P\(^{\prime}\),P\(^{\prime}\)-Di(adenosine-5')pentaphosphate, a Potent Multi-substrate Inhibitor of Adenylate Kinase

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SUMMARY

Rabbit muscle adenylate kinase is potently inhibited by P\(^{\prime}\),P\(^{\prime}\)-di(adenosine-5')pentaphosphate (Ap\(_5\)A) but not by the homologs of this compound with fewer phosphoryl groups in the polyphosphate bridge and not by adenosine 5'-pentaphosphate. The inhibition by Ap\(_5\)A is competitive with respect to both of the substrates, AMP and ATP. The association constant for the binding of Ap\(_5\)A to adenylate kinase is about 4 \times 10^8 M\(^{-1}\) at 24\(^\circ\) and pH 8.0.

Two substrate enzymatic reactions that proceed by way of a ternary complex of enzyme and both substrates should be potently inhibited by compounds that possess in an appropriate spatial relationship within 1 molecule the binding determinants for both substrates (1, 2). We have applied this principle to identify a potent inhibitor of the enzyme, adenylate kinase (EC 2.7.4.3) from rabbit muscle. This enzyme catalyzes the transfer of the terminal phosphoryl group from ATP to AMP. The kinetic mechanism of this reaction is random bi bi (3), and it is probable that the chemical mechanism involves direct displacement of ADP from ATP by a phosphoryl oxygen atom of AMP (4). The enzyme is probably a single polypeptide chain with only one active site (5). This communication describes the potent inhibition of adenylate kinase by the compound, P\(^{\prime}\),P\(^{\prime}\)-di(adenosine-5')pentaphosphate

\[
\begin{array}{c}
A \\
\text{O}^{-} \\
\text{O} \\
\text{O} \\
\text{O} \\
\end{array}
\]

where A—O = adenosine with a bond to the 5' oxygen atom.

MATERIALS AND METHODS

Samples of calcium Ap\(_5\)A\(^{\prime}\), calcium Ap\(_5\)A, calcium Ap\(_{4}\)A, and sodium Ap\(_5\)A were most generously supplied by Dr. J. G. Moffatt of the Institute of Molecular Biology at Syntex Research. Reiss and Moffatt (6) have previously described the synthesis and characterization of these compounds. Ammonium p\(_{4}\)A was kindly supplied by Dr. R. Lehman of the Salk Institute for Biological Studies. Barium p\(_{4}\)A was purchased from Sigma Chemical Co. and converted to the sodium salt by precipitation of the barium from an acidic aqueous solution with sulfate. The identity and purity of these compounds were checked by thin layer chromatography upon polyethyleneimine cellulose (PEI-cellulose F plates from EM Laboratories, Inc.) with aqueous LiCl of various concentrations as the solvent. The compounds showed the expected mobilities relative to AMP, ADP, and ATP (7) and gave only a single, ultraviolet light-absorbing spot, with the exception of p\(_{4}\)A, which contained about 90% p\(_{5}\)A. The purity of the p\(_{5}\)A was examined with especial care; the compound gave a single spot (R\(_{f}\) 0.55 to 0.68) upon chromatography in 2.0 M LiCl of an amount that was large enough to allow detection of 5% of an ultraviolet light-absorbing impurity of different mobility. Dr. E. J. Griffiths of Monsanto Industrial Chemicals Co. generously gave us samples of sodium tripolyphosphate and sodium tetra and hexametaphosphates. Sodium polyphosphates of average chain length 4 to 6 were purchased from Sigma Chemical Co. (P8385). Upon thin layer chromatography on PEI-cellulose with aqueous LiCl as the solvent the linear polyphosphates migrated with the expected mobilities relative to each other, and the same was true of the cyclic metaphosphates (8). However, in contrast to the earlier report (8), tripolyphosphate moved more slowly than hexametaphosphate and pyrophosphate moved more slowly than tetrametaphosphate.

Rabbit muscle adenylate kinase, pyruvate kinase, and lactic dehydrogenase were Boehringer Mannheim products. Crystalline rabbit muscle fructose 6-phosphate kinase was obtained from Sigma, and crystalline rabbit muscle creatine kinase was a gift from Dr. L. Noda.

The initial velocities of the adenylate kinase reaction were determined spectrophotometrically at 340 nm by coupling the formation of ADP to the oxidation of NADH via the pyruvate kinase and lactic dehydrogenase reactions (3). The reaction mixture contained 50 mM Tris-HCl buffer, pH 8.0, 10 mM cysteine-HCl (freshly dissolved and adjusted to pH 8 with 1.3 eq KOH), 0.5 mg per ml of bovine serum albumin (Sigma, crystallized and lyophilized), 1 mM potassium or sodium phosphoenolpyruvate, 10 mM MgSO\(_4\), 0.1 mM NADH, ATP, AMP, inhibitor (in some cases), 3 to 5 \mu\,g per ml of lactic dehydrogenase, 3 to 5 \mu\,g per ml of pyruvate kinase, and 0.022 to 0.035 \mu\,g per ml of adenylate kinase. The reactions were initiated by the addition of 5- or 10-\mu\,l volumes of the inhibitor and the enzymes, in the order given above, to the rest of the mixture after it had temperature-equilibrated at 24\(^\circ\) in a 1-ml cuvette in the cell compartment of the spectrophotometer. The concentrations of lactic dehydrogenase and pyruvate kinase were sufficient for efficient trapping of the ADP, since larger amounts of these enzymes did not increase the rates. The concentration of adenylate kinase in micrograms per ml given for the reaction mixtures is based upon the absorbance of a stock solution at 279 nm (9) and is equivalent to 1.0 to 1.7 \times 10^{-3} \, M (10). The specific activity of our enzyme was about 13% of that reported for the pure enzyme under similar conditions (11).

RESULTS AND DISCUSSION

The effects of various potential inhibitors upon the rate of the adenylate kinase reaction are given in Table I. Ap\(_5\)A is an extremely potent inhibitor; it inhibits the reaction by 55% at 3 \times 10^{-5} \, M. The relatively weak inhibition (8 to 15% at 330 to
Inhibition of adenylate kinase

For each inhibitor, the initial velocities of the adenylate kinase reaction were measured in the presence and absence of the inhibitor, with the same concentration of enzyme, by the method described in the text. The concentrations of AMP and ATP throughout were 0.20 and 0.15 mM, respectively. The per cent inhibition is 100 X (v0 - v1)/v0, where v0 and v1 are the initial velocities in the presence and absence of inhibitor, respectively.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration [μM]</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ap6A</td>
<td>10</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>Ap4A</td>
<td>10</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>Ap2A</td>
<td>10</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>ApA</td>
<td>10</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>10</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>Triphosphate</td>
<td>10</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>Tetraphosphate</td>
<td>10</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>Tetrahexaphosphate</td>
<td>10</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>Hexametaphosphate</td>
<td>10</td>
<td>0 ± 6</td>
</tr>
</tbody>
</table>

* At 35°.  
* Assay mixture contained 88 mm KCl.  
* From Reference 12, which describes inhibition under conditions almost identical with ours.  
* There is no inhibition by 200 μm calcium chloride.

An identical type of expression describes the relationship between Ki and the equilibrium constant (KAMP) for formation of the complex between enzyme and AMP when the concentration of MgATP2- is very low. Kuby et al. (9) have found that the values of KAMP and Kampp for rabbit muscle adenylate kinase are 10^4 M^-1 and 1.6 x 10^6 M^-1, respectively, at pH 7.9 and 3°. The value of v0/vt at very low concentrations of each variable substrate is equivalent to the ratio of the slope of the plot in Fig. 1 for the case with inhibitor present to that for the case with inhibitor absent. Using the values of KAMP and Kampp, the values of v0/vt given by the data in Fig. 1, and the two expressions for Ki, we calculate values of 2.8 and 5.0 x 10^4 M^-1 for Ki.

Although the binding of ApA to adenylate kinase is strong, the rate of dissociation of the complex is relatively fast. When a solution of 1.0 x 10^-4 M ApA and 5 x 10^-7 M adenylate kinase (assuming all of the protein is the kinase) in 10 mM MgSO4-10 mM cysteine-0.5 mg per ml of bovine serum albumin-50 mM Tris-HCl, pH 8.0, was diluted by a factor of 500 into the usual assay mixture that contained 1.0 mM AMP and 0.15 mM ATP, there was no detectable lag in the establishment of the initial velocity, which was the same as that for the same dilution of a stock solution of the enzyme alone. The lack of a measurable lag requires that the half-time for dissociation of the complex be less than 0.2 min.

The magnitude of the association constant for the binding of...
Ap₅A to adenylate kinase is about 20 times larger than the product of \( K_{\text{MgATP}} \) and \( K_{\text{AMP}} \). Since the binding of each substrate appears to strengthen somewhat the binding of the other (3), \( K_i \) for Ap₅A is less than 20 times larger than the association constant for the formation of the ternary complex from free enzyme and both substrates. Wolfenden has termed such inhibitors multisubstrate analogs (1), and this description of Ap₅A seems more appropriate than the term transition state analog for two reasons. First, the transition state for phosphoryl transfer in the adenylate kinase reaction probably resembles a trigonal bipyramid with the phosphorus atom at its center and the entering and leaving oxygen atoms at its apexes (4):

\[
\begin{align*}
O & \quad O \\
A & \quad P \quad O \quad P \quad O \cdots P \quad O \quad P \quad O \quad A \\
O & \quad O
\end{align*}
\]

Clearly, the pyrophosphate functional group of Ap₅A does not closely resemble the pentaoxy phosphorus atom of the suspected transition state. Second, a good analog of this transition state should have a much larger association constant for binding to the enzyme than the association constant for the formation of the ternary complex from enzyme and both substrates (1, 2).

Ap₅A may prove to be useful for studying the role of adenylate kinase in metabolism (4) and also for inhibiting adenylate kinase wherever its presence complicates the investigation of other reactions of the adenine nucleotides, such as in the investigation of the role of the ADP-ATP exchange reaction in oxidative phosphorylation (13). It will probably be possible to prepare potent inhibitors of other phosphoryl transferring enzymes (14) by replacing the phosphoryl group that is transferred by a pyrophosphate bridge.

Acknowledgment—We thank Dr. Lafayette Noda for sharing his knowledge of adenylate kinase with us.

REFERENCES

P1,p5-Di(adenosine-5')pentaphosphate, a Potent Multisubstrate Inhibitor of Adenylate Kinase
Gustav E. Lienhard and Isaac I. Secemski


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