Platelet AMP Deaminase

REGULATION BY Mg-ATP\textsuperscript{2-} AND INORGANIC PHOSPHATE AND INHIBITION BY THE TRANSITION STATE ANALOG COFORMYCIN\textsuperscript{*}

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Kinetic studies with platelet AMP deaminase, at pH 7.0 and 100 mM NaCl, gave cooperative initial velocity curves with AMP as substrate, with Mg-ATP\textsuperscript{2-} as an activator, and with Pi as an inhibitor. In the absence of Mg-ATP\textsuperscript{2-}, the \( k_{\text{on}} \) for AMP was 4.5 mM with a Hill coefficient approaching 2.0. In the presence of saturating Mg-ATP\textsuperscript{2-}, the \( k_{\text{on}} \) for AMP was reduced to 0.18 mM, the maximum velocity was increased by about 35%, and the Hill coefficient was 1.0. The half-activation constant for Mg-ATP\textsuperscript{2-} varied from 0.7 to 0.07 mM as the concentration of AMP was varied from 0.1 to 5.0 mM and the Hill coefficient for Mg-ATP\textsuperscript{2-} activation changed from 2.0 to 1.0 over the same range. Phosphate inhibition was competitive with AMP and with Mg-ATP\textsuperscript{2-} (\( K_i \) = 2.0 mM) and reversed the activation by Mg-ATP\textsuperscript{2-}. Coformycin inhibited the Mg-ATP\textsuperscript{2-}-activated enzyme with a \( K_i \) of 0.35 mM. Coformycin inhibition was slow, with a second order rate constant of 6.0 \( \times \) 10\textsuperscript{-4} M\textsuperscript{-1} min\textsuperscript{-1}, suggesting that the compound acts as a transition state analog according to Frieden, C., Kurz, L. C., and Gilbert, H. R. (1980) Biochemistry 19, 5303-5309. The kinetic properties of the enzyme indicate that substantial regulation can occur through changes in AMP concentration acting synergistically to enhance Mg-ATP\textsuperscript{2-} binding and displace Pi from a single type of regulatory site.

AMP deaminase (EC 3.5.3.6) catalyzes the irreversible deamination of AMP to IMP and ammonia. In platelets, the enzyme is activated in response to changes brought about by thrombin stimulation (1) and leads to irreversible loss of 30% of platelet metabolic adenine nucleotides. IMP is further converted to inosine and then to hypoxanthine which diffuses out of the cell (1). The role of AMP deamination is unclear. In some cell types, the enzyme is thought to stimulate ATP out of the cell (1). The role of AMP deamination is unclear. In platelets, we have shown that elimination of AMP deaminase activity, using the potent inhibitor coformycin, has no effect on the rate of glycolysis even when glycolysis is maximally stimulated by thrombin. AMP deamination also plays a role in maintaining the adenylate energy charge at around 0.9, which appears to be important for platelet function (4, 5). The enzyme is clearly subject to tight regulation; otherwise, adenylate nucleotide homeostasis would not be maintained.

We have previously shown (6) that the activity of purified AMP deaminase is modulated somewhat by changing from potassium- to sodium-containing media and that the difference in activity between the two cations is enhanced by GTP. Sodium-potassium exchange that accompanies platelet activation (7) may thus play a role in regulation of the platelet enzyme. We also showed (6) that ATP is virtually an obligatory activator of platelet AMP deaminase, changing the apparent Michaelis constant for ATP from around 4 mM in the absence of ATP to 1.2 mM in the presence of saturating ATP. In the present paper, we show that Mg-ATP\textsuperscript{2-} is a much more effective activator than ATP and that inorganic phosphate is an inhibitor of both the nonactivated and Mg-ATP-activated enzyme. We have also examined the effects on the Mg-ATP-activated enzyme of the nucleoside coformycin. Coformycin is a tight binding inhibitor of both adenosine deaminase (8-11) and AMP deaminase (12, 13), probably acting as a transition state analog (11, 13).

**MATERIALS AND METHODS**

**AMP Deaminase**—The enzyme was prepared from outdated platelet concentrates, obtained from the Penn-Jersey Red Cross, by slight modification of the method of Ashby and Holmsen (6). Enzyme was eluted batchwise from a phosphocellulose column, using 10 mM sodium phosphate buffer, pH 7.0, containing 3 mM NaCl, and was added directly to assay mixtures.

**Initial Velocity Measurements**—Enzyme activity was measured by a spectrophotometric assay (6) at 37 °C and 285 nm (\( \Delta A = 0.3 \) mM/cm), using a Cary 210 spectrophotometer and either a 1-cm or 0.5-cm path length. Assays were performed in duplicate in 50 mM imidazole/HCI buffer, pH 7.0, containing 100 mM NaCl and 5 mM MgCl\textsubscript{2} together with appropriate additions of AMP, ATP, and sodium phosphate. Results obtained in the presence of 100 mM KCl (not shown) were virtually identical with those obtained in NaCl. MgCl\textsubscript{2} concentrations up to 10 mM had no effect on enzyme activity in the absence of ATP. The apparent dissociation constant for Mg-ATP\textsuperscript{2-} at pH 7.0 in the presence of 100 mM NaCl was calculated to be 80 \( \mu \)M, using the method of Nanninga and Kempen (14) and values of dissociation constants obtained from Storer and Cornish-Bowden (15). Hence, under the conditions used, over 98% of the ATP was present as Mg-ATP\textsuperscript{2-}. Protein concentration was determined by the method of Lowry et al. (16).

**Chemicals**—Coformycin was a gift of Dr. Martin Black, Warner-Lambert Pharmaceutical Research Division, Ann Arbor, MI. AMP and ATP were obtained from Sigma Chemical Co., St. Louis, MO.

**RESULTS**

**Effect of Mg-ATP\textsuperscript{2-} on the Activity of AMP Deaminase**—Previous studies (6) have shown that platelet AMP deaminase is active in the absence of ATP, displaying sigmoidal kinetics with regard to AMP, with an apparent \( K_m \) of about 4 mM. Activation by ATP\textsuperscript{2-} caused the sigmoidicity to shift to hy-
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FIG. 1. Substrate and activator kinetics of AMP deaminase. Enzyme preparations had a specific activity of 294 μmol/min/mg of protein, measured at saturating AMP in the presence of 50 μM ATP. The enzyme concentration was 1.7 μg/ml in each case. Initial velocity is expressed in μmol/min. Mg-ATP₂⁻ concentration was varied by fixing MgCl₂ at 5.0 mM and varying the ATP content. Under these conditions, more than 98% of the added ATP was present as MgATP₂⁻ (see "Materials and Methods"). Reactions were run at 37 °C in 50 mM imidazole/HCl, pH 7.0, containing 100 mM NaCl. a, 1/v versus 1/AMP concentration in the presence of saturating concentrations of ATP₄⁻; the maximum velocity remained the same, and the interaction of the enzyme with ATP was more or less hyperbolic (6).

Activation by Mg-ATP₂⁻ was more complex. Plots of 1/initial rate versus 1/AMP were upward curving, becoming

FIG. 2. Substrate and activator kinetics of AMP deaminase. Enzyme concentration and assay conditions were identical with those described in Fig. 1. a, 1/v versus 1/Mg-ATP₂⁻ concentration at various concentrations of AMP. b, S₀.₅ for MgATP (millimolar) and Hill coefficient plotted against Mg-ATP₂⁻ concentration. Kinetic constants were determined as described under "Results."
linear at high concentrations of Mg-ATP$^{2-}$ (Fig. 1a). Hill plots of the same data (not shown) gave Hill coefficients varying from 1.74 to 1.9 as the concentration of Mg-ATP$^{2-}$ was increased (Fig. 1b). The $s_{0.5}$ for AMP was lowered from 4.5 mM in the absence of Mg-ATP$^{2-}$ to 0.18 mM in the presence of 1.5 mM Mg-ATP$^{2-}$ and the maximum velocity increased by about 35% over the same range (Fig. 1b). Maximum velocities were determined by extrapolation of double reciprocal plots and $s_{0.5}$ values were determined from Hill plots from the value of log [AMP] when log $v/(V - v) = 0$. Hill plots were linear with correlation coefficients better than 0.96 in each case (not shown).

Plots of 1/initial rate versus 1/Mg-ATP$^{2-}$ were also upward curving, indicating positive cooperativity of activator interaction (Fig. 2a). The activation constant for Mg-ATP$^{2-}$ varied from 0.7 mM at 0.1 mM AMP to 0.07 mM at 5 mM AMP and Hill coefficients varied from 1.95 to 1.0 over the same range (Fig. 2b). Activation constants were determined as $s_{0.5}$ values from the value of log [Mg-ATP$^{2-}$] when log $v/(V - v) = 0$ (not shown).

Inhibition by Phosphate—Platelet AMP deaminase was inhibited by inorganic phosphate, competitively with AMP (Fig. 3a). The concentration of Pi required to double the apparent $s_{0.5}$ for AMP (the apparent inhibition constant) was around 2.0 mM. Similarly, phosphate inhibition of the Mg-ATP$^{2-}$-activated enzyme was competitive with Mg-ATP$^{2-}$ (Fig. 3b). At a fixed AMP concentration of 0.5 mM, the concentration of Pi required to double the activation constant for Mg-ATP$^{2-}$ was again around 2.0 mM. The $s_{0.5}$ values for AMP and the activation constants for Mg-ATP$^{2-}$ at each Pi concentration were determined from Hill plots of the same data (not shown). Activation by Mg-ATP$^{2-}$ was cooperative in the absence and presence of Pi with Hill numbers approaching 2.0 in all cases.

Effect of Mg-ATP$^{2-}$ and Pi, Together on the Initial Velocity versus Substrate Curve—Fig. 4 shows initial velocity versus AMP concentration plots in the absence of modifiers in the presence of 2 mM Mg-ATP$^{2-}$ and in the presence of both 4 mM Pi and 2 mM Mg-ATP$^{2-}$. In the presence of Mg-ATP$^{2-}$, the inhibitory effect of Pi was apparent only at low concentrations of AMP so that the curve was altered from the hyperbolic plot in the absence of Pi to a curve that rose steeply over

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**Fig. 3. Phosphate inhibition of AMP deaminase.** Assay conditions were the same as those in Fig. 1. The enzyme concentration was 0.8 mg/ml in each assay. a, phosphate inhibition as a function of AMP concentration expressed as a double reciprocal plot. b, phosphate inhibition as a function of Mg-ATP$^{2-}$ concentration expressed as a double reciprocal plot. The AMP concentration was fixed at 0.5 mM.

**Fig. 4. Initial velocity as a function of AMP concentration.** Assay conditions were the same as those in Fig. 1. The enzyme concentration was 1.6 µg/ml. ▲, in the absence of modifiers; ●, in the presence of 2 mM Mg-ATP$^{2-}$; ○, in the presence of 2 mM Mg-ATP$^{2-}$ and 4 mM Pi; ●, in the presence of 2 mM Mg-ATP$^{2-}$ and 4 mM Pi. 2 mM Mg-ATP$^{2-}$ was the highest usable concentration in the spectrophotometric assay. Inset, Hill plots of the data in Fig. 4.: ○, in the presence of 2 mM Mg-ATP$^{2-}$; □, in the presence of 2 mM Mg-ATP$^{2-}$ and 4 mM Pi.
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Discussion

In the absence of ATP$, platelet AMP deaminase shows cooperativity with an apparent $K_a$ of 4.5 mM. In the presence of saturating ATP$, the kinetics becomes hyperbolic with a $K_a$ of 1.2 mM and no change in the maximum velocity (6). In the presence of saturating Mg-ATP$, we show here that the velocity curve is also hyperbolic but the $K_a$ is reduced to 0.18 mM and the maximum velocity is increased by about 35%.

In a previous paper (6), we attempted to explain the sigmoidicity, in the absence of ATP, in terms of a simple, noninteracting site model in which AMP bound weakly to an activator site that was otherwise specific for ATP. Although it is probably true that AMP binds to the ATP activator site, the complex kinetics of Mg-ATP interaction with the enzyme is also consistent with AMP deaminase being a multimeric enzyme with interacting sites. Plots of $v_0/initial rate vs 1/Mg-ATP$ concentration were upward curving (Fig. 2a), suggesting positive cooperative interaction of Mg-ATP$^2$ sites.

The half-saturation value for AMP varied 25-fold from 4.5 to 0.18 mM as the Mg-ATP$^2$ concentration was varied from zero to saturation. Similarly, the half-activation constant for Mg-ATP$^2$ varied with the AMP concentration. The synergistic relationship is shown simplified in Scheme 1. The interactions of both AMP and Mg-ATP with the enzyme are inhibited competitively by Pi. It seems likely that Pi is competing for binding of AMP to the same activator site that binds Mg-ATP$^2$ (AMP binding to this site is not shown in Scheme 1), rather than competing directly for the catalytic site. The fact that Pi binding is competitive with AMP rules out the possibility that AMP binding to the catalytic site enhances the binding of Pi to the regulatory site. This is apparent from Scheme 1: if $K_a$ the dissociation constant for binding of I to ES was reduced compared with $K_a$, then in order to maintain thermodynamic equilibrium the dissociation constant $K_a$ would also have to be reduced, the apparent $K_a$ of the enzyme would thus fall, and Pi would be an activator. The possibility that IES is an inactive species is ruled out since Pi has no effect on the maximum velocity.

Since Mg-ATP$^2$ is the predominant ATP species present in the cell, the results obtained with Mg-ATP$^2$ are more relevant to regulation than those obtained earlier (6) with ATP$. Competition between Mg-ATP and Pi suggests that they are major regulatory ligands of AMP deaminase. The concentration of ATP in the cytosol of resting platelets is around 4.2 mM (17) and the AMP concentration is about 1% of the ATP content. The concentration of Pi in resting platelets is around 3.6 mM (based on 2.5 mM of Pi/10$^9$ platelets (18) and a platelet volume of $7 \times 10^{12}$ ml). The inhibition constant of 2.0 mM for Pi is similar to that for activation by Mg-ATP (1.25 mM; see Scheme 1) when the AMP concentration approaches zero. In resting platelets, the regulatory sites of AMP deaminase will contain similar proportions of Mg-

![Graph](https://via.placeholder.com/150)

**Fig. 5.** Time course of Coformycin inhibition of Mg-ATP-activated AMP deaminase. 25 μg of enzyme (specific activity, 200 μmol/min/mg) were added at zero time to 10 μl of 50 mM imidazole/HCl, pH 7.0, containing 100 mM NaCl, 5 mM MgCl$_2$, 1.0 mM ATP, and various concentrations of Coformycin. At 37 °C, 800 μl samples were removed and placed in a spectrophotometer cuvette at various times and the reaction was started by addition of 45 μl of 20 mM AMP in the same buffer. The final concentrations of AMP and Mg-ATP were 1.0 mM. Straight lines were drawn by eye. The second order rate constant for Coformycin inhibition was determined as $6.0 \times 10^6$ M$^{-1}$ min$^{-1}$ by dividing each pseudo-first order rate constant by the appropriate Coformycin concentration and averaging the resulting values.

Inhibition by Coformycin—The nucleoside Coformycin has been shown to be a potent inhibitor of muscle AMP deaminase (12, 13). Coformycin also inhibited Mg-ATP-activated platelet AMP deaminase. The onset of inhibition was very slow, with a second order rate constant of $6 \times 10^6$ M$^{-1}$ min$^{-1}$ (Fig. 5), making determination of the inhibition constant impossible since the enzyme underwent time-dependent inactivation in the absence of Coformycin. However, essentially complete inhibition could be obtained with 0.25 μM Coformycin after 2.5 h (Fig. 5). The pseudo-first order plot remained linear, suggesting that this concentration of Coformycin was still well above saturation. The results suggest that Coformycin may act as a transition state analog as discussed by Frieden et al. (13).

**Scheme 1.** Kinetic constants of platelet AMP deaminase. $E$ represents enzyme, $A$ represents Mg-ATP$, $I$ represents Pi, $S$ is the substrate, AMP, and $P$ represents products. $ES$ represents substrate binding to the catalytic site, $AE$ represents Mg-ATP$^2$ binding to the activator site, and $IE$ represents binding of Pi, to the same site. **Straight lines** represent the equilibria between species. The **broken lines** represent equilibria with unknown dissociation constants that are probably very high. The scheme is simplified so that only a single interaction of each ligand is shown. The constants represent ligand concentrations required for half-maximum effect and are therefore complex. The value of 0.05 mM for interaction of Mg-ATP with the AMP complex was determined by extrapolation of a plot of $s_{0.5}$ for Mg-ATP versus I/(AMP) to infinite [AMP] (not shown). The value of $s_{0.5} = 1.25$ mM for formation of $E$-Mg-ATP in the absence of AMP was determined from the other constants according to the principle of microscopic reversibility.
ATP and P; and the catalytic sites will be nearly empty. In stimulated platelets, AMP concentration would increase due to increased ATP hydrolysis. Increasing AMP concentration would shift the equilibrium toward the enzyme-Mg-ATP complex since increasing AMP enhances the affinity of the enzyme for Mg-ATP. Hence, the enzyme may be regulated by the AMP concentration acting to enhance binding of the activator, displacing the inhibitor. This is illustrated in Fig. 4. In the absence of P, the velocity versus substrate plot at 2 mM Mg-ATP was hyperbolic. In the presence of 4 mM P, the curve was sigmoidal at low concentrations of AMP but rapidly merged with the curve obtained in the absence of P. The Hill coefficient changed from 1.7 to 1.0 when the AMP concentration reached about 0.6 mM, suggesting that all of the P1 had been displaced. The consequence of this mechanism is apparent from Fig. 4. In the presence of both P1 and Mg-ATP, the activity of the enzyme increased 20-fold as the AMP concentration changed from 0.04 to 0.4 mM compared with a 4-fold change in activity over the same range when only Mg-ATP was present. The enzyme AMP nucleosidase from Escherichia coli behaves in a qualitatively similar manner with regard to interaction with AMP, Mg-ATP, and P; and precisely the same mechanism has been proposed for regulation of this bacterial enzyme (19). AMP nucleosidase is the major enzyme of AMP catabolism in E. coli (19), whereas AMP deaminase has the same role in platelets and some other eukaryotic cells (20). It is of interest that different enzymes, fulfilling essentially the same role, may be controlled in identical ways.

Coformycin inhibition of Mg-ATP-activated platelet AMP deaminase is both slow, with an on-rate of $6 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$, and potent, with an inhibition constant $<0.25 \mu\text{M}$. Determination of the inhibition constant is difficult because, during the time required to attain full inhibition at low concentrations of coformycin (>3 h), the enzyme loses activity, even in the absence of coformycin, presumably due to slow denaturation. However, by the criteria described by Frieden et al. (13), coformycin may be regarded as a transition state analogue inhibitor that binds poorly to the active site before a shift to the complementary enzyme conformation of the transition state allows tight binding. We have already demonstrated (4) the usefulness of coformycin in examining the role of AMP deaminase in intact platelets.

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