucidate the reaction mechanism, those in conditions where allosteric kinetic appears suggest the existence of regulatory mechanisms which could act through allosteric transition directed either by NAD or by GMP One may reasonably assume that both IMP by its first place in the ordered sequence reaction mechanism and NAD by its ability to induce allosteric transition act upon the regulation of the enzyme

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