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Binding of Guanosine Triphosphate and Adenosine Triphosphate by Rabbit Muscle Adenosine Monophosphate Deaminase*

Yasuko Tomozawa and Richard Wolfenden†

ABSTRACT: Equilibrium binding studies show that crystalline adenosine monophosphate deaminase from rabbit back muscle possesses two binding sites for the allosteric effector guanosine triphosphate (GTP), and a minimum of four binding sites for the allosteric effector adenosine triphosphate (ATP). Observed dissociation constants are comparable to the concentration of each effector required to produce half-maximal modification of catalytic activity under the same conditions. Binding of ATP and binding of GTP are mutually inhibitory. Treatment of the enzyme with 6.5 molar equiv of *p*-mercuribenzoate selectively abolishes binding of GTP. Enzyme treated with mercurial retains its original sedimentation properties and its ability to bind ATP.

Adenosine monophosphate deaminase (AMP-aminohydrolase, EC 3.5.4.6) from various tissues has been found to be subject to allosteric regulation by GTP and ATP (Cunningham and Lowenstein, 1965; Setlow *et al.*, 1966; Setlow and Lowenstein, 1967, 1968a,b). Smiley *et al.* (1967) have recently devised a simple procedure for crystallization of a highly active form of this enzyme from rabbit back muscle. Activity is associated with a protein of approximate mol wt 270,000, the amino acid composition of which has been determined (Wolfenden *et al.*, 1968).

The present studies were undertaken to determine whether the enzyme possesses separate binding sites for ATP and GTP, their number, and their possible mutual interaction in the absence of substrate. Since regulation of a number of allosteric enzymes has been found to be sensitive to mercurials, and sulfhydryl groups have been implicated in the action of several nucleoside deaminases (see Discussion), it was also of interest to determine the effect of mercurials on catalysis and on the binding of allosteric modifiers by AMP-deaminase.

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Experimental Section

Frozen rabbit back muscle was obtained from Pel-Freez Biochemicals, Inc. Bio-Gel P-2 was obtained from Bio-Rad, Inc. Cellulose phosphate, AMP, ATP, and GTP were purchased from Sigma Chemical Co. ATP-8-^{14}C and GTP-8-^{14}C were obtained from Schwarz BioResearch, Inc.

AMP-deaminase was prepared from rabbit back muscle essentially by the published procedure of Smiley *et al.* (1967). After column chromatography on cellulose phosphate in the presence of 2-mercaptoethanol (10^{-3} M), enzyme was recovered from active fractions by addition of solid ammonium sulfate to a concentration of 300 g/l. The pooled fractions, adjusted to pH 6.50 with K_2HPO_4, were allowed to stand overnight at 0°, and the resulting precipitate was dissolved in a minimal volume of KCl (0.45 M) containing 2-mercaptoethanol (10^{-3} M) and adjusted to pH 6.50 at room temperature. Upon cooling in ice, the enzyme crystallized immediately. It was recovered by centrifugation and recrystallized twice under the same conditions. All kinetic and binding experiments were performed on samples of crystalline enzyme dissolved in ammonium succinate buffer (0.1 M, pH 6.50) containing 2-mercaptoethanol (10^{-3} M) and incubated for 1 hr at room temperature. Following incubation, excess 2-mercaptoethanol was removed from enzyme samples by column chromatography on Bio-Gel P-2 in buffer not containing mercaptoethanol. Enzyme prepared in this way was stable for at least 10 hr at room

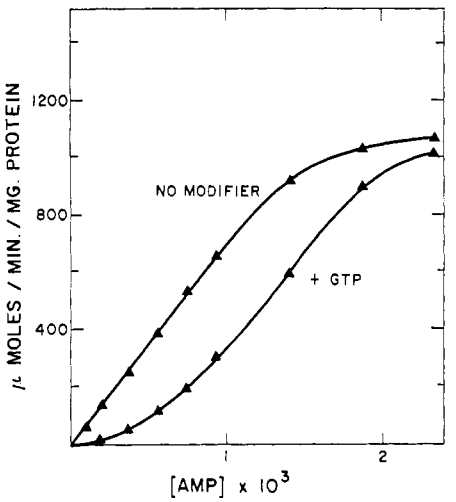

FIGURE 1: Activity of AMP-deaminase as a function of AMP concentration in ammonium succinate buffer (0.1 M, pH 6.50) at 25° in the presence and absence of GTP (1.4×10^{-6} M).

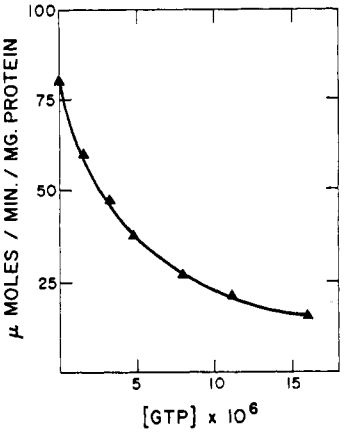

FIGURE 2: Activity of AMP-deaminase (AMP concentration = 1.2×10^{-4} M) as a function of GTP concentration (other conditions as in Figure 1).

temperature, and had a specific activity of 990 μmoles/min per mg of protein when assayed in the presence of 2.5×10^{-3} M AMP in ammonium succinate buffer (0.1 M, pH 6.50) at 25°.

Assays of enzyme activity were performed at 25° in the presence of ammonium succinate buffer (0.1 M, pH 6.50) by measuring the decrease in absorption at 260 mμ or the increase in absorption at 285 mμ, using a Zeiss PMQ spectrophotometer and cuvets of 1-cm path length. Rates were calculated as micromoles per minute per milligram of enzyme using the appropriate relative extinction coefficients for AMP and IMP at each wavelength (Smiley and Suelter, 1967). Protein determinations were made by the method of Lowry *et al.* (1951).

Binding of nucleotides was measured by gel filtration using Bio-Gel P-2, using a procedure similar to those described in the literature (Hummel and Dryer, 1962; Fairclough and Fruton, 1966). Columns (0.4 × 80 cm) of gel were preequilibrated with buffer (0.1 M ammonium succinate, pH 6.50) containing radioactive ATP or GTP diluted with nonradioactive nucleotide to the desired concentration. Samples of enzyme (0.5–0.8 ml containing 0.5–1.0 mg of protein), preequilibrated in the same solution for 30 min at room temperature, were applied to the column and eluted with the same solution at room temperature. Fractions (0.4 ml) were collected over a period of approximately 1 hr. Of these fractions, 0.1 ml was used for protein determination by the method of Lowry *et al.* (1951), and 0.2 ml was dried on a planchet for determination of radioactivity using a Nuclear-Chicago low-background gas-flow counter.

Results

Effects of GTP, ATP, and p-MB[1] *on Enzyme Activity.* The activity of rabbit muscle AMP-deaminase has been shown to be sensitive to the effector nucleotides ATP and GTP (Smiley and Suelter, 1967). Preliminary kinetic studies were undertaken to provide information concerning GTP and ATP effects on catalytic activity under conditions in which direct binding studies were to be made.

Figure 1 shows that, in ammonium succinate buffer (0.1 M, pH 6.50), GTP caused powerful inhibition of the enzyme, particularly at low concentrations of substrate. When the concentration of AMP was held constant, GTP was found to produce detectable inhibition over a concentration range from 2×10^{-7} to 1×10^{-4} M; a portion of this range is shown in Figure 2. A plot of reciprocal velocity as a function of GTP concentration (Dixon, 1953) was linear at several AMP concentrations, yielding an apparent inhibition constant of 2.9×10^{-6} M (Figure 3). This value was similar to the value obtained in direct binding studies described below.

The effect of ATP on enzyme activity was more complex. Low concentrations of ATP inhibited the enzyme, with half-maximal inhibition at approximately 1.5×10^{-5} M. Net activation of the enzyme was observed at ATP concentrations above 4×10^{-4} M (Figure 4).

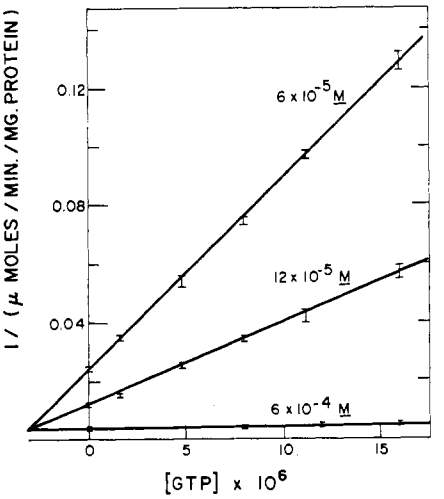

FIGURE 3: Dixon plot of the inhibition of AMP deamination by GTP at varying levels of AMP. Numbers on the graph refer to the concentration of AMP.

[1] Abbreviation used is: *p*-MB, *p*-mercuribenzoate.

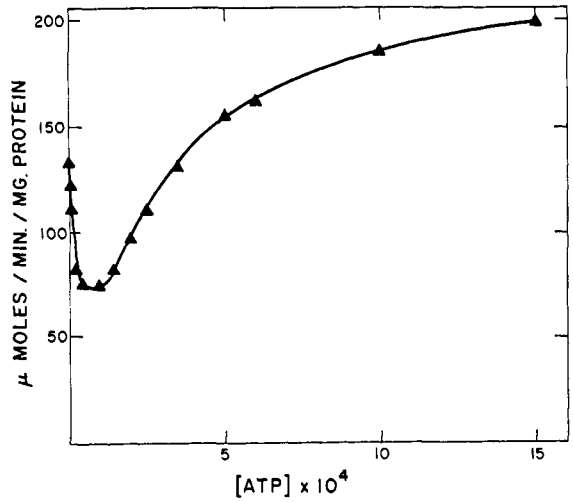

FIGURE 4: Activity of AMP-deaminase (AMP concentration = 2.0×10^{-4} M) as a function of ATP concentration (other conditions as in Figure 1).

Titration of deaminase with *p*-MB by the method of Boyer (1954) indicated the presence of 6.15 rapidly reacting sulfhydryl groups per 270,000 g of protein in ammonium succinate buffer (0.1 M, pH 6.50) (Wolfenden *et al.*, 1968). Incubation of enzyme with 4.8 equiv of *p*-MB resulted in no detectable change in enzyme activity. When 6.5 molar equiv of *p*-MB was added, enzyme activity increased markedly in the absence of modifier, and inhibition by GTP was selectively abolished (Table I). When excess *p*-MB was removed by gel filtration and the enzyme was incubated with mercaptoethanol, enzyme activity and sensitivity to GTP inhibition slowly returned to their original values (Table I). Recovery did not occur in the absence of mercaptoethanol. Sedimentation studies performed as described earlier (Wolfenden *et al.*, 1968) showed no detectable change in sedimentation or diffusion coefficient as a result of treatment with *p*-MB.

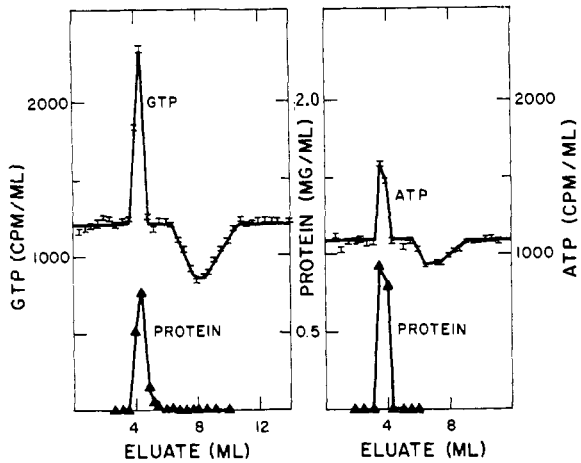

FIGURE 5: Elution profile of AMP-deaminase, radioactive GTP, and radioactive ATP from Bio-Gel P-2 in the presence of ammonium succinate buffer (0.1 M pH 6.50) containing (on the left) GTP (5.17×10^{-6} M) and (on the right) ATP (4.85×10^{-5} M). For other conditions, see Experimental Section. Bars refer to standard deviation.

TABLE I: Effects of *p*-MB and 2-Mercaptoethanol on Enzyme Activity.[a]

Enzyme	Act. (μmoles/min mg) No Additions	+GTP	+ATP	Inhibn by GTP (%)
Native	120	29	189	76
Treated with *p*-MB (5×10^{-5} M)	230	231	330	0
p-MB treated, after gel filtration	221	206	322	6
p-MB treated, after 2 hr in 2-mercaptoethanol	201	138		36
p-MB treated, after 7 hr in 2-mercaptoethanol	109	33		70

[a] AMP concentration = 2.0×10^{-4} M, GTP (when present) = 1.0×10^{-5} M, ATP (when present) = 1.5×10^{-3} M, 2-mercaptoethanol (when present) = 1.0×10^{-3} M. Assays were performed in duplicate at 25° with enzyme (0.25 μg/ml) in the presence of ammonium succinate buffer (0.1 M, pH 6.50).

Equilibrium Binding of GTP and ATP. Binding of radioactive GTP and ATP was investigated by gel filtration on Bio-Gel P-2 in the presence of ammonium succinate (0.1 M, pH 6.50). Typical results (obtained as described in Experimental Section) are shown in Figure 5. GTP showed a hyperbolic saturation curve. A double-reciprocal plot of these results (Figure 6) was linear, and indicated binding of 2.04 moles of GTP/270,000 g of protein, with an apparent dissociation constant of 2.0×10^{-6} M, in reasonable agreement with the apparent inhibition constant of GTP under the same conditions. Enzyme treated with *p*-MB was found to bind little or no GTP under same conditions (Table II).

Similar experiments performed with radioactive ATP also

TABLE II: Effect of *p*-MB, ATP, and P_i on GTP Binding in 0.1 M Ammonium Succinate.

GTP (M)	Addition (M)	moles of Bound GTP/mole of Enzyme
5.0×10^{-6}	None	1.41
5.0×10^{-6}	*p*-MB (3.1×10^{-5})	0.20
5.0×10^{-6}	P_i (5.0×10^{-3})	1.32
5.0×10^{-6}	ATP (1.4×10^{-5})	1.30
5.0×10^{-6}	ATP (2.5×10^{-5})	1.05
5.0×10^{-6}	ATP (5.5×10^{-5})	0.59
5.0×10^{-6}	ATP (9.3×10^{-5})	0.43

TABLE III: Effect of *p*-MB, GTP on ATP Binding in 0.1 M Ammonium Succinate.

ATP (M)	Addition	moles of Bound ATP/ mole of Enzyme
2.05×10^{-5}	None	2.02
2.05×10^{-5}	GTP (1.90×10^{-5} M)	1.18
4.88×10^{-5}	None	2.60
4.88×10^{-5}	*p*-MB (3.1×10^{-5} M)	2.33

showed hyperbolic saturation curves, with binding of 3.8 moles of ATP/270,000 g of protein and an apparent dissociation constant of 2.1×10^{-5} M (Figure 6), near the apparent inhibition constant determined kinetically. Enzyme treated with *p*-MB retained its ability to bind ATP at low concentrations (Table III). Unfortunately, it was not possible to examine the strength or stoichiometry of binding of ATP in the high concentration range corresponding to kinetic activation, since binding experiments would have required concentrations of enzyme well in excess of 100 mg/ml, far above the solubility of the protein under these conditions.

When binding of each of nucleoside triphosphates was measured in the presence of the other, antagonistic effects were observed. Thus, increasing concentrations of ATP virtually abolished binding of GTP, and ATP binding was reduced in the presence of GTP (Table II and III).

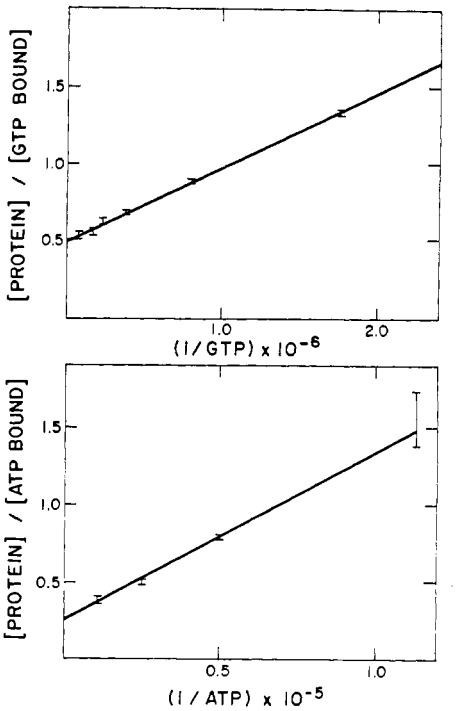

FIGURE 6: Double-reciprocal plots of GTP and ATP bound as a function of concentration in the presence of ammonium succinate buffer (0.1 M, pH 6.50). Concentration of enzyme was based on a molecular weight of 270,000. Results were obtained from duplicate experiments, and bars represent standard deviation of radioassay.

Discussion

The present results indicate that ATP and GTP, previously shown to modify the catalytic activity of AMP-deaminase from various calf tissues (Setlow *et al.*, 1966) and from rabbit muscle (Smiley and Suelter, 1967), are bound at different sites on the enzyme from rabbit muscle. This is supported by the observation that there are different numbers of binding sites for the two nucleotides, and that *p*-MB selectivity prevents binding of GTP, but not of ATP.

The number of binding sites per 270,000 g of protein appears to be 2 for GTP. There appear to be 4 binding sites for ATP at the low concentrations at which, under the conditions of the present experiments, ATP is inhibitory (see below). It is probable that another class of binding sites exists for ATP, since activation occurs at higher concentrations of ATP than those which could be attained in the present binding experiments (Figure 4). The observed stoichiometries of binding are of interest in conjunction with recent observations which suggest the presence of 4 moles of zinc/300,000 g of protein (C. H. Suelter, personal communication).

The lack of cooperativity in the binding of GTP, on the one hand, or ATP, on the other, is noteworthy in view of the mutual inhibition of binding of the two effectors. This need not be the case *in vivo*, since the present conditions are likely to be quite different from those prevailing in rabbit muscle. The catalytic and regulatory behaviors of AMP-deaminase are highly sensitive to ions present in solution, particularly alkali metals (Smiley and Suelter, 1967). We have found that the direction of the modifier effect of low concentrations of ATP actually changes from potent activation to inhibition as the concentration of ammonium succinate buffer is raised from 0.005 to 0.1 M, or when increasing concentrations of KCl are added to 0.005 M ammonium succinate buffer. GTP inhibits under all conditions which have been tested, and ATP activates at the relatively high concentrations, in excess of 10^{-3} M, found in gross analyses of rodent muscle (LePage, 1948; Threlfall, 1957; Seraydarian *et al.*, 1962). The biphasic regulation by ATP observed in ammonium succinate (Figure 4), is therefore unlikely to be physiologically significant.

The activation of AMP-deaminase by mercurial, and its densensitization to GTP, may be contrasted with the inhibitory effect of mercurials on adenosine deaminases from *Aspergillus oryzae* and from calf intestine (Wolfenden *et al.*, 1967). Other experiments have shown that inhibition of AMP-deaminase does occur at higher concentrations of mercurial than those used in the present experiments. Desensitization to modifier effects in the presence of mercurials has been observed for a number of allosteric enzymes, including aspartate transcarbamylase (Gerhart and Pardee, 1962), fructose diphosphatase (Rosen and Rosen, 1966), threonine deaminase (Freundlich and Umbarger, 1963), homoserine dehydrogenase (Patte *et al.*, 1966), and deoxy-*arabino*-heptulosonate 7-phosphate synthetase (Jensen and Nester, 1966). This appears to be the first instance in which treatment with mercurial interferes with the enzyme's ability to bind the allosteric effector.

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Binding of Substrate and Transition State Analogs to Triosephosphate Isomerase*

Richard Wolfenden

ABSTRACT: Triosephosphate isomerase from rabbit muscle is competitively inhibited by 2-phosphoglycollate, which is maximally bound below apparent $pK_a = 7.35$. K_m for the substrate glyceraldehyde 3-phosphate and K_i for the substrate analog α-glycerophosphate show little variation with pH.

V_{max} for isomerization of glyceraldehyde 3-phosphate falls off below apparent $pK_a = 7.35$. Binding of the transition state analog 2-phosphoglycollate (unlike binding of the substrate or the substrate analog) is thus sensitive to the state of ionization of a catalytic residue on the protein, its affinity changing in parallel with the changing rate enhancement produced by the enzyme as catalyst. Saturating concentrations of 2-phosphoglycollate produce virtually complete protection of the enzyme against heat inactivation under conditions where saturating α-glycerophosphate produces only partial protection. These findings suggest that in solution, as previously observed in crystals, progressive changes in enzyme structure are brought about by α-glycerophosphate and by 2-phosphoglycollate.

2-Phosphoglycollate has recently been shown to be an exceptionally powerful competitive inhibitor of triosephosphate isomerase (D-glyceraldehyde 3-phosphate keto isomerase, EC 5.3.1.1) (Wolfenden, 1969). The inhibitory power of this compound is believed to result from its resemblance to a high-energy ene–diolate intermediate earlier proposed for enzymatic isomerization of aldoses (Rose, 1962). Studies described in the present paper were undertaken in order to provide information concerning the pH dependence of the catalytic activity of triosephosphate isomerase and the pH dependence of inhibition by 2-phosphoglycollate. A variable which changes the rate enhancement produced by the enzyme as catalyst (relative to the rate of the nonenzymatic reaction under identical conditions) might be expected to produce parallel changes in the affinity of the enzyme for a transition state analog (relative to the affinity of the enzyme for substrate) (Wolfenden, 1969).

2-Phosphoglycollate brings about a reversible contraction of 5% in the unit cell volume of crystals of triosephosphate isomerase; a smaller contraction (1.7% occurs) in the presence of the substrate analog α-glycerophosphate (Johnson and

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