Adenylate deaminase from rat skeletal muscle has been studied with the objective of understanding how the activity of the enzyme is regulated in vivo. ATP and GTP inhibit the enzyme at low concentrations in the presence of 150 mM KCl. The ATP inhibition is reversed as the ATP concentration is raised to physiological levels. The GTP inhibition is reversed as the GTP concentration is raised to unphysiologically high levels. In the presence of physiological concentrations of ATP, the GTP inhibition is also greatly diminished, but inhibition by orthophosphate remains strong. The apparent affinities of the enzyme for ATP, GTP, and orthophosphate are reduced as the pH is decreased from 7.0 to 6.2. ADP also reduces the apparent affinities of the enzyme for the inhibitors. The regulatory effects of ATP, GTP, and ADP are produced primarily by their ionic forms. Comparison of the kinetic behavior of the enzyme in vitro with metabolite concentrations in vivo indicates that the major variables that regulate the activity of adenylate deaminase of muscle in vivo are the concentrations of AMP, ADP, orthophosphate, and H⁺.

Adenylate deaminase from skeletal muscle is regulated by a number of effectors. It is activated by potassium ions (1), ADP (2), and GDP (3), and is inhibited by GTP (1), ATP (3), and orthophosphate (4). Creatine phosphate was also reported to be an inhibitor (5). However, this inhibition is due mainly to pyrophosphate present as an impurity in commercial preparations of creatine phosphate (6).

The major objective of the present paper is to clarify the factors which regulate the activity of the skeletal muscle enzyme in vivo. The experiments to be described were carried out using concentrations of nucleotides and K⁺ believed to approximate those found in resting and exercising muscle. Magnesium ions influence the regulation of the enzyme by nucleotides (3) and particular attention was paid to the role of physiological concentrations of unchelated and chelated nucleotides (8) in the regulation of the enzyme.

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§ Strictly speaking, the word “chelated” applies only to the metal that is bound to the nucleotide. For simplicity of terminology, we shall use the terms “chelated” and “unchelated” to refer to the nucleotides also.
corrected accordingly. These corrections were usually less than 20%.

In the case of highly absorbing solutions, deviations in the linearity of absorbance with nucleotide concentration occurred due to stray light (for example, a 5% deviation at A262.5 = 1.1 occurred using the spectrophotometer model 356). The resulting deviations in the observed rates were corrected by calibrating the amount of stray light; however, most rates required little or no correction. The quoted rates are averages of duplicate or triplicate assays.

**Specific Activity at Low Enzyme Concentrations**—Hemp-hill et al. (15), working with adenylate deaminase from rabbit muscle, observed that the enzyme showed a decrease in specific activity at concentrations below 1 µg/ml when assayed at low AMP concentrations and pH 7.0. Such anomalous kinetics may lead to erroneous conclusions concerning the regulation of the enzyme. We therefore tested proportionality of activity to enzyme concentration. The specific activity did not decrease between 1.0 and 0.1 µg of enzyme/ml in the presence of enzyme concentration. We therefore tested proportionality of activity to enzyme concentration between the absence of effectors being varied (v0). Activation is defined by A = (v/v0) - 1, inhibition by I = 1 - (v/v0). The data were analyzed using a variation of the Hill equation (14):

\[ Q = Q_{\text{max}} \times \frac{L^n}{L^n + K^n} \]

where Q represents A or I and Q_{\text{max}} the value of Q at saturating ligand; L the concentration of ligand; n the Hill coefficient; and K (K_a or K_i) the concentration of ligand producing half-maximal effect. Q_{\text{max}} was obtained from plots of Q versus Q/L. n and K were obtained from Hill plots (log (Q/Q_{\text{max}} - Q)) versus log L).

**RESULTS**

**Specific Activity at Low Enzyme Concentrations**—Hemp-hill et al. (15), working with adenylate deaminase from rabbit muscle, observed that the enzyme showed a decrease in specific activity at concentrations below 1 µg/ml when assayed at low AMP concentrations and pH 7.0. Such anomalous kinetics may lead to erroneous conclusions concerning the regulation of the enzyme. We therefore tested proportionality of activity to enzyme concentration. The specific activity did not decrease between 1.0 and 0.1 µg of enzyme/ml in the presence of enzyme concentration. We therefore tested proportionality of activity to enzyme concentration between the absence of effectors being varied (v0). Activation is defined by A = (v/v0) - 1, inhibition by I = 1 - (v/v0). The data were analyzed using a variation of the Hill equation (14):

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**Effect of GTP, ATP, and Orthophosphate**—Inhibition of adenylate deaminase activity by GTP, ATP, and orthophosphate when assayed in the presence of 20 µM AMP and 150 mM KCl at three different pH values is shown in Fig. 1. The uninhibited rates are approximately equal at pH 6.5 and 6.2 and about 30% lower at pH 7.0. The optimum KCl concentration is 100 to 150 mM at these pH values. Each inhibitor is capable of producing complete or nearly complete inhibition. The K_i values for the inhibitors at pH 7.0 are 0.9 µM, 2.7 µM, and 1.3 mM for GTP, ATP, and orthophosphate, respectively. These increase slightly with decreasing pH. Under the conditions studied, each inhibitor shows some cooperativity (n = 1.3 to 1.6), and the cooperativity changes little with pH. The ATP curves deviate from the calculated curves at high concentrations; this is discussed later.

**Effect of ADP**—Activation of adenylate deaminase by ADP in the presence of 20 µM AMP and 150 mM KCl was studied at pH 6.2, 6.5, and 7.0. The enzyme is only weakly activated by ADP (A_{\text{max}} = 30 to 100%, K_a = 40 to 200 µM) under these conditions (not shown). However, in the presence of inhibitors such as GTP, ATP, and orthophosphate, the effect of ADP is much greater. Relief of the orthophosphate inhibition by ADP is shown in Fig. 2. Inhibition by 2 to 50 µM ATP and by 1 to 20 µM GTP was relieved by ADP in a similar manner (not shown). K_i values for these inhibitors at various ADP concentrations are listed in Table I. In each case, ADP increases the concentration of inhibitor needed to attain 50% inhibition. Note that the three inhibitors differ in the sensitivity of their K_i values to [ADP], ATP being the most sensitive and orthophosphate the least sensitive. In a similar experiment, the activating effect of ADP in the presence of 20 µM AMP and 5 mM orthophosphate was compared at pH 6.5 and 7.0 (not shown). The K_a for ADP decreases from 110 µM at pH 7.0 to...
Regulation of Adenylate Deaminase from Rat Muscle

TABLE I

<table>
<thead>
<tr>
<th>[ADP] (µM)</th>
<th>GTP (µM)</th>
<th>ATP (µM)</th>
<th>Orthophosphate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1.6</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>4</td>
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</tr>
<tr>
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<td>2</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>&gt;20</td>
<td>5</td>
</tr>
<tr>
<td>200</td>
<td>10</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>500</td>
<td>&gt;20</td>
<td></td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

FIG. 1

**Effect of ADP on Kᵢ values at 10 and 20 µM AMP, 150 mM KCl, pH 7.0.**

Kᵢ was calculated according to Equation 1. In the calculation of inhibition, v₀ was the rate in the presence of ADP and absence of inhibitor. [AMP] was 10 µM in the experiment with GTP and 20 µM in the experiments with ATP and orthophosphate.

70 µM at pH 6.5; thus the effectiveness of ADP increases as the pH is decreased.

**Effect of GDP**—Fig. 3 shows a comparison of the activating effects of GDP and ADP at 20 µM AMP, 150 mM KCl, pH 7.0, in the presence of 0, 5, or 20 µM GTP. GDP has little or no effect in the absence of GTP. It is able to relieve the inhibition produced by GTP, but is clearly much weaker in this respect than ADP. Relief of GDP, ATP, and orthophosphate inhibition of the muscle enzyme by ADP and GDP was previously noted by Ronca and co-workers (3, 5).

**Biphasic Effects of ATP**—In the concentration range of 1 to 50 µM, ATP inhibits adenylate deaminase in a manner similar to GTP (Fig. 4). However, as the concentration of ATP is increased above 100 µM, the inhibitor is relieved, less than 35% inhibition being seen above 1 mM ATP. The inhibition by GTP also begins to be relieved above 300 µM GTP (Fig. 4). The biphasic effects of ATP are not due to the presence of impurities such as ADP or AMP in the ATP. The ATP used in these experiments was purified by chromatography on Dowex 1 and was shown by enzymatic analyses to contain less than 0.3% ADP and less than 0.05% AMP, whereas our measurements show that more than 100 µM ADP is needed to relieve the inhibition produced by 50 µM ATP.

The inhibition produced by 50 µM GTP is relieved by 2 mM ATP (Table II). Even higher levels of GTP (100 to 600 µM) have little effect on the activity of the enzyme in the presence of 0.5 to 2 mM ATP (not shown). Thus, ATP is capable of relieving not only its own inhibition but also the inhibition produced by GTP. Relief of GTP inhibition by ATP was previously reported by Ronca-Testoni et al. (5). Orthophosphate remains a strong inhibitor even in the presence of 2 mM ATP (Table II).

We considered the possibility that the biphasic behavior of adenylate deaminase with respect to ATP was an artifact introduced during the purification procedure, and performed experiments on unpurified extracts. Rat hind leg muscle (14 g fresh weight) was extracted and centrifuged as described previously (13) and 3.5 ml of the resulting high speed supernatant was placed on a Sephadex G-25 column (13 cm high × 1.7 cm diameter) and eluted with a mixture of 150 mM KCl, 10 mM potassium phosphate, pH 6.5, and 1 mM dithiothreitol. Fractions of 1 ml were collected. The fraction richest in...
protein was assayed for adenylate deaminase activity at a 60-fold dilution in the presence of 20 μM AMP, 150 mM KCl, pH 7.0. Controls lacked AMP. The biphasic behavior with respect to ATP was observed even with the unpurified extract, although the maximum inhibition and apparent affinity for ATP was a little less than that observed with the purified enzyme. The GTP inhibition of adenylate deaminase was also somewhat weaker with the unpurified extract. The small differences between the pure enzyme and crude extract are probably due to interfering enzymes in the latter. Biphasic ATP inhibition curves at low K⁺ concentration have been reported (16, 17). Yoshino et al. (18), working with the enzyme from chicken erythrocytes, observed biphasic curves with both ATP and GTP. Moreover, adenylate deaminase from several other tissues is activated by ATP (19). Thus, the activating effect of ATP is a common feature of the enzyme from a variety of tissues and is not an artifact.

Effect of Mg²⁺—The influence of ATP and GTP on the activity of adenylate deaminase is modified by Mg²⁺. In the experiment shown in Fig. 4, MgCl₂ was added to give a free concentration of 0.5 mM Mg²⁺ and the mixture contained 150 mM KCl at pH 7.0. It can be calculated that under these conditions 75% of the nucleoside triphosphate is chelated to Mg²⁺. If only the unchelated forms of ATP and GTP are inhibitory, in the presence of 0.5 mM Mg²⁺ the inhibition curves should be shifted to nucleotide concentrations about 100/(100 - 75) = 4 times higher. The curves obtained in the absence of Mg²⁺ were replotted at 4 times higher concentrations (Fig. 4, dashed lines). The experimental results obtained in the presence of 0.5 mM free Mg²⁺ (Fig. 4, open symbols) agree quite well with these curves. The best fit would have been obtained with a MgATP stability constant of 2.4 x 10⁴ M⁻¹.

The concentrations of MgCl₂ added in the experiments shown in Figs. 4 and 5 were based on the following equilibria (20-22):

\[
\frac{[\text{MgATP}^2^-]}{[\text{Mg}^2+][\text{ATP}^4-]} = 2.0 \times 10^6 \text{M}^{-1} \quad (2)
\]

\[
[\text{HATP}^3+] = 8.9 \times 10^6 \text{M}^{-1} \quad (3)
\]

\[
[\text{KATP}^3+] = 1.0 \times 10^6 \text{M}^{-1} \quad (4)
\]

\[
[\text{MgADP}^2^-] = 1.0 \times 10^9 \text{M}^{-1} \quad (5)
\]

\[
[\text{HADP}^3+] = 7.6 \times 10^6 \text{M}^{-1} \quad (6)
\]

The MgATP stability constant of 1.0 x 10⁴ M⁻¹ quoted in Ref. 20 was determined in the presence of 0.1 mM KCl. In order to obtain the constant in the absence of K⁺, one must take into account the formation of KATP. The constant given in Equation 2 is based on the data from Ref. 20 corrected using the constant given in Equation 4. The constants for GTP were assumed to be the same as those shown in Equations 2 to 4.

The value of 2.0 x 10⁶ M⁻¹ for the stability constant of magnesium-ATP is based on results of Martell and Schwarzenbach (20) under conditions (0.1 mM KCl, 20°C) comparable to our experimental conditions. A somewhat higher value of 5 x 10⁶ M⁻¹ at 20°C is given by Kuby and Noldmann (23); it is based on determinations in the absence of alkali metal ions. Using the higher value, one would expect 88.1% of the ATP and GTP to be chelated in the presence of 0.5 mM free Mg²⁺ and 150 mM KCl, pH 7.0. This would shift the inhibition curves to concentrations 8.4 times higher if only free ATP and GTP are inhibitory; however, the observed shifts in concentration are only 4 to 5 times higher concentrations. This suggests either that the applicable stability constant is about 2.4 x 10⁶ M⁻¹ and that only unchelated ATP and GTP are inhibitory, or that the chelated forms of ATP and GTP make some contribution to the inhibition. In the latter case, if the stability constant of 5 x 10⁹ M⁻¹ is used, it can be calculated that the chelated nucleotides are only 10 to 15% as effective as inhibitors as the free nucleotides; otherwise smaller shifts of the inhibition curves would occur. Thus, even if the high value for the stability constant is correct, the free nucleotides are still much stronger inhibitors than the chelated nucleotides.

**DISCUSSION**

The experiments reported here were designed to yield information on the regulation of adenylate deaminase in rat muscle under physiological conditions. Taken in conjunction with metabolite measurements, they indicate that the major factors causing an increase in the activity of the enzyme during muscular work are increases in [AMP], [ADP], and [H⁺]. Orthophosphate acts as an inhibitor in vitro. ATP, GTP, and GDP do not appear to cause a change in the activity of the enzyme.
enzyme under physiological conditions, but they may help to set the basal activity of the enzyme.

The AMP content of rat muscle at rest is about 100 nmol/g fresh weight; this increases by about 20% during exercise (24). However, in muscle, most of the AMP appears not to be freely available. If it is assumed that the creatine kinase and myokinase reactions are at equilibrium, the calculated concentrations of freely available AMP are only 0.2 and 1 μM in resting muscle and after exercise, respectively (24). Even though these substrate concentrations are very low, the uninhibited activity of adenylate deaminase is more than sufficient to account for the rates of IMP accumulation observed in vivo (24).4 Although the AMP levels used in the experiments reported here are higher, they are still far below the \( K_{\text{app}} \) for AMP, namely 1 mM at 150 mM KCl and pH 7.0. At such low concentrations, the rates of adenylate deaminase are proportional to [AMP].

ADP exerts a relatively small activating effect in the presence of 150 mM KCl. However, ADP relieves the inhibition produced by GTP, ATP, and orthophosphate (Fig. 2 and Table I). Most of the ADP in muscle is bound to actin (26), and calculations of the concentration of freely available ADP in muscle give values of about 40 μM and 70 μM at rest and during exercise, respectively (24). Only unchelated ADP activates (Fig. 5). Its concentration can be calculated by multiplying freely available ADP (in this case, 40 and 70 μM) by the fraction that is unchelated (58% at 1.3 mM free \( \text{Mg}^{2+} \) and pH 7.0, calculated from Equations 5 and 6). The resulting unchelated ADP concentrations of about 25 and 40 μM, respectively, are sufficient to produce some relief of the inhibition under the conditions shown in Fig. 2 and Table I. GDP also relieves inhibition of the enzyme, but it is less effective than ADP (Fig. 3). Since it is present in much lower concentrations than ADP, it is unlikely that GDP plays a physiologically significant role as regulator.

An interesting feature of the regulation of adenylate deaminase is its biphasic response to changes in ATP. The concentration of unchelated ATP can be calculated to be 1.2 mM, at which much of the inhibitory effect of unchelated ATP has been overcome (Fig. 4). Moreover, 2 mM ATP is able to relieve most of the inhibition produced by physiological concentrations of free GTP (Table II). Thus, if our kinetic results are applicable to conditions in vivo, inhibition of adenylate deaminase by GTP and ATP is relatively weak.5

4 The calculations presented in Ref. 24 refer only to IMP accumulation (0.04 μmol/g fresh weight/min) and do not consider reamination. An upper limit on reamination can be estimated from the maximum activity of adenylouacase, which is about 0.4 μmol/g fresh weight/min at 30°C (25), corrected to 0.6 μmol/g fresh weight/min at 38°C. If reamination occurs at the same time as deamination, the maximum rate of deamination in vivo is 0.04 μmol/g fresh weight/min, which corresponds to a rate that uninhibited adenylate deaminase would exhibit at about 3 μM AMP.

5 The concentration of ADP in muscle is about 100 μM at rest and 200 μM during exercise. This concentration is much higher than the concentration of AMP (10 μM at rest and 20 μM during exercise) and is therefore likely to be the limiting factor in the inhibition of adenylate deaminase by ADP.

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REFERENCES

good agreement with the stability constant used, and assuming a higher value merely changes the relative contributions of chelated and unchelated ATP (see Footnote 3).

9 J. J. Aragon and J. M. Lowenstein, unpublished observations.
10 Yoshino et al. (18) interpreted the biphasic behavior of adenylate deaminase from erythrocytes in terms of the model of Monod et al. (31). They assumed that an unequal number of activator and inhibitor sites was necessary for biphasic behavior, but this assumption is unnecessary. If one considers nonexclusive binding of ligands to both R and T states, as discussed by Kuban and Changeux (32), biphasic behavior can result from a combination of dissociation constants of a ligand for activator and inhibitor sites in the two states. For example, consider a ligand \( X \) binding to inhibitor sites with \( K_I = 1 \) and 0.1 and to activator sites with \( K_A = 1 \) and 100 for the R and T states, respectively, and an equal number of both types of site. Ligand \( X \) will act as an inhibitor for \( 0 < [X] < 8.9 \), with maximum inhibition seen at \( [X] = 0.82 \), and as an activator for \( [X] > 8.9 \).
Adenylate deaminase from rat muscle. Regulation by purine nucleotides and orthophosphate in the presence of 150 mM KCl.

T J Wheeler and J M Lowenstein


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