The Inhibition of Acetate, Pyruvate, and 3-Phosphoglycerate Kinases by Chromium Adenosine Triphosphate

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SUMMARY

The inert coordination complex, chromium ATP, has been used to study the steady state kinetic mechanisms of acetate, pyruvate, and 3-phosphoglycerate kinases. With pyruvate and 3-phosphoglycerate kinases, CrATP competes with both substrates and has a dissociation constant of 200 μM. This behavior is consistent with a random sequential mechanism for these enzymes. The fact that saturation with the non-nucleotide substrate eliminates most or all of the CrATP inhibition makes these enzymes useful as coupling enzymes (together with lactate dehydrogenase or glyceraldehyde-P dehydrogenase) in assays for other kinases when CrATP inhibition is being studied. With acetate kinase, CrATP competes with MgATP and is noncompetitive versus acetate. As a product inhibitor, acetyl phosphate is noncompetitive versus either MgATP or acetate. Initial velocity studies show the enzyme to have a sequential mechanism. A random mechanism is proposed which accounts for the kinetic data, and for the isotope exchanges and the phosphoenzyme isolated by other workers.

The reactions catalyzed by adenosine triphosphate:pyruvate phosphotransferase (EC 2.7.1.40, pyruvate kinase), adenosine triphosphate:3-phospho-D-glycerate-l-phosphotransferase (EC 2.7.2.3, phosphoglycerate kinase), and adenosine triphosphate:acetate phosphotransferase (EC 2.7.2.1, acetate kinase) are respectively:

\[ \text{MgATP} + \text{pyruvate} \rightarrow \text{MgADP} + \text{phosphoenolpyruvate} \]
\[ \text{MgATP} + 3\text{P-glycerate} \rightarrow \text{MgADP} + 1,3\text{-di-P-glycerate} \]
\[ \text{MgATP} + \text{acetate} \rightarrow \text{MgADP} + \text{acetyl-phosphate} \]

Rabbit muscle pyruvate kinase has been postulated to have a rapid equilibrium random mechanism on the basis of product inhibition studies (1, 2). However, Robinson and Rose (3) have recently shown by tritium-labeling studies that the equilibrium assumption should be re-evaluated since catalysis is faster than release of either product.

Yeast phosphoglycerate kinase showed sequential kinetics and the mechanism has been postulated to be rapid equilibrium random from ATP product inhibition and AMP inhibition studies (4). However, the question of a ping-pong mechanism has been raised by the observation with rabbit muscle enzyme of ADP-ATP exchange and the isolation of a phosphoenzyme which phosphorylates ADP (5).

Escherichia coli acetate kinase is reported by Purich and Fromm (6) to have a ping-pong Bi Bi mechanism with acetyl phosphate and enzyme reacting to form a phosphoenzyme and MgADP reacting with the phosphoenzyme to form MgATP. This mechanism was postulated on the basis of initial velocity, product, and dead-end inhibitions in the thermodynamically favored direction (acetyl phosphate + MgADP). Further evidence for a ping-pong mechanism comes from the work of Spector (7) who showed that a phosphoenzyme intermediate does exist and will phosphorylate ADP and acetate under proper conditions. The enzyme catalyzes a fast ADP-ATP exchange, but a much slower acetate-acetyl phosphate exchange.

The present work was undertaken as part of a program to use the inert coordination complex chromium ATP (8) as a dead-end inhibitor to determine kinetic mechanisms. Initial velocity and product inhibition experiments have also been run to determine kinetic constants needed to analyze the dead-end inhibition data.

Pyruvate kinase, phosphoglycerate kinase, and hexokinase are enzymes commonly used in coupled assays to study other kinase reactions. Hexokinase has been shown to be strongly inhibited by CrATP (9), and thus can be used only when the kinase being studied is also strongly inhibited by CrATP (such as glycerokinase (10)). Pyruvate kinase and phosphoglycerate kinase were included in the present study partly to determine their usefulness in coupled assays in the presence of CrATP.

MATERIALS AND METHODS

Acetyl phosphate, DPNH, and TPN were from Sigma. Other nucleotides were from P-L Biochemicals. All enzymes were from Boehringer. Piperazine-N,N'-bis(2-ethanesulfonic acid) was added to water and dissolved by adjusting the pH to 7.0 with 5% KOH.

CrATP was prepared by heating 10 mM chromium perchlorate and 10 mM disodium ATP for 12 min at 80° (8) and cooling. The solution was placed on a column (1 × 10 cm) of Dowex 50-X2-H⁺ and eluted by extensive washing with water at 4° (time on column,
8 to 10 hours). The first of the two major bands eluted was used for these experiments after concentration by rotary evaporation at 35-40°C. (Cr:ATP = 1.0) The material was stored at 4°C at pH 3.5 for 6 months without appreciable loss in activity.

All velocities were measured using a Beckman DU monochromator with a deuterium lamp, a Gilford optical density converter, and a 10-nm recorder. Thermospacers were used to keep the cell compartment at 25°C, and a full scale sensitivity of 0.2 A was used with chart speeds varying from 0.6 to 6.0 inches per min. Reaction mixtures were made to 2.9 ml total volume in a 1.0-cm cuvette and equilibrated at 25°C in the cell compartment. Reaction was started by adding 0.1 ml of enzyme solution with an added mixer and the initial velocity determined from the slope of the recorded line. All measurements were made in 50 mM piperezine-N,N'-bis(2-ethanesulfonic acid) buffer, pH 7.0, in the presence of 3 mM free Mg2+; concentrations of Mg2+ and nucleotides were calculated by assuming 80% of the ADP present to be MgADP and all ATP to be MgATP. With acetate kinase magnesium acetate was replaced by magnesium chloride.

The pyruvate kinase assay contained 2 μg of pyruvate kinase, 10 μg of lactate dehydrogenase, 0.10 mM DPNH, and required substrates and inhibitors in a total volume of 3.0 ml. The phosphoglycerate kinase assay contained 2 μg of 3-phosphoglycerate kinase, 100 μg of glyceraldehyde-3-P dehydrogenase, 0.25 mM DPNH, and substrates and inhibitors as required in a total volume of 3.0 ml. The acetate kinase assay in the forward direction contained 0.25 mM DPNH, 0.7 mM P-enolpyruvate, 100 μg of pyruvate kinase, 50 μg of lactate dehydrogenase, 1.0 μg of acetate kinase, and substrates and inhibitors as required in a total volume of 3.0 ml. The acetate kinase assay in the reverse direction contained 2 mM glucose, 0.3 mM TPN, 100 μg of hexokinase, 50 μg of glucose-6-P dehydrogenase, 1.6 μg of acetate kinase, and substrates and inhibitors in a total volume of 3.0 ml.

Data Processing—Reciprocal velocities were plotted graphically against reciprocals of substrate concentrations, and when such plots were linear the data were fitted to Equation 1, assuming equal variance for the velocities and using the FORTRAN program of Cleland (11). Data for a sequential initial velocity pattern were fitted to Equation 2, for a ping-pong initial velocity pattern to Equation 3, for linear competitive inhibition to Equation 4, for noncompetitive inhibition to Equation 5, and for competitive substrate inhibition in a sequential pattern to Equation 6.

\[
v = \frac{VA}{K + A} \quad (1)
\]

\[
v = \frac{VA}{K_iA + K_B + K_B + K_B + AB} \quad (2)
\]

\[
v = \frac{VA}{K_B + K_B + AB} \quad (3)
\]

\[
v = \frac{VA}{K(1 + I/K_{iS}) + A} \quad (4)
\]

\[
v = \frac{VA}{K(1 + I/K_{iS}) + A(1 + I/K_{ii})} \quad (5)
\]

\[
v = \frac{VA}{K_I + I/K_{iI} + A} \quad (6)
\]

For the noncompetitive substrate inhibition of acetate kinase the data for each line were fitted to Equation 1. The lines on all graphs are from fits to the appropriate equations while data points are experimental values. Velocities in all figures are ΔA per min.

Pyruvate Kinase—The initial velocity pattern with phosphoenolpyruvate as the variable substrate is shown in Fig. 1. Mg-ADP causes competitive substrate inhibition; the data were fitted to Equation 6 with MgADP as B and the constants were: K_MgADP = 80 ± 10 μM, K_MgADP = 220 ± 10 μM, K_MgADP = 80 ± 30 μM, K_MgADP = 700 ± 200 μM, V = 180 units per mg, where PEP is P-enolpyruvate. Equation 6 assumes that the dissociation constants of B as an inhibitor at the combining sites for A on E and EB are equal. If this assumption is not valid, the separate dissociation constants for the inhibitions cannot be calculated from the data.

CrATP gave linear noncompetitive inhibition versus both MgADP and phosphoenolpyruvate as shown in Figs. 2 and 3, although the slope effects are more prominent in both cases. Varying both substrates in a constant fixed ratio gave parabolic reciprocal plots showing noncompetitive inhibition by CrATP (K_H = 1.5 mm), indicating that neither substrate at saturation can overcome the inhibition.

Veraet Phosphoglycerate Kinase—The initial velocity pattern is shown in Fig. 4 with 3-phosphoglycerate as the variable substrate. The data were fitted to Equation 2 and the constants were: K_MgATP = 140 ± 10 μM, K_MgATP = 700 ± 100 μM, K_PgA = 80 ± 10 μM, K_PgA = 370 ± 50 μM, V = 200 units per mg, where PGA is P-glycerate. CrATP gave linear competitive inhibition versus both MgATP and 3-phosphoglycerate as shown in Figs. 5 and 6 and the apparent K_M values from fits to Equation 4 were 200 ± 10 μM in both cases.

Escherichia coli Acetate Kinase—The initial velocity patterns are shown in Figs. 7 and 8 for the forward and reverse reactions. Data from these and similar experiments were fitted to Equation 2 and the results are shown in Table 1. The data for the reverse reaction were also fitted to Equation 3, but the fit was poorer than to Equation 2. Data for experiments with MgITP and MgGTP are also shown in Table 1 for comparison, although the precision of the MgGTP data is not sufficient to determine some of the constants. At high levels of MgADP, noncompetitive substrate inhibition is seen with an inhibition constant for MgADP of about 1 mM (Fig. 9).

An experiment to determine the ratio of maximum velocities was run by varying both substrates at the constant fixed ratios of MgATP:acetate = 0.002 and MgADP:acetyl phosphate = 2. The resulting parabolic reciprocal plots gave a V_max in the forward direction of 70 units per mg and in the reverse direction of 68 units per mg.

CrATP showed competitive inhibition versus MgATP and noncompetitive inhibition versus acetate (Figs. 10 and 11). Acetyl phosphate showed noncompetitive product inhibition versus both acetate and MgATP (Figs. 12 and 13). The inhibition constants from fits to Equations 4 and 5 are given in Table II.

Discussion

Pyruvate kinase and phosphoglycerate kinase show similar patterns of inhibition by CrATP. With pyruvate kinase CrATP is noncompetitive with both substrates with K_M values versus MgADP and phosphoenolpyruvate of 0.4 ± 0.1 and 0.38 ± 0.08 μM, and K_M values of 2.1 ± 0.8 and 1.5 ± 0.2 μM. The K_M for CrATP when both substrates were varied together is also 1.5 μM. The intercept effects must be due to combination of CrATP with the enzyme at some place outside the active site since saturation with both substrates does not eliminate the inhibition. With phosphoenolpyruvate as the variable substrate,
**Fig. 1** (left). Initial velocity pattern for pyruvate kinase with phosphoenolpyruvate as the variable substrate and showing competitive substrate inhibition by MgADP. Data fitted to Equation 6.

**Fig. 2** (center). Inhibition of pyruvate kinase by CrATP with MgADP as the variable substrate. Data fitted to Equation 5.

**Fig. 3** (right). Inhibition of pyruvate kinase by CrATP with phosphoenolpyruvate as the varied substrate. Data fitted to Equation 5.

**Fig. 4** (left). Initial velocity pattern for phosphoglycerate kinase with 3-phosphoglycerate as the varied substrate. Data fitted to Equation 2.

**Fig. 5** (center). Competitive inhibition of phosphoglycerate kinase by CrATP with MgATP as the varied substrate. Data fitted to Equation 4.

**Fig. 6** (right). Competitive inhibition of phosphoglycerate kinase by CrATP with 3-phosphoglycerate as the varied substrate. Data fitted to Equation 4.

**Fig. 7** (left). Initial velocity pattern for acetate kinase with MgADP as the varied substrate. Data fitted to Equation 2.

**Fig. 8** (center). Initial velocity pattern for acetate kinase with acetate as the varied substrate. Data fitted to Equation 2.

**Fig. 9** (right). Initial velocity pattern for acetate kinase showing noncompetitive substrate inhibition by MgADP. Data for each line fitted to Equation 1.

### Table I

**Kinetic constants for acetate kinase from initial velocity studies**

<table>
<thead>
<tr>
<th>Substrate A</th>
<th>Substrate B</th>
<th>$K_a$</th>
<th>$K_b$</th>
<th>$K_{ia}$</th>
<th>$K_{ib}$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgADP</td>
<td>Acetyl-P</td>
<td>360 ± 100</td>
<td>0.34 ± 0.04</td>
<td>50 ± 20</td>
<td>0.047 ± 0.020</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>MgATP</td>
<td>Acetate</td>
<td>20 ± 8</td>
<td>5.8 ± 2.0</td>
<td>350 ± 100</td>
<td>100 ± 50</td>
<td>70 ± 3</td>
</tr>
<tr>
<td>MgGTP</td>
<td>Acetate</td>
<td>60 ± 10</td>
<td>60 ± 10</td>
<td>30 ± 10</td>
<td>30 ± 10</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>MgADP</td>
<td>Acetate</td>
<td>70 ± 20</td>
<td>60 ± 10</td>
<td>30 ± 10</td>
<td>30 ± 10</td>
<td>102 ± 9</td>
</tr>
</tbody>
</table>

* The data were fitted to Equation 2.

* MgGTP data not precise enough to determine these constants.
Fig. 10. Competitive inhibition of acetate kinase by CrATP with MgATP as the varied substrate. Data fitted to Equation 4.

Fig. 11. Noncompetitive inhibition of acetate kinase by CrATP with acetate as the varied substrate. Data fitted to Equation 5.

Fig. 12. Product inhibition of acetate kinase by acetyl phosphate with acetate as the variable substrate. Data fitted to Equation 5.

Fig. 13. Product inhibition of acetate kinase by acetyl phosphate with MgATP as the varied substrate. Data fitted to Equation 5.

MgADP at 100 μM was equal to its dissociation constant and likewise when MgADP was varied, phosphoenolpyruvate at 33 μM was equal to its dissociation constant. The identical values of 400 μM for K_j in these experiments thus suggest that CrATP is competitive versus both substrates at the active site and has a dissociation constant of 200 μM. This is not surprising if the mechanism is random since CrATP should occupy part of the phosphoenolpyruvate site and all of the MgADP site and be displaced from the active site by either substrate. The competitive substrate inhibition by MgADP is consistent with a random mechanism where MgADP can combine at the phosphoenolpyruvate site as well as at its own site.

With phosphoglycerate kinase the same inhibition patterns are seen as for pyruvate kinase, but without the intercept effect. CrATP competes with both substrates with K_ij values of 200 μM. (Since MgATP and 3-phosphoglycerate were held at levels well below their dissociation constants in these experiments, the apparent K_ij values are the true dissociation constants of CrATP.) In experiments not shown, saturation with either substrate eliminates inhibition. These results suggest a random sequential mechanism for this enzyme.

The specific activity for the muscle phosphoglycerate kinase was reported to be 310 units per mg (5) while the ADP−ATP exchange rate was 0.18 units per mg. If the exchange in the yeast enzyme used in the present work has a similar rate, a ping-pong pathway cannot be the major reaction path. The mechanism is sequential but this does not prove that the phosphoglycerate is not part of the catalytic mechanism (see discussion below concerning acetate kinase).

Both pyruvate and phosphoglycerate kinases are useful in coupled assays for other kinases. Since CrATP is competitive with both substrates (ignoring the nonspecific intercept effect with K_i = 1.5 to 2 mm seen with pyruvate kinase), using a high level of phosphoenolpyruvate for pyruvate kinase and 3-phosphoglycerate for phosphoglycerate kinase should minimize CrATP inhibition of these enzymes and thus permit their use in the presence of high levels of CrATP.

The intersecting initial velocity pattern shown in Fig. 8 demonstrates that E. coli acetate kinase has a sequential kinetic mechanism in which both substrates must add to the enzyme before either product is released, in contrast to the ping-pong mechanism previously postulated on the basis of the nearly parallel pattern seen in the reverse direction (6). Product inhibition by acetyl-P is noncompetitive versus both acetate and MgATP, but as was the case with CrATP inhibition of pyruvate kinase, the K_ij values are much smaller than the k'ii values and are of the size expected from the dissociation constant (Table I) for a molecule occupying part of the adsorption sites of both acetate and MgATP. The intercept effect may represent inhibition by combination elsewhere than the active site. Since CrATP is competitive versus MgATP and noncompetitive versus acetate, it appears to occupy only the MgATP site. These patterns are most easily explained by a random sequential mechanism. The noncompetitive substrate inhibition by very high levels of MgATP (Fig. 9) is not explained by this model, and presumably results from nonspecific combination elsewhere on the enzyme.

If the mechanism is random, the dissociation constants of CrATP in the presence and absence of acetate can be calculated

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Variable substrate</th>
<th>Fixed substrate</th>
<th>Type of inhibition*</th>
<th>K_i</th>
<th>K_ij</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CrATP</td>
<td>MgATP</td>
<td>Acetate, 40 mm</td>
<td>C</td>
<td>60 ± 10</td>
<td>700 ± 100</td>
<td></td>
</tr>
<tr>
<td>CrATP</td>
<td>Acetate</td>
<td>MgATP, 100 μM</td>
<td>NC</td>
<td>60 ± 10</td>
<td>800 ± 300</td>
<td></td>
</tr>
<tr>
<td>Acetyl-P</td>
<td>MgATP</td>
<td>Acetate, 40 mm</td>
<td>NC</td>
<td>90 ± 10</td>
<td>630 ± 80</td>
<td></td>
</tr>
<tr>
<td>Acetyl-P</td>
<td>Acetate</td>
<td>MgATP, 200 μM</td>
<td>NC</td>
<td>90 ± 10</td>
<td>630 ± 80</td>
<td></td>
</tr>
</tbody>
</table>

* C, competitive, data fitted to Equation 4. NC, noncompetitive, data fitted to Equation 5.
from the apparent $K_{i}$ and $K_{\alpha}$ values for the noncompetitive inhibition of CrATP versus acetate (Table II). The apparent $K_{i}$ value is the dissociation constant in the presence of acetate multiplied by $(1 + A/K_{\alpha})$, where $A$ is MgATP, and $K_{\alpha}$ its Michaelis constant (12). Likewise $K_{\alpha}$ is the dissociation constant in the absence of acetate multiplied by $(1 + A/K_{\alpha})$, where $K_{\alpha}$ is the dissociation constant of MgATP in the absence of acetate. With the values of $K_{a}$ and $K_{\alpha}$ from Table I, the dissociation constants of CrATP in the presence and absence of acetate are thus $120 \pm 40 \mu M$ and $46 \pm 8 \mu M$. These two values can then be used to calculate the apparent $K_{i}$ expected for the competitive inhibition of CrATP versus MgATP by using the equations of Morrison and James (12). The resulting value of $66 \mu M$ agrees well with the observed $K_{i}$ of $90 \pm 10 \mu M$. In binding more tightly in the absence of acetate CrATP thus resembles MgADP, which binds 5-fold tighter in the absence of acetyl-P, more than it resembles MgATP, which binds 20-fold tighter in the presence of acetate.

The kinetic data thus require a sequential mechanism for the majority of the reaction flux while the rapid ADP-ATP exchange suggests that a ping-pong pathway is possible. These data suggest a mechanism such as that in Scheme 1.

The reaction $E \cdot MgATP + EP \rightarrow MgADP$ must be fast to account for the ADP-ATP exchange while the reaction $E \cdot acetyl-P \rightarrow EP \cdot acetate$ is very slow. Thus the enzyme form EP, while rapidly formed by reaction with MgATP, is not a major intermediate in the chemical reaction. However, the enzyme form $EP \cdot MgADP$ acetyl-P may be an obligatory intermediate between $E \cdot MgATP$-acetate and $E \cdot MgADP$-acetyl-P during the catalytic reaction, which would suggest that the presence of a nucleotide is necessary for rapid reaction of phosphorylated enzyme with acetate or free enzyme with acetyl-P. This mechanism accounts for the exchanges observed, the existence of a phosphoenzyme, and for the random sequential kinetic mechanism.

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The Inhibition of Acetate, Pyruvate, and 3-Phosphoglycerate Kinase by Chromium Adenosine Triphosphate
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