Size-dependent Allosteric Effects of Monovalent Cations on Rabbit Liver Fructose-1,6-bisphosphatase*

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Effects of monovalent cations on the neutral rabbit liver fructose-1,6-bisphosphatase are multifunctional and dependent on their nonhydrated ionic size. (a) The maximal velocity is increased by addition of monovalent cations with the optimum stimulation occurring with a nonhydrated ionic radius of 1.2 Å in the presence of a chelating agent such as EDTA. (b) Activation curves are sigmoidal with n values varying from 1.5 to 2.3 as ionic radius of monovalent cation increases. The apparent $K_a$ values from 16.0 to 180 mM, obtained for various monovalent cations, have a linear relationship to ionic radii of cations. (c) At lower concentrations of fructose 1,6-bisphosphatase monovalent cations show the inhibitory effect and the apparent $K_i$ for fructose 1,6-bisphosphate is increased as the concentration of monovalent cation is increased. A linear relationship is obtained between the slopes of increase in the $K_i$ and the reciprocals of ionic volume of monovalent cations. (d) The apparent $K_a$ for Mg$^{2+}$ is also increased as the concentration of monovalent cation is increased, and a linear relationship is obtained again between the increases in $K_a$ and the reciprocals of ionic volume of monovalent cations. The cooperative nature for Mg$^{2+}$ saturation is decreased as the $K_a$ increases. (e) The apparent $K_i$ for AMP is also linearly altered as the concentration of monovalent cation is varied. However, the alteration of the $K_i$ is unusual, that is, the smaller cations than K$^+$ increase the $K_i$ (Li$^+$ > Na$^+$ > NH$_4^+$), whereas the larger cations decrease the value ((CH$_3$CH$_2$OH)$_2$NH$^+$ > Ca$^+$ > Rh$^+$). The effect of K$^+$ is insignificant. Alterations in the $K_i$ are also linearly related to the reciprocals of ionic volume of monovalent cations. The cooperative nature for AMP inhibition is decreased or increased as the $K_i$ increased or decreased. (f) In the absence of the chelating agent, the curves for Mg$^{2+}$ saturation and AMP inhibition were hyperbolic without monovalent cations. By addition of monovalent cation the $K_a$ for Mg$^{2+}$ or $K_i$ for AMP is increased and cooperative nature for binding of both ligands are induced.

For nonspherical monovalent cations, the application of "functional ionic radius" is proposed. Functional ionic radii of NH$_4^+$, (CH$_3$OH)$_2$CNH$_3^+$, and (CH$_3$CH$_2$OH)$_2$N$^+$ are estimated to be 1.17, 2.55, and 2.87 Å, respectively.

The presence of two distinct sites for the actions of monovalent cations is suggested.

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Fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) catalyzes Reaction 1 in the presence of a divalent cation Mg$^{2+}$ (1) or Mn$^{2+}$ (2).

\[
\text{d-Fructose-1,6-P$_2$} + \text{H$_2$O} \rightarrow \text{d-fructose-6-P} + \text{P$_i$} \tag{1}
\]

Hubert et al. (3) reported that this enzyme is activated by monovalent cations such as K$^+$ or NH$_4^+$. A large number of other enzymes have also been reported to be activated by monovalent cations (4,6). Regarding the action of monovalent cations, Eisenman (7) has predicted that the key to the cation selectivity is the anionic field strength of the binding site, and Melchoir (8), Lowenstein (9), and Sueltzer (5) suggest that the monovalent cation acts as a bridge between the enzyme and the substrate. McClure et al. (10) observed that not only anionic field strength but also nonhydrated ionic size of the monovalent cation is an important factor in activation of rat liver pyruvate carboxylase. On the other hand, Evans and Sorger (4) proposed that monovalent cations maintain a protein conformation necessary for optimum catalytic efficiency.

Recently, fructose-1,6-bisphosphatase was shown to be readily modified during the purification procedure (11) or under certain conditions in vivo (12,13) by an endogenous protease which was identified with lysosomal cathepsin B (14,15). Modified fructose-1,6-bisphosphatase expresses the alkaline pH optimum, while the native enzyme shows the neutral pH optimum (neutral fructose-1,6-bisphosphatase) which has recently been purified from rabbit kidney (16), liver (17), and fish liver (18).

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The abbreviations used are: d-fructose-1,6-P$_2$, d-fructose-1,6-bisphosphate; d-fructose-6-P, d-fructose 6-phosphate; glucose-6-P, glucose-6-phosphate; M$^+$, monovalent cation.
To clarify the mode of action of monovalent cations on neutral rabbit liver fructose-1,6-bisphosphatase, the kinetic parameters of the enzyme in the presence of cations are investigated and the sequence specificity of monovalent cations is analyzed. Evidence is presented for size-dependent actions of cations on two distinct sites of enzyme.

**EXPERIMENTAL PROCEDURE**

**Materials**—Glucose-6-P isomerase and glucose-6-P dehydrogenase were obtained from Boehringer Mannheim and mixed. Before use, these enzyme preparations were dialyzed against a thousand volumes of 0.1 M triethanolamine-HCl, pH 7.4, for 18 hours at 4°C. Na-fructose-1,6-P\(_2\), NaNADP\(^+\), and NaAMP were purchased from Sigma. P-cellulose (P11) was obtained from Whatman and washed with 1 N NaOH and HCl, and then extensively with water. All other chemicals were reagent grade.

**Methods**—Neutral fructose-1,6-bisphosphatase was purified from fresh male rabbit liver according to the methods of Tashima et al. (16) and Traniello et al. (17) with the following modifications. The animal was killed by a blow on the head, and the liver was perfused with cold 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4, before removal, and was chilled in the same solution. The liver was chopped into small pieces and homogenized with 5 volumes (v/w) of the perfusion medium at 4°C in a Potter-Elvehjem type homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 12,000 x g for 20 min, and to each liter of the supernatant fraction 243 g of (NH\(_4\))\(_2\)SO\(_4\) was added and kept in (NH\(_4\))\(_2\)SO\(_4\) solution of 80% saturation. For experiments, enzyme suspension was diluted to the concentration of 1 mg fructose-1,6-P\(_2\) per ml with 10 mM triethanolamine-HCl buffer, pH 8.0, and was dialyzed against a thousand volumes of the same buffer for 18 hours at 4°C. Dialyzed enzyme preparation was diluted with 50 mM triethanolamine-HCl, pH 7.4, to desired concentrations before use.

Fructose-1,6-bisphosphatase activity was assayed spectrophotometrically by following the formation of NADPH at 340\(\text{nm}\) in the presence of excess glucose-6-P isomerase and glucose-6-P dehydrogenase. The standard assay system (1 ml) contained 0.1 mM fructose-1,6-P\(_2\), 2.5 mM MgCl\(_2\), 0.1 mM EDTA, 0.2 mM NADP\(^+\), 7 units of glucose-6-P isomerase, 5 units of glucose-6-P dehydrogenase, and 40 mM triethanolamine-HCl, pH 7.4. Glucose-6-P isomerase and glucose-6-P dehydrogenase were added before the addition of fructose-1,6-bisphosphatase to the cuvettes, in which other reagents including monovalent cation were added monovalent cations in this buffer.

**Statistical Estimation of Kinetic Parameters**—Data giving linear relationships were fitted to Equation 2, and the kinetic parameters were calculated using a least squares method as described by Wilkinson (18).

\[
y = a + bx
\]

To estimate the Michaelis constant (\(K_m\)) and maximal velocity (\(V\)), when the concentration of fructose-1,6-bisphosphatase is varied, Michaelis equation was fitted to Equation 2 (19).

To estimate Mg\(^{2+}\) concentration for 50% activation (\(K_c\)), Hill equation (20) was fitted to Equation 2. For estimation of the \(K_m\) for monovalent cation, \(\log (V - V_0)/(V - V)\) and \(\log ([M]^{-1})\) are written for \(y\) and \(x\), respectively, where \([M]^{-1}\) is monovalent cation. AMP concentration for 50% inhibition (\(K_c\)) was obtained using the modified Hill equation (20) for the calculation.

**RESULTS**

**Activation by Monovalent Cation**—Monovalent cation activation was observed only in the presence of concentrations of fructose-1,6-P\(_2\) higher than 0.025 mM (Fig. 1). With respect to Li\(^+\), activation was not observed at any concentration. The activity in the absence of alkali metal ions or NH\(_4^+\) corresponds to that in the presence of 40 mM triethanolamine alone. The enzyme showed a partial dependency on monovalent cation, and at lower concentrations of triethanolamine than 25 mM, enzyme activity became constant to approximately 40% of that observed in the presence of saturating NH\(_4^+\). Moreover, activation by 40 mM triethanolamine, which was used for the buffer solution in the studies of the effects of monovalent cations, was almost negligible (less than 3%). Thus it was practicable to analyze the kinetic parameters in the presence of added monovalent cations in this buffer.

Activation curves were found to be sigmoidal, and this cooperative nature was increased as ionic size of the monovalent cation increased. The estimated \(n\) values for Na\(^+\), NH\(_4^+\), K\(^+\), Rb\(^+\), Cs\(^+\), Tris, and triethanolamine were 1.5, 1.5, 1.6, 1.6, 1.7, 2.1, and 2.3, respectively.

Although activation was observed with potassium salt of any anion tested, the sulfate salt was most effective as was previously observed with kidney enzyme (3), whereas phosphate salt diminished the activating effect by about 40%. In the following experiments, sulfate salts of monovalent cations were used throughout.

These activating effects of monovalent cations were not evident in the absence of a chelating agent such as EDTA (Table I). Unless otherwise noted, 0.1 mM EDTA was added in the assay system throughout the experiments.

**FIG. 1.** Activation of rabbit liver fructose-1,6-bisphosphatase by monovalent cations obtained from the concentrations for 50% activation in Fig. 1 were 16.0, 33.6, 48.3, 58.2, 75.6, 152, and 180 mM for Na\(^+\), NH\(_4^+\), K\(^+\), Rb\(^+\), Cs\(^+\), (CH\(_3\)OH)\(_2\)CNH\(_2^+\), and (CH\(_3\)CH\(_2\)OH)\(_2\)N\(^+\), respectively. A linear relationship was observed between the \(K_v\) values for Na\(^+\), K\(^+\), Rb\(^+\), and Cs\(^+\) and nonhydrated ionic radii of these cations (Fig. 2A).

When the reported nonhydrated ionic radius of 1.43 Å (21) or 1.48 Å (22) is employed for NH\(_4^+\), the position in the figure deviates from the line, probably because of the nonspherical

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**Fig. 1.** Activation of rabbit liver fructose-1,6-bisphosphatase by monovalent cations.
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**TABLE I**

| Concentration (mm) | (CH$_2$CH$_2$OH)$_2$N$^+$ | K$^+$ | Na$^+$ |
|-------------------|---------------------------|------|-------|
|                   | -EDTA                     | +EDTA | -EDTA | +EDTA |
| 15                | 0.14                      | 0.80  | 0.14  | 0.96  |
| 40                | 0.14                      | 0.82  | 0.14  | 1.37  |
| 150               | 0.14                      | 1.13  | 0.12  | 1.72  |

a Contained 40 mM triethanolamine.

b At the concentration of 0.1 mm.

**Figure 2.** Ionic radius dependency of monovalent cation activation. A, apparent activation constants ($K_a$) estimated statistically from the data of Fig. 1 as described under "Experimental Procedure" were plotted against nonhydrated ionic radius (21) of monovalent cations. B, the maximal velocities in the presence of monovalent cations were obtained from double reciprocal plots of monovalent cation concentration versus ($v - v_0$) at high cation concentrations, where $v_0$ is the velocity without added monovalent cation, and was expressed as percent of $v_a$, ionic radii of NH$_4^+$, Tris, and triethanolamine (TEA) were obtained from A.

form of the ion. To satisfy the linear relationship in this biological system, "functional radius" was postulated for NH$_4^+$, being 1.17 Å from the line in Fig. 2A. This value was found to be appropriate in cases of alteration in the $K_a$ for fructose-1,6-P$_2$, $K_a$ for Mg$^{2+}$, and $K_c$ for AMP (see below). Functional nonhydrated ionic radii of Tris and triethanolamine were also estimated to be 2.55 and 2.87 Å, respectively, from their $K_a$ values.

Maximal Activation and Ionic Radius—When the maximal velocities in the presence of various monovalent cations were plotted against ionic radii of cations, optimal ionic radius for the maximal activation was observed at about 1.2 Å (Fig. 2B).

Fructose-1,6-P$_2$ Saturation and Monovalent Cation—The saturation kinetics of the enzyme for fructose-1,6-P$_2$ was affected by the presence of a monovalent cation, and at lower concentrations of fructose-1,6-P$_2$, the monovalent cation showed an inhibitory effect (Fig. 3). The Michaelis constant for fructose-1,6-P$_2$, which was estimated from the data at concentrations lower than 0.02 mm, was linearly increased as the concentration of monovalent cation increased (Fig. 4). The $K_m$ in the absence of alkali metal ions or NH$_4^+$ corresponds to the value of 6.1 × 10$^{-4}$ M for 40 mM triethanolamine and 40 mM diethanolamine (10). From Fig. 4, the $K_m$ of neutral rabbit liver enzyme for fructose-1,6-P$_2$, which would be obtained in the absence of monovalent cations, was estimated to be 4.0 × 10$^{-4}$ M.

$K_m$ and Ionic Volume—Increase in the $K_m$ was linearly correlated with the reciprocal of ionic volume of monovalent cation (Fig. 4, inset). Ionic volumes obtained for NH$_4^+$ and (CH$_2$CH$_2$OH)$_2$N$^+$ from the data of Fig. 2A are also in good correlation to the increases in the $K_m$ value.

These effects of monovalent cations on the $K_m$ were also observed in the absence of a chelating agent.

Mg$^{2+}$ Saturation and Monovalent Cation—Monovalent cations altered the affinity of neutral rabbit liver fructose-1,6-bisphosphatase for the cofactor divalent cation, Mg$^{2+}$, increasing its apparent $K_a$ value obtained as the concentration for 50% activation. Fig. 5 shows the effect of Na$^+$ on Mg$^{2+}$ saturation curves in the presence of 0.1 mM EDTA. The curves were sigmoidal and the increased $K_a$ values were obtained by addition of Na$^+$ as previously reported for 150 mM K$^+$ (3).

Similar effects were observed with other monovalent cations, and the $K_a$ for Mg$^{2+}$ linearly increased as the concentration of each monovalent cation increased (Fig. 6). The $K_a$ in 40 mM triethanolamine buffer alone was 0.20 mM, and the basic $K_a$ for Mg$^{2+}$, which would be obtained in the absence of any monovalent cation, was estimated to be 0.16 mM.

The sigmoidal nature of Mg$^{2+}$ saturation curves for other fructose-1,6-bisphosphatases had been reported previously (3). Neutral rabbit liver enzyme also showed the cooperative nature in Mg$^{2+}$ saturation with the n value of 1.95 in 40 mM triethanolamine, and this value was decreased in the presence of Li$^+$ or Na$^+$. With 30 mM Li$^+$ and 300 mM Na$^+$, the n values of 0.33 and 1.12 were obtained, respectively. On addition of larger monovalent cations, decrease in the value was very slight.

$K_a$ for Mg$^{2+}$ and Ionic Volume—The increase in the $K_a$ for Mg$^{2+}$ was also found to be related to ionic volume of monovalent cation. A linear relationship was observed between the rates of increase in the $K_a$ and the reciprocals of ionic volume of monovalent cations (Fig. 7).

Effect of EDTA—The $K_a$ and n values for Mg$^{2+}$ saturation were influenced by removal of the chelator EDTA. In 40 mM triethanolamine buffer containing no EDTA, the Mg$^{2+}$ saturation curve was found to be almost hyperbolic showing the n value and $K_a$ of 1.02 and 0.42 mM, respectively (Fig. 8). High concentrations of the monovalent cation, however, induced the sigmoidal nature of the Mg$^{2+}$ saturation curve in the absence of EDTA, and with 150 mM K$^+$ the n and $K_a$ values were increased to 1.25 and 2.1 mM, respectively. Thus in the absence of EDTA, increase in the $K_a$ for Mg$^{2+}$ by addition of monovalent cation was more marked, whereas increase in the maximal velocity was very slight.

AMP Inhibition and Monovalent Cation—Effect of monovalent cation on the affinity of rabbit liver fructose-1,6-bisphos-
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Fig. 4 (right). Effect of monovalent cations on the Michaelis constant for fructose-1,6-P. The apparent \( K_m \) values were estimated using a least squares method as described under “Experimental Procedure,” and were plotted as a function of monovalent cation concentration. The slopes and intercepts of the figure were also obtained with a least squares method, writing the \( K_m \) value and (the cation concentration + 0.1) for \( y \) and \( x \), respectively, in Equation 2. The slopes were replotted against the reciprocals of ionic volume of monovalent cations in the inset. Ionic radii of \( \text{NH}_4^+ \) and \( (\text{CH}_3\text{CH}_2\text{OH})_3\text{N}^+ \) were obtained from their \( K_m \) values as described in the text.

The apparent \( K_m \) values for AMP were interesting. Larger cations than \( K^+ \) increased the affinity, whereas smaller cations decreased it (Fig. 9). The \( n \) for AMP inhibition of 1.76 was also altered slightly in the presence of high concentrations of monovalent cations. Larger cations than \( K^+ \) increased the value and smaller ones decreased it.

The apparent \( K_i \) value for AMP was also linearly altered as the concentration of monovalent cations increased (Fig. 10). \( K^+ \) exhibited almost no effect on the \( K_i \) value at any concentration. This may be the reason why Hubert et al. (3) could not observe any significant effect of the monovalent cation on the \( K_i \) of rabbit liver enzyme testing \( K^+ \). The \( K_i \) in the absence of alkali metal ions of 1.16 \( \times 10^{-5} \) M corresponds to the value with 40 mM triethanolamine alone. The \( K_i \) value in the absence of monovalent cations was estimated to be 1.32 \( \times 10^{-5} \) M.

These effects of monovalent cations on AMP inhibition were observed in the absence of the chelator EDTA, too. AMP inhibition curve in the absence of EDTA was almost hyperbolic, and by the addition of 150 mM Na\(^+\) the \( K_i \) and \( n \) values were altered from 1.66 \( \times 10^{-5} \) M and 1.1 to 4.37 \( \times 10^{-4} \) M and 1.4, respectively. That is, the monovalent cation in the absence of a chelator induces the cooperativity for AMP inhibition, as well as for Mg\(^{2+}\) saturation.

\( K_i \) and Ionic Volume—When the slopes of alteration in the \( K_i \) were plotted against the reciprocals of ionic volume of monovalent cations, a linear relationship was obtained again as shown in Fig. 11. Critical ionic volume, which would cause no alteration in the \( K_i \), was estimated to be 9.80 Å\(^3\) corresponding to ionic radius of 1.33 Å.

DISCUSSION

The kinetic constants for all ligands, namely, the \( K_m \) for fructose-1,6-P\(_2\), \( K_a \) for Mg\(^{2+}\), and \( K_i \) for AMP, were increased or altered as the concentration of monovalent cations increased. As has been discussed previously by McGregor et al. (23), a general conformational change in enzyme protein may result in the alteration of the constants for all substances. One of the effects of a monovalent cation on rabbit liver fructose-1,6-bisphosphatase may be a general conformational change in enzyme protein. Linear relations are observed between the rates of alteration in these constants and the reciprocals of ionic volumes of monovalent cations (Figs. 4, 7, and 11). A possible explanation of the mechanism for these phenomena would be that the extent of conformational change is dependent on the amount of ions which could get into a specific "pocket," distinct from the catalytic site or AMP binding site. The alteration in the \( K_i \) for AMP is, however, different from that for the \( K_m \) for fructose-1,6-P\(_2\), or the \( K_a \) for Mg\(^{2+}\); that is, \( K^+ \) shows the neutral effect and smaller cations than \( K^+ \) increase the constant whereas larger ones decrease the value. Probably a general conformational change may give different effects on different loci of enzyme protein.
The presence of a chelator such as EDTA (Table I) whereas the monovalent cation with the optimum radius of 1.2 Å. It is of pation in enzymatic catalysis at the catalytic site. The increase of AMP inhibition in the presence of a chelating agent EDTA are decreased or increased as the $K_1$ and $K_2$ increased or decreased, respectively, by addition of monovalent cations. In the absence of the chelator, however, Mg$^{2+}$ activation and AMP inhibition are noncooperative and the monovalent cation induces the cooperativity for both cases. All these observations indicate that the monovalent cation acts as a potent allosteric effector on rabbit liver fructose-1,6-bisphosphatase activating system, it may be possible to obtain the "functional" radii of various monovalent cations.

Another role of the monovalent cation in the regulation of fructose-1,6-bisphosphatase activity may be the direct participation in enzymatic catalysis at the catalytic site. The increase in the maximal velocity is dependent on ionic radius of the monovalent cation with the optimum radius of 1.2 Å. It is of interest that this activation by a monovalent cation requires the presence of a chelator such as EDTA (Table I) whereas the conformational effect of the cation mentioned above can occur even in the absence of the chelator. These results suggest that in the presence of a chelating agent the monovalent cation is also participating in the direct catalysis as a bridge between ligands, or between enzyme and a ligand as has been suggested in other enzyme reactions (8–10, 23–25). The activating effects of chelating agents such as EDTA, histidine, citrate, malonate, pyridoxal 5' phosphate, cysteine, β mercaptoethanol, or oleate on fructose-1,6-bisphosphatase have been extensively investigated and discussed. It is now established that activation of fructose-1,6-bisphosphatase by a chelator is not due to the removal of trace heavy metal contaminants (26–31). The activation mechanisms suggested so far are: (a) the direct involvement in catalysis probably in such a chelate form as Mg$^{2+}$EDTA (28–32) which would be acting on a different form of enzyme from that for free Mg$^{2+}$ (29–31), (b) prevention or removal of ATP inactivation (33, 34), and (c) of GSSG inactivation (35). We now present evidence for additional actions of the chelating agent, that is, (d) participation in monovalent cation activation and (e) induction of cooperativities in Mg$^{2+}$ saturation and AMP inhibition in the absence of monovalent cations.

In the activation reaction of rabbit liver fructose-1,6-bisphosphatase, saturation curves for monovalent cations are sigmoidal showing that the binding of a monovalent cation to the catalytic site is also cooperative (Fig. 1), while hyperbolic saturations are generally observed in other enzymes (6, 23, 36). The extent of cooperativity and the apparent activation constant possess the tight relationships to ionic radius of the monovalent cation. When the previously reported ionic radius is employed, NH$_4^+$ deviates from the linear relationship. Non-metal cations such as NH$_4^+$, Tris, or (CH$_3$OH)$_3$N$^+$ are not completely spherical, so that their effective ionic radii or ionic volumes in the biological system may differ from the physically reported values. Using fructose-1,6-bisphosphatase activating system, it may be possible to obtain the "functional" radii of various monovalent cations.

Thus the presence of two distinct sites for the actions of the monovalent cation is suggested. One is the catalytic site in which the monovalent cation is promoting the enzymatic catalysis. Another is the allosteric site which is involved in the alteration of kinetic constants for the substrate and effectors indicating a general conformational change. The action of the monovalent cation on the former site can be distinguished from that on the latter by (a) the requirement for a chelating agent, (b) the cooperative nature for its binding, and (c) the correlation to ionic radius of the monovalent cation whereas ionic volume is an important factor for the latter.

Similarly, two distinct effects of monovalent cation are recently observed in the studies on rat liver pyruvate carboxylase. A monovalent cation is required for its activity (10), while...
FIG. 9 (left). AMP inhibition of fructose-1,6-bisphosphatase in the presence of alkali metal ions. The enzyme activities were determined at varying concentrations of AMP with the fixed concentrations of monovalent cations, and expressed as per cent of the values in the absence of AMP (A). The concentration of monovalent cation was 150 mM for Na+, K+, Rb+, and Cs+ or 2.5 mM for Li+. Each point represents the mean value of duplicated determinations. Hill plots of the data are shown in B. The lines were drawn using the n and Ki values calculated as described under "Experimental Procedure." V is and v are the uninhibited and inhibited rates, respectively.

FIG. 10 (right). Effect of monovalent cation on the Ki for AMP. The apparent Ki values, obtained as described under "Experimental Procedure," were plotted against the concentration of monovalent cations. The lines were drawn using the slopes and intercepts estimated as described in the legend for Fig. 4. The data for Li+ are in the inset.

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