CALF THYMUS GMP REDUCTASE : CONTROL BY XMP

by

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Summary Calf thymus GMP reductase was found to differ in its control properties from previously reported bacterial GMP reductases. XMP, instead of ATP, was found to be a strong inhibitor of the mammalian enzyme.

GMP reductase (EC 1.6.6.9) activity was first demonstrated (1) in extracts of Aerobacter aerogenes, Salmonella typhimurium and Escherichia coli. The enzyme was soluble, required GMP, NADPH and a thiol compound for activity,and produced IMP, ammonia and $NADP^+$. ATP was found to be a strong inhibitor, and this was proposed as a metabolic control for the interconversion of purine nucleotides. ATP has since been shown to be an allosteric inhibitor of the Aerobacter enzyme (2).

GMP reductase activity has also been demonstrated in rabbit red blood cells (3), but no control properties have been reported for the enzyme from a mammalian source. Experiments by Mah and Ackerman (4) suggest that thyroid hormone may be implicated.

The present report describes experiments with calf thymus gland GMP reductase which show that while ATP has no effect on the enzyme from this source, XMP is a strong competitive inhibitor. A control mechanism for the interconversion of purine nucleotides in mammals is proposed.

MATERIALS AND METHODS

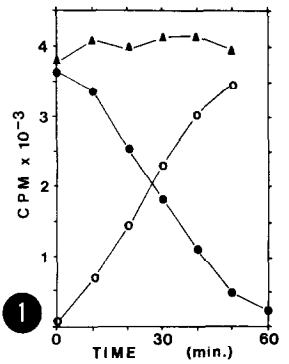
All nucleotides, nucleosides and bases were obtained from the Sigma Chemical Company, St. Louis, Missouri. GMP-(8-^{14}C) (specific

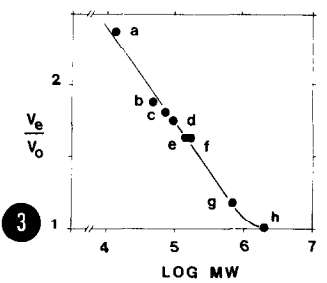
activity 61 mCi/mmole) was purchased from the Radiochemical Centre, Amersham, U.K.

The mixture for the assay of enzyme activity contained 5 µl of 0.25 mM GMP (61 mCi/mmole), 0.1 ml of assay buffer at pH 7.5 containing 0.05 M Triethanolamine-HCl (TEA-HCl) in 10 mM EDTA, 0.2 ml of 1.0 mM NADPH in 1.0 mM KOH with 0.35 M 2-mercaptoethanol, and 0.2 ml of enzyme. The reaction was started by the addition of enzyme and incubated at 37°C for 30 min. HCl (0.1 ml; 10 M) was then added to stop the reaction, followed by 0.05 ml of 10 mM IMP in 10 mM GMP to provide carriers for the subsequent separation. If necessary, the mixture was then centrifuged to remove protein precipitate. The nucleotides were hydrolysed to bases by boiling the acidic mixture for 30 min., and the hydrolysate was evaporated to dryness over conc. H_2SO_4 in a vacuum dessicator.

After taking up the residue in 0.1 ml of 1.0 M HCl, 10 µl of this was spotted onto Whatman 3 MM paper for ascending chromatography with 5% KH_2PO_4 (adjusted to pH 7.0) overlayered with iso-amyl alcohol (5). Purine spots were located using a U.V. lamp and identified by R_f. They were cut into fine pieces and placed in counting vials with 10 ml of toluene scintillant containing PPO (4 g/l) and dimethyl-POPOP (0.1 g /l).

The enzyme was prepared from calf thymus glands obtained within two hours of death. All steps were carried out at 4°C. The washed glands (120 g) were homogenised for two min. at high speed in a Waring Blendor with 180 ml of buffer. The homogenising buffer (pH7.5) contained 0.05 M TEA-HCl, 1.0 mM EDTA and 10 mM 2-mercaptoethanol. The crude homogenate was centrifuged at 20,000 x g for 15 min. and the supernatant fluid thus obtained made 0.12% w/v with respect to protamine sulfate. After centrifuging at 50,000 x g for 30 min., the clear supernatant fluid (290 ml) was fractionated by the slow addition of





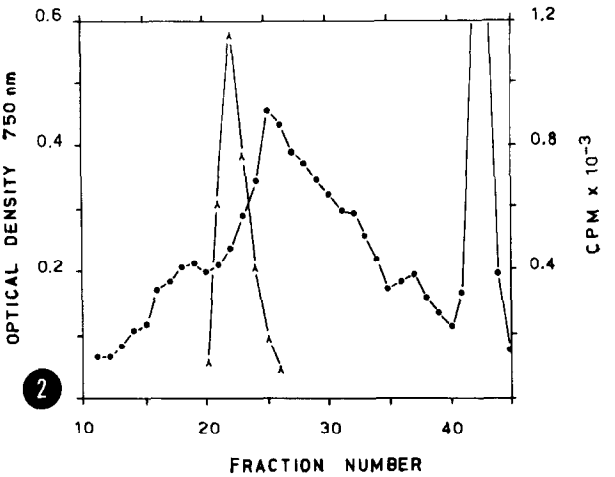


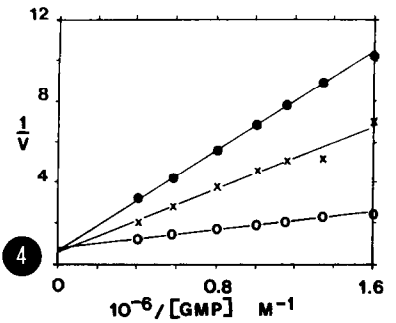
Figure 1 Time course of the reaction catalysed by GMP reductase. Total counts per minute ▲ --▲, guanine ● -- ●, hypoxanthine o -- o .

Figure 2 Purification of GMP reductase on an agarose A-0.5m gel column. Protein ● -- ●, activity as hypoxanthine counts per minute ⅄ -- ⅄.

Figure 3 Molecular weight estimation of GMP reductase on agarose A-1.5m gel. The standards were (7): a) cytochrome c, b) ovalbumin, c) bovine serum albumin, e) dimer of bovine serum albumin, f) bovine gamma globulin,g) bovine thyroglobulin and h) blue dextran. Point d is calf thymus GMP reductase.

a saturated (4°C) solution (pH 7.5) of $(NH_4)_2SO_4$ containing 0.05 M TEA-HCl and 1.0 mM EDTA. The precipitate between 55 and 65% saturation was collected by centrifugation and redissolved in 12 ml of 0.02 M TEA-HCl (pH 7.5) containing 1.0 mM EDTA and 10 mM 2-mercaptoethanol. The $(NH_4)_2SO_4$ was dialysed out against a bath of the same buffer.

A sample of 8 ml of the dialysate was then applied to an agarose A-0.5m, 200-400 mesh (Bio-Rad) column (80 x 2.1 cm) previously equilibrated with buffer (pH 7.5) containing 0.05 M TEA-HCl, 0.15 M



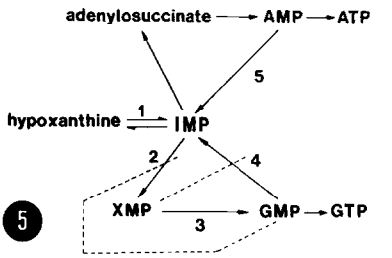


Figure 4 XMP inhibition of GMP reductase. The GMP-(8-^{14}C) substrate concentration was varied for each of the fixed concentrations of XMP as follows: no XMP O--O; 2.5 x 10^{-8} M XMP x--x; 5 x 10^{-8} M XMP ●--●. Velocity was measured as a function of the micromolar change in concentration of hypoxanthine (hydrolysed IMP) occurring during 30 min. with the standard assay system.

Figure 5 Reactions involved in the interconversion of purine nucleotides. Numbered reactions are catalysed by : 1) hypoxanthine-guanine phosphoribosyl transferase, 2) IMP dehydrogenase, 3) XMP aminase, 4) GMP reductase and 5) AMP deaminase. Broken lines indicate control by inhibition.

NaCl, 1.0 mM EDTA and 10 mM 2-mercaptoethanol. Elution was continued with the same buffer and 8 ml fractions collected. Protein content (6) and enzyme activity were measured for each fraction.

The molecular weight of the enzyme was estimated (7) using an agarose A-1.5m, 200-400 mesh column (80 x 2.1 cm). The elution buffer was the same as that used in the purification of the enzyme.

RESULTS

A summary of the enzyme purification is presented in Table 1, and the profile for the agarose column is shown in Fig.1.

Dilution of the preparation showed that the activity was directly proportional to the concentration of enzyme. The time course of the reaction (Fig. 2) was linear up to 75% conversion of the substrate, and IMP was produced at the same rate as GMP was utilized. Guanine-(8-^{14}C) was not a substrate.

The optimum pH for activity was in the region of pH 7.5. No detectable activity occurred in the absence of NADPH, and NADH would

not function as a cofactor. With 3.2×10^{-6} M GMP, the K_m for NADPH was 3.1×10^{-5} M, and with 4×10^{-4} M NADPH the K_m for GMP was 1.4×10^{-6} M. The Lineweaver-Burk (8) plots used for the determination of these values were linear. The molecular weight of the enzyme was estimated to be 90,000 (Fig. 3).

ATP was tested as an effector at concentrations ranging from one fifth to eight times the concentration of substrate in the assay (2.5×10^{-6} M). No significant change in activity was found. AMP, GTP, guanosine, inosine, hypoxanthine, xanthine and xanthosine at similar concentrations also had no effect, but IMP and XMP were definite inhibitors (Table 2). When unlabelled substrate was added to the normal assay mixture so that the total GMP concentration was trebled (specific activity lowered three fold), the expected theoretical decrease of approximately 60% occurred in the incorporation of counts into IMP.

The inhibition by XMP was shown to be purely competitive with respect to GMP and devoid of any allosteric effects (Fig. 4). The apparent inhibitor constant for XMP was 1×10^{-8} M.

l-thyroxine (4) did not effect enzyme activity when present at an equivalent concentration to substrate.

DISCUSSION

In cells having the de novo pathway for purine biosynthesis, two levels of control are found which ensure the normal metabolic ratio between adenine and guanine nucleotides : (a) at the de novo level, the independent control of amidophosphoribosyl transferase by AMP and GMP (9); (b) at the level of interconversion, GMP feed-back inhibition of IMP dehydrogenase and ATP inhibition of GMP reductase (1).

However, it has been shown that most mammalian cells are dependent upon an exogenous supply of hypoxanthine (10) transported by the red blood cells (11) from the liver (12). While the liver has the de novo pathway, the peripheral tissues do not, and are dependent upon salvage.

Table 1 Summary of the purification of calf thymus GMP reductase.

Fraction	Volume (ml)	Protein (mg)	Specific Activity cpm Hx/mg protein	Purification (fold)
protamine supernatant	290	10,000	480	(1)
65% $(NH_4)_2SO_4$ pellet	14	840	1,770	3.7
agarose pool	48	44	45,000	94

Table 2 Inhibitors of calf thymus GMP reductase.

Inhibitor	Concentration	% Inhibition
IMP	1×10^{-5} M	25
XMP	2.5×10^{-8} M	40
	5×10^{-8} M	62
	5×10^{-7} M	89

It may also be expected that the control at the nucleotide interconversion level may be different.

It has already been pointed out by Murray et al (10) that ATP activation and GTP inhibition of AMP deaminase (13) is unlikely to function as an important metabolic control in mammals in view of the high levels of ATP and low levels of GTP normally found there (14). Further, ATP does not inhibit GMP reductase in calf thymus, as shown above. Thus an alternative control for the interconversion of IMP and GMP must be sought.

The experiments reported here with calf thymus GMP reductase show that XMP offers a more likely means of control. XMP may be produced by hypoxanthine-guanine phosphoribosyl transferase, but xanthine is a very poor substrate for this enzyme (15). It is more likely that XMP is principally produced by IMP dehydrogenase (Fig. 5), which is weakly inhibited by GMP (K_i 5 x 10^{-5} M; Stephens, unpublished). It may be seen then that a low level of GMP will allow production of sufficient XMP to inhibit (K_i 1 x 10^{-8} M) conversion of GMP to IMP by GMP reductase. Thus purine rings from hypoxanthine may be channelled into GMP via hypoxanthine-guanine phosphoribosyl transferase, IMP dehydrogenase and XMP aminase. If the level of GMP is markedly raised, then IMP dehydrogenase production of XMP will be inhibited, allowing conversion of GMP into IMP and thence AMP.

Thus the removal of GMP for the synthesis of AMP will only proceed until IMP dehydrogenase is no longer inhibited by GMP.

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