Kinetic Studies of the Activation of Adenosine Triphosphate-Lombricine Phosphotransferase by Magnesium Ions

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Some general properties of the enzyme, ATP-lombricine phosphotransferase (lombricine kinase), which catalyses the reaction:

were described by Gaffney, Rosenberg & Ennor (1964). In common with other ATP-guanidino phosphotransferases (Ennor & Morrison, 1958) this enzyme is activated by Mg^{2+} , Mn^{2+} , Ca^{2+} and Co^{2+} ions. The role of the activating metal ion in enzymic reactions concerned with the transfer of the terminal phosphoryl group of ATP to an acceptor molecule has been difficult to elucidate. Evidence has been presented that for ATP-creatine phosphotransferase (EC 2.7.3.2) the function of the metal ion is to combine with the nucleotide (Kuby, Noda & Lardy, 1954). On the other hand Griffiths, Morrison & Ennor (1957) have suggested that the activity of ATP-arginine phosphotransferase (EC 2.7.3.3) depends on the formation of an active enzyme-metal ion complex. More recent studies on ATP-creatine phosphotransferase indicate that it is unlikely that the mechanism is simple and that it is necessary to consider the possible interaction of all of the species, free metal ion, free nucleotide and metal ion-nucleotide complex, with the enzyme (Kuby & Noltmann, 1962).

In the present paper, some observations on the activation of lombricine kinase by Mg^{2+} ions are presented. The approach used was that outlined by Morrison, O'Sullivan & Ogston (1961) whereby all possible pathways leading to the formation of an active enzyme-metal ion-substrate complex were considered. The results are consistent with the reaction of both free nucleotide and metal ion-nucleotide complex with the enzyme and also with a possible, but much weaker, interaction between Mg^{2+} ion and the enzyme.

MATERIALS AND METHODS

Materials. Buffers, MgCl_2 , substrates and enzyme were as described by Gaffney *et al.* (1964).

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Removal of bivalent cations from reaction components. Chelex 100 (Na^+ form; 200-400 mesh) was obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Before use, the resin was made into a column and treated successively with N-HCl and N-NaOH , being washed with water after each step. The final washing was continued until the pH of the effluent had fallen to pH 7.5. The resin was stored wet. All components of the reaction systems (water, ADP, ATP, lombricine, phospholombricine, buffers and the glycerol used to stabilize the lombricine-kinase preparation) were treated with chelating resin as follows. To each of the solutions (about 50 ml.) chelating resin (approx. 1.0 g. dry wt.) was added and the mixture stirred for 4 hr. at 4° . After filtration, the pH of the solution was checked and readjusted, if necessary, with 0.1 N-HCl or 0.1 N-KOH . The concentration of the solution was adjusted with metal ion-free water to the final required value.

Determination of the initial rate of enzymic reaction. The methods for determining the initial rate, both in the forward and reverse directions, were as described by Gaffney *et al.* (1964). The reaction was carried out in a total volume of 1.0 ml., but, to increase the sensitivity of the assays, the final volume in which the released lombricine or phospholombricine was measured was reduced from 10 ml. to 3 ml. and the volumes of the reagents were scaled down accordingly, essentially as described by Morrison *et al.* (1961) for the reverse reaction catalysed by ATP-creatine phosphotransferase. Under these conditions, 0.01 μmole of lombricine and 0.1 μmole of orthophosphate gave extinctions of 0.045 at 535 μm and 0.1 at 750 μm respectively, when measured in cells of 1 cm. light-path.

Calculation of K and V . Values for K and V were obtained by the weighted-regression procedure of Wilkinson (1961).

THEORY

The theoretical basis of the kinetic experiments has been elaborated by Morrison *et al.* (1961) and only some of the details are reproduced. The reaction was assumed to occur via the formation of an enzyme- Mg^{2+} ion-ADP-phospholombricine (or enzyme- Mg^{2+} ion-ATP-lombricine) complex, which can be formed by three sets of binary reactions, all of which are in rapid equilibrium. The activation by Mg^{2+} ions was studied in relation to ADP^{3-} or ATP^{4-} while the phospholombricine or lombricine concentration was kept constant. It was supposed that the reaction velocity was determined by the concentration of the enzyme-metal ion-

substrate complex and that the concentration of phospholombricine or lombricine altered the velocity only by a constant factor. The following binary reactions were assumed to be involved in the formation of an active enzyme-metal ion-substrate complex, as shown in Scheme 1.

Scheme 1. E, Enzyme; M, metal ion; S, substrate.

Under equilibrium conditions, the concentration of the enzyme-metal ion-substrate complex will be independent of the pathway by which it is formed, so that:

$$K_1 K_2 = K_3 K_4 = K_5 K_6 \quad (1)$$

The velocity equation for the general case may be written

$$v = \frac{ke}{\frac{K_1 K_2}{\sigma \mu} + \frac{K_2}{\mu} + \frac{K_4}{\sigma} + 1} \quad (2)$$

where k is the velocity constant for the breakdown of the enzyme-metal ion-substrate complex, e is the total enzyme concentration, σ is the concentration of free nucleotide (ADP^{3-} or ATP^{4-}) and μ is the concentration of free metal ions (Mg^{2+}).

Eqn. (2) may be rearranged in reciprocal form, so that, if the above assumptions are valid, when $1/v$ is plotted against $1/\sigma$ at various fixed values of μ (μ'), a series of straight lines should be obtained from which values may be calculated for $K_{\mu'}$ and $V_{\mu'}$, as defined previously (cf. Morrison *et al.* 1961). Further, plots of $1/V_{\mu'}$ against $1/\mu'$ should yield a straight line with an intercept on the abscissa equal to $-1/K_2$, and a plot of the function $K_{\mu'}[(K_2/\mu') + 1]$ against $1/\mu'$ should also give a straight line with intercepts K_4 and $-K_4/K_1 K_2$ on the ordinate and abscissa respectively. By use of eqn. (1), values may then be assigned to K_1 and K_3 and, as K_5 is determined independently, to K_6 .

The total nucleotide and total metal ion concentrations required to maintain μ at a fixed value (μ'), while σ is varied, are determined from:

$$[\text{Total nucleotide}] = \frac{K_5[\text{total metal ion}]}{\mu} - K_5 + [\text{total metal ion}] - \mu$$

Thus it is necessary to know the value of K_5 , the inverse of the stability constant of MgADP^- or MgATP^{2-} . In the present experiments the stability constant of MgADP^- was taken as 2000 M^{-1} ($K_5 = 0.5 \text{ mM}$) at pH 7.0 in 0.1 M-*N*-ethylmorpholine-HCl buffer at 30° , and that of MgATP^{2-} as 70000 M^{-1} ($K_5 = 0.014 \text{ mM}$) at pH 8.6 in 0.1 M-triethanolamine-HCl buffer at 30° . These particular values were adopted since they were obtained under conditions closely approximating those used in the present kinetic experiments (Morrison *et al.* 1961; O'Sullivan & Perrin, 1961). No allowance was made for the interaction of the HADP^{2-} present at pH 7.0 with Mg^{2+} ion to form MgHADP . The stability constant of the latter complex may be considered to be negligible (Bock, 1960). Also, HADP^{2-} was regarded as being inert in the reaction. The latter assumption would appear to be justified by the finding that linear plots were obtained (cf. Fig. 1).

RESULTS

Determination of residual enzymic activity. Preliminary experiments on the reverse reaction showed that the residual activity, i.e. the enzymic activity that occurs in the absence of added metal ions, was 30% of the maximal activity obtained with 0.24 mM- Mg^{2+} ion, when none of the reaction components had been treated with chelating resin. This residual activity was eliminated by 1 mM-EDTA. Treatment of lombricine kinase with chelating resin for 4 hr. at pH 7.0 did not cause any appreciable alteration in the residual activity. However, treatment of the other reaction components with chelating resin decreased the residual activity to less than 2% of the maximal velocity obtained with 0.4 mM- Mg^{2+} ion, 0.7 mM-ADP and 10 mM-phospholombricine. No significant residual activity was observed when the reaction was studied in the forward direction.

Kinetics of the reverse reaction. The activation of lombricine kinase by Mg^{2+} ions in relation to ADP^{3-} at pH 7.0 is shown in Fig. 1 as plots of $1/v$ against $1/\sigma$, with the concentrations of free Mg^{2+} ions varied from 0.08 to 0.4 mM. A linear relationship was obtained with each plot, indicating that v and σ can be related by the Michaelis-Menten equation. The lines shown in Fig. 1 were drawn from calculated values of $K_{\mu'}$ and $V_{\mu'}$, and the individual points shown were experimental findings. The calculated values of $K_{\mu'}$ and $V_{\mu'}$ are collected in Table 1.

A plot of $1/V_{\mu'}$ against $1/\mu'$ (Fig. 2) gave a straight line from which K_2 was calculated to be 0.24 mM. This plot also gave a value of 0.28 μmole of lombricine/ $\mu\text{g.}$ of lombricine kinase/min. (28000 moles/ 10^5 g. of enzyme/min.) for the maximum

Fig. 1. Double-reciprocal plots of the initial velocities in the reverse reaction, at various concentrations of free Mg^{2+} ions (μ'), against concentrations of free ADP^{3-} (σ). The reaction mixtures contained 0.1 M *N*-ethylmorpholine-HCl buffer, pH 7.0, 10 mM-phospholombricine and 2 μg . of lombricine kinase, as well as the indicated amounts of ADP^{3-} and Mg^{2+} ions. The total volume was 1.0 ml.; the temperature was 30°. Velocity is expressed as the change in extinction at 535 m μ /min. Concn. of Mg^{2+} ions: ■, 0.4 mM; △, 0.2 mM; ●, 0.133 mM; ○, 0.1 mM; ▲, 0.08 mM.

Table 1. Summary of $K_{\mu'}$ and $V_{\mu'}$ values for the reverse reaction catalysed by lombricine kinase

Values were calculated from the plots of $1/v$ against $1/\sigma$ at various fixed values of μ' (the concentration of free Mg^{2+} ions) at pH 7.0 (Fig. 1). All $K_{\mu'}$ values are expressed in terms of mM, and those for $V_{\mu'}$ are given in terms of μmoles of lombricine released/ μg . of lombricine kinase/min.

μ' (mM)	$V_{\mu'}$	$K_{\mu'}$
0.4	0.167	0.179
0.2	0.137	0.322
0.133	0.101	0.347
0.1	0.068	0.341
0.08	0.066	0.402
(0.066)*	(0.055)*	(0.326)*

* Plot not shown in Fig. 1.

Table 2. Summary of the dissociation constants for the various reactions leading to the formation of the active enzyme-metal ion-substrate complex

All values are expressed in terms of mM. The values in parentheses have been corrected for the presence of HADP^{2-} at pH 7.0, the $\text{p}K_a$ for the terminal phosphate group of ADP being assumed to be at pH 6.65.

	Reverse reaction	Forward reaction
K_1	0.42 (0.29)	0.13
K_2	0.24	0.1
K_3	6.7	0.45
K_4	0.018 (0.013)	0.03
K_6	0.2	0.9
K_5	0.5	0.014

velocity at pH 7.0, with the enzyme saturated with ADP^{3-} and Mg^{2+} ions and with phospholombricine at a concentration of 10 mM. A plot of the calculated values of $K_{\mu'}[(K_2/\mu') + 1]$ against $1/\mu'$ (Fig. 3) again gave a straight line, with intercepts K_4 (0.018 mM) and K_4/K_1K_2 (150 M^{-1}) on the ordinate and abscissa respectively. Then, as K_5 had been determined independently, values for all the constants could be calculated from eqn. (1) (Table 2).

Kinetics of the forward reaction. The general kinetic approach used for the reverse reaction was also applied to the forward reaction. However, the practical difficulties inherent in the assay procedure, i.e. in the determination of the amount of phospholombricine formed, reduced the sensitivity of the method and severely restricted the range over which accurate results could be obtained.

Plots of $1/v$ against $1/[\text{ATP}^{4-}]$ (where $[\text{ATP}^{4-}]$ is σ) at pH 8.6 at various concentrations of Mg^{2+} ion (μ') ranging from 0.05 to 0.1 mM are shown in Fig. 4.

Fig. 2. Double-reciprocal plot of the maximum velocities ($V_{\mu'}$) obtained in the reverse reaction at various fixed concentrations of free Mg^{2+} ions (μ') against the concentration of free Mg^{2+} ions. The calculated maximum velocities, obtained from the plots shown in Fig. 1 and reported in Table 1, are expressed as μmoles of lombricine released/ μg . of lombricine kinase/min.

Fig. 3. Plot of the calculated values for $K_{\mu'}[(K_2/\mu') + 1]$ against the reciprocals of the concentrations of Mg^{2+} ions in the reverse reaction.

Fig. 4. Double-reciprocal plots of the initial velocities in the forward reaction, at various concentrations of free Mg^{2+} ions (μ'), against concentrations of free ATP^{4-} (σ). The reaction mixtures contained 0.1 M-triethanolamine-HCl buffer, pH 8.6, 10 mM-D-lombricine and 10 μg . of lombricine kinase, as well as the indicated amounts of ATP^{4-} and Mg^{2+} ions. The total volume was 1.0 ml.; the temperature was 30°. Velocity is expressed as the change in extinction at 750 m μ /min. Concn. of Mg^{2+} ions: ■, 0.1 mM; △, 0.066 mM; ●, 0.05 mM.

Table 3. Summary of $K_{\mu'}$ and $V_{\mu'}$ values for the forward reaction catalysed by lombricine kinase

Values were calculated from the plots of $1/v$ against $1/\sigma$ at various fixed values of μ' (the concentration of free Mg^{2+} ions) at pH 8.6 (Fig. 4). All $K_{\mu'}$ values are expressed in terms of mM, and those for $V_{\mu'}$ are given in terms of μmoles of phospholombricine released/ μg . of lombricine kinase/min.

μ' (mM)	$V_{\mu'}$	$K_{\mu'}$
0.1	0.051	0.078
0.066	0.042	0.09
0.05	0.034	0.095

Again the lines were drawn from calculated values of $K_{\mu'}$ and $V_{\mu'}$, but the experimental points are shown. The values of $K_{\mu'}$ and $V_{\mu'}$ are collected in Table 3.

A secondary plot of $1/V_{\mu'}$ against $1/\mu'$ (Fig. 5) gave the values of 0.1 mM for K_2 and 0.10 μmole of phospholombricine/ μg . of lombricine kinase/min. (10000 moles/10⁵ g. of enzyme/min.) for the maximum velocity at pH 8.6, with the enzyme saturated with ATP^{4-} and Mg^{2+} ions and with lombricine at a concentration of 10 mM. A tertiary plot of $K_{\mu'}[(K_2/\mu') + 1]$ against $1/\mu'$ (Fig. 6) gave K_4 and K_4/K_1K_2 as 0.03 mM and $2.2 \times 10^3 \text{ M}^{-1}$ respectively, so that all the constants could be determined. The values obtained are included in Table 2.

DISCUSSION

The residual activity exhibited by lombricine kinase, in the presence of reaction components that had not been treated with Chelex resin, was eliminated by the presence of EDTA or by pretreatment

Fig. 5. Double-reciprocal plot of the maximum velocities ($V_{\mu'}$) obtained in the forward reaction at various fixed concentrations of free Mg^{2+} ions (μ') against the concentration of free Mg^{2+} ions. The calculated maximum velocities, obtained from the plots shown in Fig. 4 and reported in Table 3, are expressed as μmoles of phospholombricine released/ μg . of lombricine kinase/min.

Fig. 6. Plot of the calculated values for $K_{\mu'}[(K_2/\mu') + 1]$ against the reciprocals of the concentrations of Mg^{2+} ions in the forward reaction.

of the reaction components with Chelex. It may therefore be concluded that this enzyme has an absolute requirement for a bivalent metal ion. Further, the activation of the enzyme by Mg^{2+} ions is in accordance with the rate laws predicted for a metal ion-activated enzyme, so that lombricine kinase may be classified as a true metal-enzyme (Malmström & Rosenberg, 1959).

In the present investigation certain assumptions were made about the reaction mechanism. These included the supposition that all the possible reactions leading to the formation of the active enzyme-metal ion-substrate complex are in rapid equilibrium and that the concentration of the guanidine substrate affects the rate of breakdown of the enzyme-metal ion-complex only by a constant factor. On this basis, and from study of the activation of lombricine kinase by Mg^{2+} ions in relation to the concentration of the free nucleo-

tide, we have obtained dissociation constants for each of the complexes leading to the formation of the enzyme-metal ion-substrate in both directions of the reaction. Thus the results are consistent with the reaction of Mg^{2+} ions and free nucleotide, as well as Mg^{2+} ion-nucleotide complex, with the enzyme, indicating that all the pathways considered can lead to the formation of the enzyme-metal ion-substrate complex.

There is, however, some doubt about the magnitude of the dissociation constant (K_3) of the enzyme-metal ion complex. This value is obtained indirectly from the dissociation constant (K_4) of the reaction:

where E is the enzyme, M is the metal ion, and S is the substrate, and, since this latter value is small and its graphical determination subject to large error, there cannot be any certainty that it is not zero. If K_4 were zero, then under equilibrium conditions the value for K_3 would be infinity, which would indicate that there is no reaction between Mg^{2+} ions and the enzyme. Hence the conclusion that pathway II is operative must be regarded as tentative.

The kinetic behaviour of lombricine kinase is similar to that of ATP-creatine phosphotransferase (Kuby & Noltmann, 1962; Morrison *et al.* 1961).

SUMMARY

1. Kinetic studies of the activation of ATP-lombricine phosphotransferase by Mg^{2+} ions have been made in both the forward and reverse reactions:

2. On the assumption that a ternary enzyme-metal ion-substrate complex is involved in the enzymic reaction and that all the reactions leading to its formation are in rapid equilibrium, the dis-

sociation constants of all the possible intermediate steps have been determined.

3. The kinetic results are compatible with the view that the enzyme can react with free metal ion, free nucleotide and metal ion-nucleotide complex. However, the interaction between Mg^{2+} ion and the enzyme is weak and, taking the experimental error into account, it is possible that the dissociation constant for an enzyme-metal ion complex would be indistinguishable from infinity, which would indicate that this complex does not participate in the formation of an active enzyme-metal ion-substrate complex.

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