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PURIFICATION AND PROPERTIES OF RAT LIVER AMP DEAMINASE

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SUMMARY

1. The enzyme AMP aminohydrolase (EC 3.5.4.6) was purified about 90-fold from the 105 000 × *g* supernatant of rat liver. The procedure consisted of Li_2SO_4 precipitation, heat treatment, chromatography over DEAE-cellulose, and gel filtration over Sepharose 4B.

2. The purified enzyme had a pH optimum between 6.0 and 6.2.

3. ATP and alkali metal ions strongly activated the enzyme. GTP activated the enzyme at low concentrations of substrate, but was inhibitory at high substrate concentrations. When GTP and ATP were added to the reaction mixture simultaneously, it was found that GTP was a potent inhibitor of ATP activation.

4. Comparison of the properties of the enzyme isolated from rat liver are made with that isolated from other tissue, especially muscle and brain, and differences noted.

5. The data are consistent with the hypothesis that this is an allosteric enzyme, and that its intracellular regulation may be accomplished by the ratio of AMP–ATP–GTP.

INTRODUCTION

Previous studies established that a specific deaminase exists in rat liver which catalyzes the conversion of AMP to IMP[1,2]. The enzyme from brain and muscle tissue was purified[3–5], and kinetic studies indicated the enzyme had regulatory properties similar to those of allosteric enzymes first described by MONOD *et al.*[6]. The enzyme from rat liver has not been purified nor has its regulation by effectors been studied in detail. Therefore, it is the purpose of this paper to describe the purification of this enzyme from rat liver, and to describe some of its properties.

MATERIALS AND METHODS

Female Holtzman rats weighing 160–180 g were used in this study, and were fed Rockland Mouse and Rat diet *ad libitum*.

The nucleotides used in this study were obtained from P–L Biochemicals, Mil-

waukee, Wisc., except for Tris–ATP and GTP (sodium salt), which were purchased from Sigma, St. Louis, Mo. For Tris–GTP, GTP (sodium salt) was converted to the free acid by percolating through a Dowex-50 column at 4°. The pH was then adjusted to 6.0 with Tris base. Dextran-500 and Sepharose 4B were purchased from Pharmacia Fine Chemicals, Piscataway, N.J. DEAE-cellulose was purchased from Reeve Angel, Clifton, N.J. Freunds complete adjuvant was a product of Difco Laboratories, Detroit, Mich. All other chemicals were of reagent grade.

Determination of enzyme activity

Assay 1. This assay was used to monitor activity during purification. Reaction mixtures contained: 40 mM AMP; 4 mM ATP; 0.2 M sodium citrate buffer (pH 6.0); and enzyme. Total volume was 0.6 ml. Incubation was for 30 min at 37°. Nucleotides were the sodium salts. Usually three levels of enzyme were used to assure that enzyme activity was proportional to the amount of protein present.

Assay 2. This assay was used in studies on modulation of enzyme activity by various effectors. The buffer was 0.01 M Tris–citrate (pH 6.0); the substrate was AMP neutralized with Tris base. Nucleotide effectors were the Tris salts or if only sodium salts were available, Na^+ effects on activity were compensated for by its addition to control reaction mixtures. The enzyme source was the best purified preparation dialyzed for 1 h against 50 vol. of deionized water and added to give 40–60 μg protein per reaction mixture.

Enzyme activity was determined by following the amount of NH_3 produced. NH_3 was collected by the diffusion apparatus described by SELIGSON AND SELIGSON[7], and measured by Nesslers' reagent. One unit of enzyme activity is defined as 1 μmole of NH_3 produced in 30 min.

Purification of the enzyme

AMP deaminase was partially purified by modification of a procedure described by SETLOW AND LOWENSTEIN[3]. The procedure is outlined below and the results are tabulated in Table I. All steps were performed at 4° unless noted.

Step 1: High speed centrifugation. Rat livers (200 g) were homogenized in 4 vol of 0.05 M Tris buffer (pH 7.4) containing 0.1% mercaptoethanol. The homogenate was centrifuged at 105 000 $\times g$ for 60 min. The precipitate was discarded.

Step 2: Li_2SO_4 precipitation. Solid Li_2SO_4 was slowly added to the supernatant with stirring to a final concentration of 1.75 M. The precipitate was collected by centrifugation at 20 000 $\times g$ for 20 min and solubilized by overnight stirring in 0.2 M Tris buffer (pH 7.4) containing 10 mM ATP. The excessive yield resulted from slight activation of the enzyme by Li^+.

Step 3: Heat treatment. The solution from Step 2 was diluted 2-fold with cold, deionized water containing ATP to give a final concentration of 15 mM. The solution was then heated in a 55° water bath for 5 min. The temperature was rapidly returned to 0–4° by immersion in ice water. The precipitate which formed was removed by centrifugation.

Step 4: DEAE-cellulose chromatography. The enzyme solution (approx. 255 ml) from Step 3 was dialyzed 2 h against 15 l of 1 mM Tris–HCl (pH 7.4) containing ATP at 10 μM. After dialysis, the enzyme was embedded on a 4.3 cm $\times$ 39.5 cm column packed with DEAE-cellulose which had been washed previously with at least 1 vol of

Solution A. (Solution A contained 0.05 M Tris–HCl (pH 7.4), 0.1% mercaptoethanol and 0.25% Dextran-500.) The enzyme was eluted from the column with a linear gradient permitting a decrease in pH and an increase in ionic strength. This was accomplished by mixing 750 ml each of two solutions. The first chamber contained 750 ml of a 1:1 mixture of Solutions A and B (for composition of Solution A, see above; Solution B contained 0.2 M sodium citrate (pH 6.5), 0.1% mercaptoethanol and 0.25% Dextran-500). This mixture flowed into a second chamber containing 750 ml of Solution A alone. Fractions containing enzyme activity were pooled and the enzyme was precipitated by addition of solid Li_2SO_4 to a final concentration of 2 M. The precipitate was taken up in 20 ml of 0.2 M Tris buffer (pH 7.4) containing 10 mM ATP. This procedure yielded a specific activity of 83.10 with 196 mg protein.

Step 5: Gel filtration. The enzyme from Step 4 was placed directly into a 2.5 cm × 80 cm column packed with Sepharose 4B. The column was eluted with 0.05 M Tris buffer (pH 7.4) containing 0.1 M KCl, 0.05 M LiCl, and 2 mM ATP at a flow rate of 27 ml/h. Fractions containing enzyme were pooled and concentrated as described in Step 4. After concentration, the enzyme was taken up in 0.2 M Tris buffer (pH 7.4) and stored at −50°. The enzyme has been stored up to 2 months in this manner with no appreciable loss in activity. The final specific activity was 139.0 with an overall yield of 34%. The final purification was 93-fold.

Preparation of antiserum and immunochemical titration of AMP deaminase

5 mixed-breed rabbits were each injected subcutaneously with an emulsion of 0.25 ml (7.89 mg/ml) of the best purified enzyme and an equal volume of Freunds complete adjuvant at 3-week intervals. 1 rabbit was injected with adjuvant alone as a control. Antibody production was monitored by the double diffusion technique of OUCHTERLONY[8] of test bleeding 1 week after the third and fourth injections. Under these conditions, maximal titers were obtained 9–10 weeks after the initial antigen injections.

Immunochemical titration of AMP deaminase was performed essentially as described by JOST *et al.*[9]. Dilutions of enzyme purified through Step 5 were incubated for 30 min at 37° with dilutions of antiserum (0.2 ml of each), and held 24 h at 4°. Controls were appropriate dilutions of enzyme incubated with serum from rabbits injected only with adjuvant. After centrifugation at 1200 × *g*, enzyme activity in supernatants was determined using Assay 1. NH_3 was determined by a direct colorimetric procedure[10] on aliquots of supernatants after precipitation of proteins with 10% trichloroacetic acid.

Disc electrophoresis

Acrylamide gel disc electrophoresis on Step 5 enzyme was accomplished essentially in the manner described by WILLIAMS AND REISFELD[11]. The apparatus and all chemicals were purchased from Canal Industrial Corp., Rockville, Md.; and preparation of gels and buffers was accomplished exactly as described in their instructions except that the separation gel contained 5% acrylamide. Protein bands were stained with 0.5% aniline black in 7% acetic acid.

RESULTS

Disc gel electrophoresis

When 100–250 μg of enzyme from Step 5 were subjected to electrophoresis for 30–35 min at 5 mA, 8 stained bands were readily visible; 6 of these bands were in the upper (cathode) half of the gel, while 2 were in the lower (anode) half. Attempts to determine which protein bands had enzyme activity were unsuccessful, for the enzyme irretrievably lost catalytic activity during electrophoresis.

TABLE I

PURIFICATION OF AMP DEAMINASE

The reaction mixture for enzyme assay contained 40 mM AMP, 4 mM ATP dissolved in 0.2 M Tris–citrate buffer (pH 6.0), and enough enzyme to make a final volume of 0.6 ml. The reactions were incubated for 30 min at 37°, and the NH_3 determined by diffusion[7] and reaction with Nesslers' reagent.

Step	*Total vol. (ml)*	*Total protein (mg)*	*Total units**	*Specific activity (units/mg protein)*	*Yield (%)*	*Purification (-fold)*
(1) 105 000 × *g* supernatant	630	18 144	27 034	1.49	—	—
(2) Li_2SO_4 precipitation	138	2 981	30 257	10.15	119	6.8
(3) Heat treatment	255	1 945	25 791	13.26	95	8.9
(4) DEAE-cellulose	30	158	16 288	83.10	60	56
(5) Gel filtration	7	67	9 257	139.0	34	93

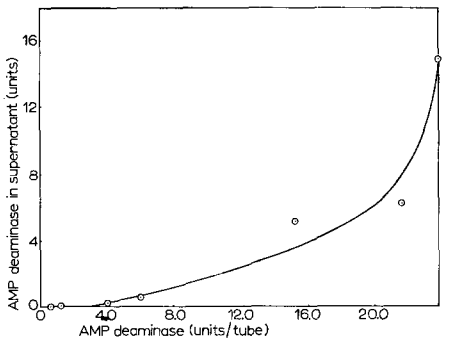
* 1 unit of enzyme activity is defined as 1 μmole of NH_3 produced in 30 min.

Inhibition of enzyme activity by antiserum

As shown in Fig. 1, the antigen–antibody reaction resulted in destruction of enzyme activity below detectable levels, suggesting that the antiserum contained antibodies to the enzyme. After disc electrophoresis of the enzyme, gel columns were embedded in agar and subjected to double diffusion against antisera. Under these conditions, two precipitin bands were observed in the lower (anode) region of the gel. Since enzyme activity was lost upon incubation with antibody, it was possible that one or both of the precipitin bands contained enzyme–antibody complexes, but, loss of catalytic activity during electrophoresis precluded experiments to investigate this possibility.

General properties of the enzyme

When the enzyme was incubated with 40 mM AMP and 4 mM ATP in sodium citrate buffer (pH 6.0) for various time intervals, it was found that enzyme activity was linear for at least 2 h. On the other hand, although activity was proportional to the amount of protein in the reaction mixture, extensive dilution of the enzyme resulted in the loss of linear relationship between activity and protein concentration. We attributed this loss of linearity to dissociation of the enzyme. Neither bovine serum albumin (0.5 or 1.0 mg/mg enzyme) nor glutathione (GSSG or GSH at 1 mg/mg enzyme) prevented the loss of linearity. The pH optimum of the enzyme lies between 6.0 and 6.2. This agrees closely with the enzyme isolated from brain tissue[3].



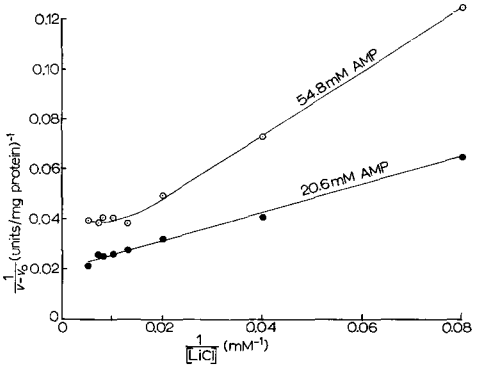


Fig. 1. Quantitative precipitation of purified AMP deaminase by antibody. Quantitative precipitation of the purified enzyme was carried out by direct titration with a specific amount of antiserum in 0.9% NaCl. Serial dilutions of the enzyme were made with 0.9% NaCl containing 1 mM ATP. The diluted enzyme and antiserum (0.2 ml each) were incubated at 37° for 30 min, and then for 24 h at 4°. Precipitates were collected by centrifugation at 105 000 × *g* for 15 min and the supernatant was assayed for enzyme activity. Reaction mixtures contained 40 mM AMP, 4 mM ATP, 150 mM LiCl and 0.05 M sodium citrate (pH 6.0).

Fig. 2. Activation of AMP deaminase by LiCl. Reaction mixtures contained either 20.6 mM AMP or 54.8 mM AMP in 0.01 M Tris–citrate buffer (pH 6.0) and 67 μg protein in a final volume of 0.6 ml. All assay procedures were as described in Table I. ●, 20.6 mM AMP; ○, 54.8 mM AMP.

Effect of alkali metal ions

Effects of Li^+, K^+, and Na^+ on the purified deaminase from rat liver were evaluated, and the data are summarized in Table II. In the presence of 20.6 mM AMP, all three monovalent cations tested stimulated activity of the enzyme at both 12.5 and 150 mM. There appeared to be no appreciable specificity for any one of the three cations.

As illustrated in Fig. 2, Li^+ was a potent activator, but the extent of activation was influenced by substrate concentration. For instance, at substrate concentrations of 20.6 and 54.8 mM, addition of LiCl resulted in a 3- and 1.5-fold increase, respectively, in enzyme activity. It has been reported that alkali metal ions activated AMP de-

TABLE II

ACTIVATION OF AMP DEAMINASE BY ALKALI METAL IONS

Reaction mixtures contained 20.6 mM AMP; either NaCl, KCl, or LiCl at 12.5 or 150 mM in 0.01 M Tris–citrate buffer (pH 6.0); 50 μg protein in a total volume of 0.6 ml. All other procedures were as described in Table I. All assays were performed in the absence of ATP.

Protocol	*Specific activity (units/mg protein)*	*% Control*
20.6 mM AMP	24.25	100
+ 12.5 mM NaCl	38.73	160
+ 150 mM NaCl	55.08	227
+ 12.5 mM KCl	31.02	128
+ 150 mM KCl	54.14	223
+ 12.5 mM LiCl	38.92	161
+ 150 mM LiCl	58.28	240

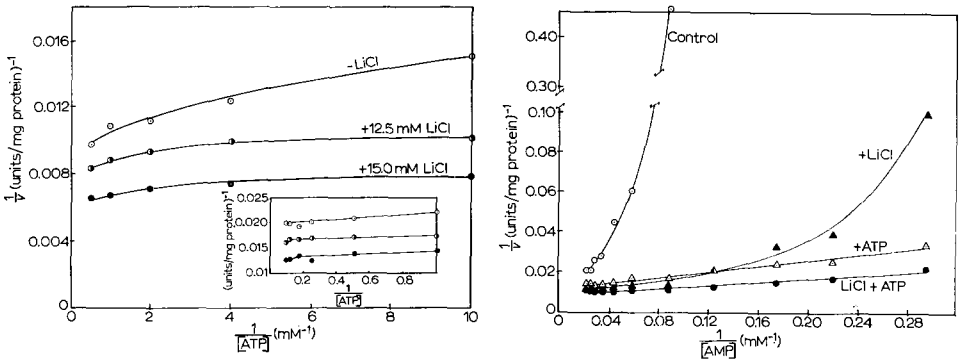


Fig. 3. Velocity *versus* concentration of ATP. The reaction mixtures contained 0.01 M Tris–citrate buffer (pH 6.0); 20.6 mM Tris–AMP; the indicated concentrations of Tris–ATP adjusted to pH 6.0; 50 μg protein; and 150 mM LiCl (●), 12.5 mM LiCl (◑), or no LiCl (○) in a final volume of 0.6 ml. All other procedures were as described in Table I.

Fig. 4. Effect of ATP and LiCl on the activity of AMP deaminase. The incubation mixtures contained the various concentrations of Tris–AMP; either 4 mM Tris–ATP (△), 150 mM LiCl (▲), 4 mM Tris–ATP + 150 mM LiCl (●), or no effector (○) in 0.01 M Tris–citrate buffer (pH 6.0); and 50 μg protein in a final volume of 0.6 ml. All assay procedures were as described in Table I.

aminase of brain[3], muscle[4,5], Ehrlich ascites tumor cells[12], and human erythrocytes[13]; and in several instances, specificity for certain of these ions over others was encountered[3,5].

ATP and enzyme activity

Activation of the purified deaminase from rat liver with ATP is shown in Fig. 3. ATP at various concentrations was added to reaction mixtures containing 20.6 mM AMP and 0, 12.5 or 150 mM LiCl. ATP alone stimulated activity about 2-fold, but the combination of 2 mM ATP and 150 mM LiCl stimulated activity about 3-fold at this concentration of substrate. As can be seen in the insert, 2 mM ATP appears to approach the minimum concentration required to obtain maximum stimulation, however, 4 mM ATP was routinely used in subsequent assays in order to insure maximum stimulation. These observations are in general agreement with results of previous studies in which ATP activation of AMP deaminase was demonstrated[2–4,12–19].

A comparison of the velocity at various levels of substrate in the presence of ATP, LiCl, or the combination is shown in Fig. 4. When ATP was present in reaction mixtures either alone or with LiCl, activity varied as a hyperbolic function of substrate concentration. When it was absent, activity varied as a sigmoidal function of substrate concentration. Whether maximum velocity was altered by these conditions cannot be established because of limited solubility of the substrate, but curves suggested a common v_{max}.

Modification of activity by GTP

It has been reported that GTP inhibits the activity of AMP deaminase of brain[16,20]. When the effect of Tris–GTP was studied on the purified enzyme from rat liver, data shown in Fig. 5 were obtained. At a substrate concentration of 8 mM, GTP

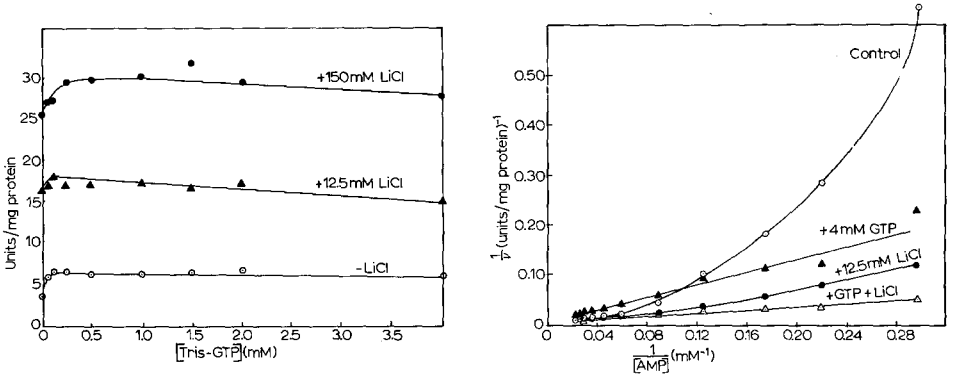


Fig. 5. Velocity *versus* concentration of GTP. The reaction mixtures contained 8 mM Tris–AMP; 50 μg protein; the various concentrations of Tris–GTP; and either 150 mM LiCl (●), 12.5 mM LiCl (▲), or no LiCl (○) in 0.01 M Tris–citrate buffer (pH 6.0) in a final volume of 0.6 ml. All other procedures were as described in Table I.

Fig. 6. Effect of GTP and LiCl upon the activity of AMP deaminase. Each reaction mixture contained 50 μg protein; the indicated concentrations of Tris–AMP; either 4 mM Tris–GTP (▲), 12.5 mM LiCl (●), 4 mM Tris–GTP + 12.5 mM LiCl (△), or no effector (○) in 0.01 M Tris–citrate buffer (pH 6.0) in a final volume of 0.6 ml. All other assay procedures were as described in Table I.

alone stimulated enzyme activity 1.5–2-fold. Addition of LiCl diminished the stimulatory effect of GTP, but stimulation was still detectable.

Velocity *vs.* substrate curves in the presence of GTP, LiCl, and the combination are shown in Fig. 6. Again, in the absence of the nucleotide, activity varied as a sigmoidal function of the AMP concentration. Upon addition of 4 mM Tris–GTP, sigmoidicity was lost. At low AMP concentrations, Tris–GTP slightly stimulated activity, but at higher concentrations it was inhibitory.

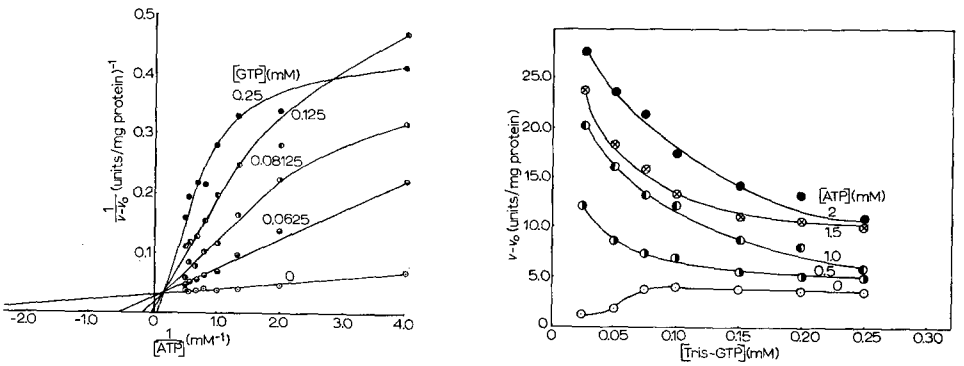


Fig. 7. Competition of ATP by GTP. Reaction mixtures contained 6 mM AMP, the indicated concentrations of Tris–ATP and Tris–GTP and 67 μg protein in a final volume of 0.6 ml with 0.01 M Tris–citrate buffer (pH 6.0). The procedures for assays were as described in Table I.

Fig. 8. Inhibition of the effect of ATP on AMP deaminase. Reaction mixtures contained 6 mM AMP, the indicated concentrations of Tris–ATP and Tris–GTP, 67 μg protein and 0.01 M Tris–citrate buffer (pH 6.0) in a final volume of 0.6 ml. Details for assay procedures are described in Table I.

Competition experiments with GTP and ATP

When various levels of Tris–GTP were incubated with increasing concentrations of ATP, it was found that GTP was a strong inhibitor of ATP (Fig. 7). It apparently inhibits ATP by decreasing the capacity of the enzyme to bind ATP, since the apparent K_m for ATP is increased 10-fold or more with GTP in the reaction mixture. Some conception of the ratio of the two nucleotides required in order to regulate the activity of the enzyme can be seen in Fig. 8. For example, when the concentration of ATP is 2 mM, GTP at 0.2 mM lowers the activity of the enzyme approx. 2-fold. In the absence of ATP, GTP additions resulted in increased enzyme activity. Data graphically depicted in Figs. 7 and 8 were obtained using reaction mixtures devoid of alkali metal ions, however, in other experiments where the metal ions were added, the same qualitative results were obtained.

TABLE III

NUCLEOTIDE SPECIFICITY OF AMP DEAMINASE

All reaction mixtures except two contained Na^+ concentration of 8 mM ,the Na^+ of the various nucleotides being taken into account. The two exceptions were the nucleotides ITP and TTP, in which the concentration of the Na^+ was 12 mM. The concentration of the nucleotides was 4 mM in all assays. In addition to the various nucleotides, each reaction mixture contained 8 mM Tris–AMP, 50 μg protein and 0.01 M Tris–citrate buffer (pH 6.0). All other procedures were as described in Table I.

Nucleotide	*Specific activity (units/mg protein)*	*% Control*
None	28.3	100
ATP	60.1	212
ADP	44.6	158
ITP	34.5	122
CTP	35.7	126
TTP	29.2	103
GTP	35.7	126
GDP	12.9	46
3'(2')-AMP	14.2	50

Nucleotide specificity

In order to test the effect of several other nucleotides upon the activity of this enzyme, reaction mixtures containing the sodium salts of the nucleotides adjusted to pH 6.0 with Tris-base were incubated with the enzyme at Tris–AMP concentrations of 8 mM. NaCl was added to all assay tubes to a final concentration of 8 mM with respect to the alkali metal ion, except in the reaction mixtures containing ITP and TTP, where the final alkali metal ion concentration was 12 mM. Table III shows that ATP and ADP were potent stimulators of activity, while 3'(2')-AMP and GDP inhibited the enzyme. GTP, CTP and ITP were slightly stimulatory, while TTP had no appreciable effect.

DISCUSSION

The AMP deaminase from rat liver was similar in many respects to the AMP deaminase from calf brain[3]. For instance, activity of the enzyme from both sources was potentiated by ATP and alkali metal ions, but displayed no absolute requirements for any of these. Plots of enzyme activity *vs.* substrate concentration yielded sigmoidal curves in the absence of nucleotides. Sigmoidicity persisted with the enzyme from brain[3] or liver (Fig. 4) in the presence of LiCl alone. Addition of ATP alone or with LiCl to the enzyme from either brain[3] or liver (Fig. 4) yielded curves that were essentially hyperbolic in shape and lowered the apparent affinity constant more than 25%.

GTP also appeared to be a modifier of AMP deaminase, but the kind of modication was dependent upon substrate concentration and upon whether other modifiers were present. At low substrate concentrations, GTP activated the enzyme, but at higher substrate concentrations (8–10 mM), activation was not seen. ATP activation was strongly inhibited by GTP at concentrations approx. 1/10 those of ATP. From these considerations it would appear that intracellular regulation of AMP deaminase might be achieved by interactions between the enzyme, its substrate AMP, and the two modifiers, ATP and GTP. Regulation of AMP deaminase may depend upon the AMP–ATP–GTP ratio rather than absolute concentrations of the 3 nucleotides.

Inhibition of ATP activation by GTP appeared to be overcome when the ATP concentration was increased. The data suggested competition by both nucleotides for the same site, however, confirmation of this suggestion must await greater purification of the enzyme.

SETLOW *et al.*[16] previously reported GTP inhibition of rat liver AMP deaminase at low substrate concentrations. This result conflicts with our consistent observation of stimulation (Figs. 6 and 8). Since the magnitude of effect is small in both cases, the discrepancy in results may rest with differences in pH of reaction mixtures, *viz.*, pH 7.2 for SETLOW *et al.*[16], pH 6.0 in our experiments (Figs. 6 and 8). ATKINSON AND MURRY[12] reported that GTP inhibited the activity of the deaminase from Ehrlich ascites tumor cells, but only in the presence of ATP.

The enzyme from liver differed from the enzyme from calf brain in at least two instances: (a) in the absence of other modifiers, Na^+ and K^+ were less efficient activators than Li^+ for brain AMP deaminase[3], whereas the enzyme from rat liver was readily activated by all three alkali metal ions (Table II); (b) GTP inhibited calf brain AMP deaminase throughout a substrate range from 0–50 mM (ref. 20), while on the other hand, GTP stimulated activity of the rat liver enzyme at lower substrate concentrations, and inhibition was not observed until the substrate concentration exceeded 8–10 mM (Fig. 6).

SETLOW AND LOWENSTEIN[3] proposed a model for AMP deaminase from brain. This model seems appropriate for our data, in that there appears to be at least three distinct functional sites on the enzyme. The first site binds the substrate and is responsible for the catalytic function of the molecule. The other two sites bind ATP and the alkali metal ion, and are the "allosteric sites" of the enzyme. These are responsible for the regulation of the catalytic activity as first described by MONOD *et al.*[6], however, recently additional models have been proposed (*e.g.* refs. 21–23), and regulation of AMP deaminase may prove to be more closely related to these. Although GTP modifies

the activity of AMP deaminase in the absence of ATP, its most striking effect is that of inhibiting ATP. It seems unlikely that a separate binding site exists for GTP, but support for this contention must await greater purity of the enzyme.

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