Kinetic Properties and Equilibrium Constant of the Adenosine Triphosphate-Creatine Transphosphorylase-catalyzed Reaction*

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As has been shown in a previous paper (1), the forward course of the enzymic reaction,

\[
\text{ATP} + \text{creatine} \rightleftharpoons \text{ADP} + \text{creatine-P} \tag{1}
\]
catalyzed by adenosine triphosphate-creatine transphosphorylase can be described by a reasonable kinetic scheme. It was shown that MgATP$^-$ is the "true substrate," that the Michaelis constant is the dissociation constant of the enzyme-substrate complex, and that the pH-activity curve resembles a single ionization curve with pH ~ 6.5.

It was of interest to study the kinetics of the reverse process, the formation of ATP and creatine. Using ADP and creatine phosphate as the substrates, we found the effects of Mg$^{++}$ and pH on the enzymic activity to be analogous to their effects on the forward process, and the equilibrium constant of Reaction 1, which has been previously determined at various magnesium ion concentrations (2), satisfied the Haldane equation (3), when magnesium nucleotide was considered as the "true substrate."

Furthermore, experiments with inosine and cytidine nucleotides as substrate and the effect of various anions as competitor with creatine phosphate gave some information about the active site of the enzyme.

EXPERIMENTAL PROCEDURE

Throughout this work, the enzyme solution and the reagents used for the experiments were prepared and stored as described previously (1). Creatine phosphate was prepared by the method reported by Ennor and Stocken (4). Analysis for acid-labile phosphate (5), inorganic phosphate, and creatine (6) showed that the product was 99.3% pure. Enzymatic assay of the forward reaction was performed as reported (1). The initial rate of the enzyme-catalyzed reaction in the direction of ATP and creatine formation was measured by determining creatine concentrations in four or five aliquots of reaction mixture pipetted into α-naphthol and diacetyl solution. Measurements were made on samples taken before 10% or less of the substrate had reacted. To maintain the desired pH, a buffer of histidine-acetate adjusted with NaOH was used.

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RESULTS

Enzymic Activity in Reverse Reaction—Studies of the enzymic activity measured in the reverse reaction revealed properties similar in many respects to the results reported for the forward reaction. Under the conditions in which the concentration of magnesium ion exceeds the concentration of ADP added, the relations between the reverse reaction velocities and the concentrations of substrates, ADP and creatine-P, were found to follow the Michaelis-Menten equation.

Fig. 1 shows the effect of pH at 30° on the $K_m$ values for ADP, at a fixed concentration of 5 mM creatine-P. The value of $K_m$ was determined as 0.18 mM, a constant independent of the pH values in the range of 5.4 to 7.4. The $K_m$ value for ADP in the presence of excess magnesium ion, at pH 6.4 was also independent of variations in concentration of creatine-P in the range from 1.0 to 5 mM (Fig. 2).

As for the relation of the concentration of creatine-P to the reaction rate, experiments at different pH values with fixed ADP concentration, or with various concentrations of accompanying substrate, [ADP], at fixed pH, e.g., pH 6.4, gave results as shown in Figs. 3 and 4. It was found that the $K_m$ value for creatine-P is a constant, 0.8 mM, independent of the pH values from 5.0 to 7.4, and also invariant to change of [ADP].

The foregoing experiments were carried out in the presence of excess magnesium ion, where magnesium ion was supplied as magnesium diacetate to avoid specific anion inhibition (see below). Under such circumstances, most of the added ADP molecules can be assumed to be the MgADP$^-$ complex.

Since MgATP$^-$ is the only effective substrate among the possible species of ATP for the forward reaction (1), we may assume for the moment (and later amply justify) that MgADP$^-$ is the true substrate for the reverse reaction. And if it is supposed that the equilibria among ATP, H$^+$, and Mg$^{++}$ are established very rapidly compared with any enzymic processes, then [MgADP$^-$] can be calculated as described in (1):

\[
[\text{ADP}^+] + [\text{HADP}^+] + [\text{MgADP}^-] = A_o
\]
\[
[\text{Mg}^{++}] + [\text{MgADP}^-] = M_o
\]
\[
\frac{[\text{HADP}^+]}{[\text{ADP}^+]} = [K_d][\text{H}^+] = K_d^*
\]
\[
\frac{[\text{MgADP}^-]}{[\text{Mg}^{++}][\text{ADP}^+]} = K_{M_d}
\]
FIG. 1. Lineweaver-Burk plots at various pH values. Creatine-P, 0.005 M; magnesium acetate, 0.015 M; histidine-acetate, 0.05 M (pH adjusted with NaOH); 30°; V is expressed in micromoles of creatine per μg per minute; pH values are indicated.

FIG. 2. Lineweaver-Burk plots at various fixed concentrations of creatine-P. Magnesium acetate, 0.015 M; histidine-acetate, pH 6.4, 0.05 M; numbers show [creatine-P] in mM and V is expressed in micromoles of creatine per μg per minute.

in which \( A_0 \) and \( M_0 \) are the total concentrations of ADP and Mg\(^{2+}\), respectively, and \( K_A \) and \( K_{M_0} \) are independently measured equilibrium constants. The reaction velocity is assumed to be related to [MgADP\(^{-}\)] by the Michaelis-Menten equation. V versus \( M_0 \) curves shown in Fig. 5 were calculated with the values of \( K_{M_0} = 2 \times 10^4 \) M\(^{-1}\), (7, 8), \( K_A = 10^{-6.1} \) M (9), and \( K_A = 1.18 \times 10^{-3} \) M at \( A_0 = 0.5 \times 10^{-2} \) M. The experimental values plotted in Fig. 5 are seen to be in good agreement with the calculated curves. At pH 6.8, however, there is good agreement (——) with the plotted experimental points if ADP\(^{-}\) be considered a competitive inhibitor (analogous to HATP\(^{-}\) for the forward reaction), but not (——) if the ADP\(^{-}\) be considered inert (i.e. neither substrate nor inhibitor). Analogous to the method used in the previous paper (1), the equation,

\[
\frac{1}{V_{\text{apparent}}} - \frac{1}{V_{\text{relate}}} = \frac{K_i}{[\text{ADP}^{-}]} (1 + 1/K_{M_0}) [\text{MgADP}^{-}]
\]

could be used to determine the value of \( K_i \), the ADP\(^{-}\) inhibi-

FIG. 3. Lineweaver-Burk plots at various pH values. ADP, 0.0002 M; magnesium acetate, 0.010 M; histidine-acetate (pH adjusted with NaOH), 0.05 mM; 30°; V is expressed in micromoles of creatine per μg per minute; pH values are indicated. CrP, creatine-P.

FIG. 4. Lineweaver-Burk plots at various fixed concentrations of ADP. Magnesium acetate, 0.015 M; histidine-acetate, pH 6.4, 0.05 M; numbers show [ADP] in mM and V is expressed in micromoles creatine per μg per minute. (Note: top curve should be labeled 0.12 and bottom curve 0.5.)
MgADP$^{-}$ and creatine-P, are each independent of the concentration of the other substrate, and are not influenced by pH change over the range from 5 to 8. Consequently, these Michaelis constants are considered as the true dissociation constants of the enzyme-substrate complex (see previous discussion (1)).

**Effect of pH on Reaction Velocity**—Since the binding of neither substrate is affected by $[H^+]$ in the range pH 5 to 8, the pH-activity curve will represent the effect of $[H^+]$ on the other step(s) subsequent to the binding of substrates to the enzyme. Fig. 7 shows the relative reaction velocities at various pH values, which were measured under given concentrations of ADP (0.5 × 10$^{-3}$ M), creatine-P (5 × 10$^{-3}$ M), and magnesium acetate (0.015 M) at 30° and 20°. From the figure, the apparent pK is seen to be about 6.6 at 30°, and heat of ionization, -1.9 Kcal per mole, both in agreement with those values of the forward process. The pH-activity curve of the forward process at 30° is also shown in the figure for comparison.

**Relation between Kinetic Constants and Over-all Equilibrium Constant**—Taking into consideration the mutual independence of $K_m$ for each substrate and the absence of pH effect on the $K_m$ values, the whole reaction sequence can be assumed to occur in the following manner (with $E$ representing enzyme):

$$[\text{MgATP}^+] + [E] = [\text{MgATP} \cdot E]$$  \hspace{1cm} (3)

$$[\text{creatine}] + [E] = [\text{creatine} \cdot E]$$  \hspace{1cm} (4)

$$[\text{MgATP} \cdot E] + [\text{creatine}] = [\text{MgATP} \cdot \text{creatine} \cdot E]$$  \hspace{1cm} (5)

$$[\text{creatine} \cdot E] + [\text{MgATP}^+] = [\text{MgATP} \cdot \text{creatine} \cdot E]$$  \hspace{1cm} (6)

$$[\text{MgATP} \cdot E] + [\text{creatine} \cdot P^+] = [\text{MgATP} \cdot \text{creatine} \cdot P \cdot E]$$  \hspace{1cm} (7)

$$[\text{creatine} \cdot P \cdot E] + [\text{MgADP}^{-}] = [\text{MgADP} \cdot \text{creatine} \cdot P \cdot E]$$  \hspace{1cm} (8)

$$[\text{MgADP}^{-}] + [E] = [\text{MgADP} \cdot E]$$  \hspace{1cm} (9)

$$[\text{creatine} \cdot P^+] + [E] = [\text{creatine} \cdot P \cdot E]$$  \hspace{1cm} (11)

These are the same type of equations which Botts and Morales (10) and more recently Hearon et al. (11) have treated theoretically.
In our case, Y is the stoichiometric number.

According to Horiiuchi's theory (13), the equilibrium constant, \( K \), is expressed as:

\[
K = \left( \frac{k}{k'} \right)^{\nu}
\]

in which \( k \) and \( k' \) are the rate constants for the forward and reverse direction, respectively, and \( \nu \) is the stoichiometric number.

The text further discusses the inhibition of creatine-P by various anions and the measurement of equilibrium constants for MgADP, MgATP, and creatine-P, with Table I and Table II providing data for these measurements.

Table I lists the comparisons between \( K_{\text{apparent}} \) values measured directly at high concentrations of MgATP and ADP and those calculated by Equation 13. The results show reasonable agreement, thus lending further support to the hypothesis that Reaction 7 is the rate-determining step.

Table II lists the inhibition constants of anions measured at 30°C in the presence of excess magnesium ion. The reaction rates were measured at pH 9 for the forward reaction and at pH 6.5 for the reverse reaction at 30°C. Results are shown in Table III.

According to Horiiuchi's theory (13), the equilibrium constant, \( K' \), will be

\[
K' = \frac{V_f}{V_r}
\]

in which \( V_f \) and \( V_r \) denote the maximal velocities obtained by extrapolating to infinite concentrations of both nucleotide- and creatine-substrate, in the forward and reverse process, respectively.

This suggests strongly that added anions interact with the same active group of the enzyme molecule, more specifically where the \( \gamma \)-phosphate of ATP and the phosphoryl group of creatine-P interact.

In Table II are listed the \( K_i \) values of various substances for the reverse reaction together with those determined for the forward reaction. For any one anion, the \( K_i \) values agree within a factor of four, although the measurements were made at different pH values.

Anions as competitive inhibitors of creatine-P-The previous paper (1) described the inhibitory effect of various inorganic anions such as SO\(_4\)\(^2-\), Cl\(^-\), PO\(_4\)\(^3-\) and structural analogues that compete with MgATP\(^2-\). As shown in Fig. 8, the anions are competitive inhibitors of creatine-P. Other experiments at fixed [creatine-P] showed the anions not to be competitive inhibitors of MgADP\(^-\). In Table II are listed the \( K_i \) values of various substances for the reverse reaction together with those determined for the forward reaction. For one anion, the \( K_i \) values agree within a factor of four, although the measurements were made at different pH values.

This suggests strongly that added anions interact with the same active group of the enzyme molecule, more specifically where the \( \gamma \)-phosphate of ATP and the phosphoryl group of creatine-P interact.

Fig. 8. Anions as competitive inhibitors of creatine-P. ADP, 0.0005 M; magnesium acetate, 0.015 M; histidine-acetate, pH 6.4; 0.05 M; A, control; B, 0.05 M NaCl; C, 0.05 M Na\(_2\)SO\(_4\).

The stoichiometric number is unity by definition of the reaction sequence.
TABLE III
Kinetic constants of different nucleotide-polyphosphates

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgATP</td>
<td>0.4</td>
<td>0.08</td>
</tr>
<tr>
<td>MgITP</td>
<td>130.0</td>
<td>0.1</td>
</tr>
<tr>
<td>MgCTP</td>
<td>9.5</td>
<td>0.03</td>
</tr>
<tr>
<td>MgADP</td>
<td>0.18</td>
<td>1.3</td>
</tr>
<tr>
<td>MgIDP</td>
<td>12.3</td>
<td>1.2</td>
</tr>
<tr>
<td>MgCDP</td>
<td>100.0</td>
<td></td>
</tr>
</tbody>
</table>

* Concentrations of the accompanying substrate, creatine or creatine-P, were 0.024 M and 0.005 M, respectively, so that $V_{max}$ represents the apparent maximal velocity measured under this limitation.

$K_m$ values for inosine polyphosphates are 100-fold larger than those of adenine compounds, although the maximal velocities are approximately the same. The value of $K_m$ for CDP is much greater than that of IDP, but $V_{max}$ is still of the same order. CTP showed no activity as a substrate of this enzyme in the forward reaction, even when its concentration was raised to 10 mM in the presence of 15 mM magnesium acetate.

Assuming the $K_m$ values represent the equilibrium constant of the binding reaction between enzyme and substrate, the affinities of nucleotides to this enzyme may be arranged in the order $MgADP^-> MgATP^-> MgIDP^-> MgCDP^-> MgCTP^-> MgITP^-> MgATP^-> MgADP^-> MgITP^-> MgCTP$. 

DISCUSSION

In a manner similar to that which established the forward reaction as a "Michaelis-Menten" type reaction, the reverse process has been shown to be the same type of reaction where the substrates are MgADP and creatine-P. The smaller value of the Michaelis constant for MgADP than for MgATP might presumably be caused by a difference in electrostatic attraction or repulsion.

Because the main role of magnesium ion seems to be to form the true substrate, the magnesium dependence of the apparent over-all equilibrium constant, reported by Noda et al. (2), follows from the present mathematical analysis; in other words, it is required that both of the nucleotides, ATP and ADP, be magnesium complexes in order to act as effective substrates. These effective substrates must have a definite configuration to bind with the enzyme and to transfer the phosphate group. The order of binding affinity to the enzyme of the species of adenosine polyphosphates is: MgADP$^->$ MgATP$^->$ MgIDP$^->$ MgCDP$^->$ MgCTP$^->$ MgITP$^-> MgATP$^-> MgADP$^-> MgITP$^-> MgCTP$.

It was reported by Cho et al. (14) that the hydrogen ion release occurs proportionally to the phosphate transfer from ATP to creatine. When the Mg-nucleotide complex is taken as the substrate, this enzymic reaction may be expressed as:

\[
\text{MgATP}^+ + \text{creatine} \rightleftharpoons \text{MgADP}^+ + \text{creatine-P}^+ + H^+
\]

in which the over-all equilibrium constant, $K$, is:

\[
K = \frac{[\text{MgADP}^+][\text{creatine-P}^+][H^+]}{[\text{MgATP}^+][\text{creatine}]} \quad (14)
\]

If the effect of hydrogen ion concentration is not on the enzyme itself, $K$ as defined above must be a constant at any pH. Thus the equilibrium constant, $K$, becomes $K_{apparent} \times [H^+]$ and Table I (last column) indicates that $K$ as a function of pH is approximately constant for experimentally determined values previously reported (2) as well as for calculated values. To calculate $K_{apparent}$ in Table I, the reaction,

\[
[\text{MgATP}^+]+[\text{creatine}] \rightleftharpoons [\text{MgADP}^+]+[\text{creatine-P}^+] \quad (15)
\]

(sum of Equations 3 to 11) was assumed. In this equation the electric charges of the reaction system are not balanced. Therefore, the ratio $V':V$ does not include the hydrogen ion concentration which must be involved in the true equilibrium constant, $K$.

In calculation of the dependence of $K_{apparent}$ on $[H^+]$ and on $[Mg^{2+}]$, it is interesting to employ the method used by Podolsky and Morales (15) for the ATP hydrolysis equilibrium. One assumes the following equilibria,

\[
\frac{[\text{MgATP}^+]}{[\text{Mg}^{2+}] [\text{ATP}^+]^a} = \alpha
\]

\[
\frac{[\text{MgADP}^+]}{[\text{Mg}^{2+}] [\text{ADP}^+]^b} = \beta
\]

Setting $\alpha' = \alpha[Mg^{2+}]$ and $\beta' = \beta[Mg^{2+}]$ then

\[
[\text{ATP}^+] = (1/\alpha')[\text{MgATP}^+]
\]

\[
[\text{ADP}^+] = (1/\beta')[\text{MgADP}^+]
\]

also

\[
[\text{HATP}^+] = (1/K_s^a)[\text{ATP}^+]
\]

\[
[\text{HADP}^+] = (1/K_s^a)[\text{ADP}^+]
\]

[creatine-P$^+\] = (1/$K_s^a$)[creatine-P$^+$]

in which

\[
K_s^a = K_s/[H^+]
\]

\[
K_s^a = K_s/[H^+]
\]

\[
K_s^a = K_s/[H^+]\]

and $K_s$, $K_s^a$, and $K_s^b$ and $K_s^a$ are the dissociation constants, respectively. Then, for total concentrations of reactants, one has:

\[
[\text{ATP}] = [\text{MgATP}^+][1 + 1/\alpha' + 1/(\alpha'K_s^a)]
\]

\[
[\text{ADP}] = [\text{MgADP}^+][1 + 1/\beta' + 1/(\beta'K_s^a)]
\]

\[
[\text{creatine P}^+] = [\text{creatine P}^+]\times[1 + 1/K_s^a]
\]

\[
[\text{creatine}] = [\text{creatine}]
\]

Therefore, the equilibrium constant as measured will be,

\[
K^* = \frac{[\text{ADP}] [\text{creatine-P}^+]}{[\text{ATP}] [\text{creatine}]}
\]

\[
= K_{apparent} \frac{[1 + 1/\beta' + 1/(\beta'K_s^a)][1 + 1/K_s^a]}{[1 + 1/\alpha' + 1/(\alpha'K_s^a)]} \quad (16)
\]

in which $K_{apparent} = [\text{MgADP}^+] [\text{creatine-P}^+] / [\text{MgATP}^+] [\text{creatine}]$ (Equation 13), i.e. the $K_{apparent}$ calculated with the kinetic constants. Table IV shows the values of $K^*$ at diff-
The binding of Mg$^{2+}$ to histidine near neutral pH values may be assumed to be almost the same as the binding of Mg$^{2+}$ to glycine (16). Monk (17) has reported the logarithm of the stability constant of magnesium-glycine complex as 2.06. Differences in the values of the affinity constants of Mg-nucleotide is not the reason for different $K_M$ values for various nucleotides since the concentration of Mg$^{2+}$ was high enough to bring [Mg$^{2+}$-nucleotide] to nearly the same value as the total nucleotide concentration. Consequently, it may be that the 6-amino group of the purine moiety has a direct effect on the binding reaction between the enzyme and nucleotide. Likewise, the pyrimidine nucleotide, even though 4-amino group is present, bears much less affinity to the enzyme than does the corresponding purine nucleotide.

**SUMMARY**

1. Effects of Mg$^{2+}$ and pH on the activity of adenosine triphosphate creatine transphosphorylase (creatine kinase) are of the same character in both the forward and reverse processes.

2. Inorganic anions such as Cl$^-$ and SO$_4^{2-}$ are inhibitors which compete with creatine phosphate in the back reaction. Inhibitor constants related to the creatine phosphate are of the same order of magnitude which were measured related to adenosine triphosphate.

3. The over-all equilibrium constant, $K$, is to be expressed as,

$$K = \frac{\bar{V} K_{MgADP} K_{Creatine-P}}{\bar{V} K_{MgATP} K_{Creatine}}$$

in which $\bar{V}$ and $\bar{V}$ are the maximal reaction velocities of forward and back reaction, respectively, and the $K$ values with subscripts are the Michaelis constants.

4. Inosine and cytidine polyphosphates may substitute for adenosine polyphosphate, with similar maximal velocity but are low in affinity to the enzyme.

**REFERENCES**

December 1961

T. Nihei, L. Noda, and M. F. Morales
