Kinetic Studies of the Mechanism and Allosteric Activation of the Reaction Catalyzed by Nucleoside Diphosphatase*

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ABSTRACT: A highly purified preparation of nucleoside diphosphatase has been used to study the kinetics of the reaction at pH 8.5 under conditions where magnesium–inosine diphosphate was the variable substrate and the concentration of inosine diphosphate was controlled. Double-reciprocal plots of initial velocity as a function of the magnesium–inosine diphosphate concentration at a relatively low, fixed concentration of inosine diphosphate yielded concave-up nonrectangular hyperbolas. When present at a sufficiently high concentration, the allosteric activator, magnesium–adenosine triphosphate, caused this type of plot to become linear. The reaction velocities at lower substrate concentrations were increased by the addition of magnesium–adenosine triphosphate while those at relatively high substrate concentrations were decreased. Studies of the activation showed that the velocity increased as a hyperbolic function of the modifier concentration.

The results are in agreement with, and have been interpreted according to, a rapid equilibrium, random mechanism that allows for the interdependent reaction of two molecules of substrate, at two identical catalytic sites on the enzyme, to yield complexes that give rise to products at different rates. The mechanism also assumes that magnesium–adenosine triphosphate combines at a single, distinct site on the enzyme, so that it can react with free enzyme and all the enzyme–substrate complexes. It is further proposed that the modifier can influence the binding of substrate as well as the rate of product formation. Mathematical analysis of the data has shown that the experimental results are consistent with the suggested mechanism and values have been determined for the various kinetic constants. The results have also been discussed in relation to other theories for the mechanism of allosteric enzyme action.

Although many investigations on allosteric enzymes have been undertaken, no general conclusions can be reached about their mechanism of action. The majority of kinetic studies have been primarily qualitative and thus suffer from a number of weaknesses. Prime among these is the failure to obtain sufficient, accurate experimental data so that an analysis can be made by fitting the data to a particular rate equation. It has been more usual to attribute the results of kinetic studies with allosteric enzymes to their existence in isomeric forms as postulated by Monod *et al.* (1965), Koshland *et al.* (1966), and Kirtley and Koshland (1967) or to their ability to undergo polymerization reactions as proposed by Nichol *et al.* (1967). These hypotheses assume that the kinetically observed allosteric data are due only to the thermodynamic effects of the interactions of enzyme and substrate and give no consideration to the possibility that they arise because of changes in the rate of product formation from different enzyme–substrate complexes. Thus it was of interest to undertake a detailed kinetic investigation of a reaction catalyzed by an allosteric enzyme to determine if the resulting data were in accord with one of the aforementioned hypotheses and to ascertain if such data could be explained in terms of a kinetic theory (*cf.* Frieden, 1964, 1967; Dalziel, 1968) for which there was no necessity to postulate the nature of the enzyme–substrate complexes.

The enzyme chosen for study was nucleoside diphosphatase which catalyzes the hydrolysis of a number of metal–nucleoside diphosphate complexes and which is activated by $MgATP^{2-}$ (Yamazaki and Hayaishi, 1965, 1968; Schramm and Morrison, 1968). It has been found that the results of the kinetic studies are consistent with a mechanism which involves the interdependent reaction of two molecules of substrate with the enzyme, the combination of $MgATP^{2-}$ at a separate site and the formation of product at different rates from the various enzyme–substrate complexes. The kinetic data could not be accounted for by the hypotheses of Monod *et al.* (1965), Koshland *et al.* (1966), or Nichol *et al.* (1967).

Materials and Methods

All materials and the methods for estimating the reaction velocity have been described previously (Schramm and Morrison, 1968). Particular care was taken to ensure that initial reaction rates were measured since the failure to determine initial velocities at the lower concentrations of $MgIDP^-$ would result in misleading, nonlinear double-reciprocal plots of velocity as a function of substrate concentration. The calculations of weighted mean values of the kinetic constants, together with their standard errors, were made as elaborated by Morrison and Uhr (1966).

Analysis of Data. The type of curve obtained was determined by graphical analysis of the data which were then analyzed by an appropriate computer program. Data that gave linear double-reciprocal plots of initial velocity as a function of the reactant concentration were fitted to eq 1; data that conformed

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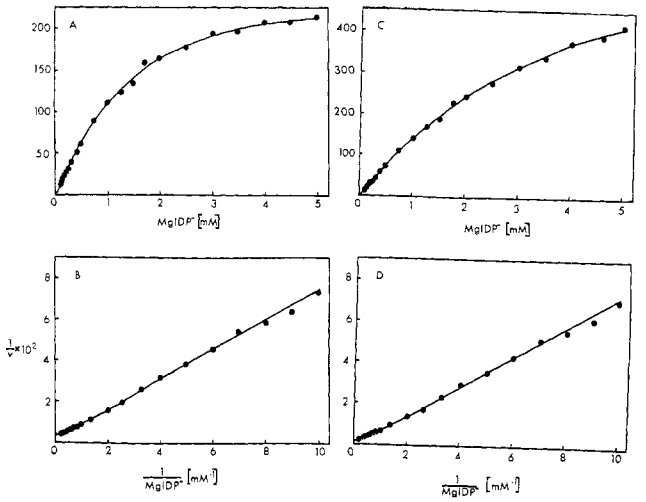


FIGURE 1: The effect of the concentration of $MgIDP^-$ on the initial velocity of the reaction before (A and B) and after (C and D) correction for the inhibition by free Mg^{2+}. The concentration of free IDP^{3-} was held constant at 0.1 mM and corrections for the inhibition by free Mg^{2+} were made as described in Table II using the constants given in Table III. A weighted fit of the data to eq 4 was made and the constants so obtained were used to draw the theoretical curves which are represented by the solid lines. v is expressed as millimicromoles per minute per microgram of protein.

to linear, noncompetitive inhibition were fitted to eq 2; data which showed that the initial velocity varied as a hyperbolic function of the modifier concentration were fitted to eq 3. For these purposes, the computer programs of Cleland (1963a) were used

$$v = \frac{VA}{K + A} \tag{1}$$

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \tag{2}$$

$$v = V\left(\frac{1 + \frac{M}{K_N}}{1 + \frac{M}{K_D}}\right) \tag{3}$$

Data that gave curvilinear double-reciprocal plots of velocity against substrate concentration were fitted to eq 4 using the computer programs of both

$$v = \frac{V(A^2 + dA)}{A^2 + bA + c} \tag{4}$$

Cleland (1963a) and Kowalik and Morrison (1968) which were modified so as to allow for the inclusion of weighting factors (see Results). In connection with the use of the latter two programs, it should be mentioned that it is first necessary to plot the experimental data in double-reciprocal form so that estimates can be made of the initial slope of the curve, the slope of the asymptote, the intercept of the curve, and the extrapolated intercept of the asymptote. The values so obtained provide starting values for the nonlinear analysis. Because the program of Cleland (1963a) utilizes a Gaussian method, reasonably accurate estimates of the above parameters are required, whereas this is not the case with the gradient minimization method used by Kowalik and Morrison (1968). Since these estimates are sometimes difficult to obtain, the procedure adopted was to make a preliminary fit of the data by means of the Kowalik and Morrison program and to use the output of refined estimates from this analysis as the starting point for the final analysis by the Cleland program. With good starting values, both programs gave the same minimum value for the residual sum of squares. All analyses were performed on an I.B.M. 360 computer, and the values of the parameters so obtained were used in the drawing of the lines for the figures.

TABLE I: Standard Deviations of Mean Velocity Values at Four Different Substrate Concentrations.

Substrate Concn (mM)	Initial Velocity[a]	Std Dev	Std Dev (%)	No. of Determinations
0.1	10.8	0.35	3.2	10
0.3	32.6	1.01	3.1	10
1.5	119	3.58	3.0	10
5.0	179	5.80	3.2	9

[a] Velocities were not corrected for inhibition by free Mg^{2+} and are expressed as millimicromoles per minute per microgram of protein.

Results

Initial Velocity Studies and Analysis of Data. When initial velocities were determined over a relatively narrow range of substrate concentrations, the data did not give good fits to eq 4. However, when analyses were made using a large number of initial rates that were determined over a wide (50-fold) range of $MgIDP^-$ concentrations, then it appeared that, for a plot of velocity against substrate concentration, there was good agreement between the theoretical and experimental points. But a plot of the same data in double-reciprocal form showed that the theoretical curve did not pass through the experimental points at low substrate concentrations. Furthermore, the standard errors of some of the kinetic parameters were relatively high.

In the computer programs used for the fitting of data to eq 4, no weighting factors were incorporated since the usual assumption was made that the variance associated with the velocity determinations was constant. But an analysis of the relationship between the variance and magnitude of the reaction velocities (Table I) showed that the standard deviation (square root of the variance) of the mean velocity values at four different substrate concentrations was proportional to the velocity value. Thus there was justification for making a weighted least-squares fit of the data to eq 4 by the inclusion in the computer program of weighting factors equal to the reciprocal of the square of each initial velocity (Cleland, 1967).

TABLE II: Values for the Kinetic Parameters of the Reaction As Determined from Initial Velocity Studies.

Kinetic Parameter	Value[a]	
	I[b]	II[c]
V	217 ± 12 mμmoles/min per μg	637 ± 38 mμmoles/min per μg
b	1.61 ± 0.12 mM	3.97 ± 0.33 mM
c	1.61 ± 0.60 mM	2.17 ± 0.72 mM
d	0.72 ± 0.32 mM	0.33 ± 0.14 mM
K_m	1.27 ± 0.12 mM	3.80 ± 0.40 mM

[a] Values are the weighted means of those obtained by fitting four sets of data, including that of Figure 1, to eq 4. [b] Values obtained by analysis of data which are not corrected for the inhibition by free Mg^{2+}. [c] Values obtained by analysis of data after correction for the inhibition by free Mg^{2+} (I) using the relationship

$$\text{true velocity} = \text{apparent velocity} \frac{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)}{K + A}$$

and the values for K, K_{is}, and K_{ii} given in Table III.

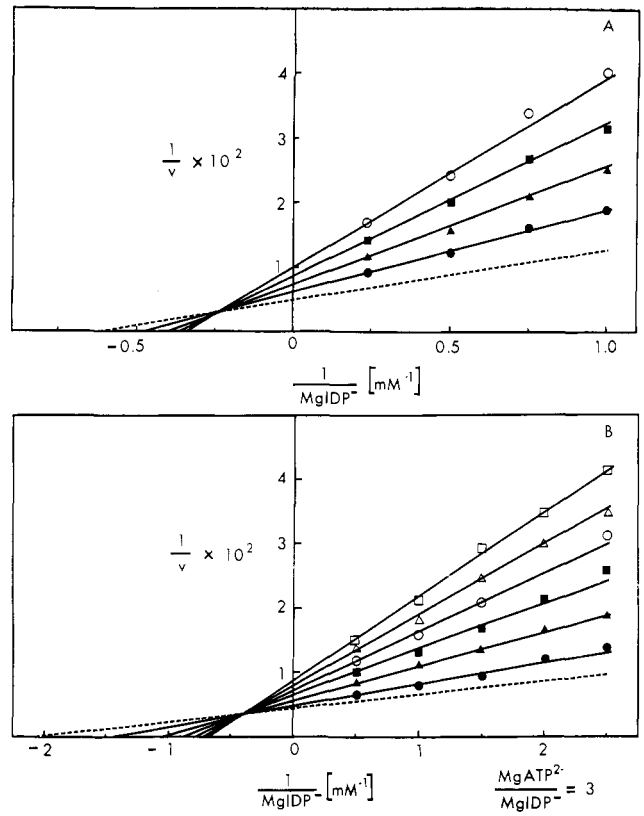


FIGURE 2: Inhibition of the reaction by free Mg^{2+} in the absence (A) and presence (B) of $MgATP^{2-}$. For part B the concentrations of $MgIDP^-$ and $MgATP^{2-}$ were varied simultaneously while maintaining their ratio constant. The concentrations of free Mg^{2+} for part A were: 5 (●), 10 (▲), 15 (■), and 20 mM (○) while the concentrations for part B were: 5 (●), 15 (▲), 25 (■), 35 (○), 45 (△), and 55 mM (□). The dashed lines represent the theoretical plots that would be obtained at zero concentration of free Mg^{2+}. The data were fitted to eq 2. v is expressed as millimicromoles per minute per microgram of protein.

This results in each velocity value making an equal contribution to the residual sum of squares. The results of the analysis of the data illustrated in Figure 1 (A and B) demonstrates the good agreement that is obtained between the theoretical curves and the experimental points for plots of velocity against substrate concentration (Figure 1A) and for plots of the reciprocals of the velocity against the reciprocals of the substrate concentrations (Figure 1B). The data did not fit well to an equation for a parabola, as would be obtained when the d term of eq 4 was zero, and it should be noted that the asymptote of Figure 1B will cut the vertical ordinate at a negative value. The weighted mean values for the kinetic parameters of eq 4 are listed in Table II, together with the K_m value for $MgIDP^-$.

Inhibition of the Reaction by Mg^{2+}. Since it has been shown that, at lower substrate concentrations, free IDP^{3-} activates the reaction (Schramm and Morrison, 1968), the above experiments were performed by holding the free IDP^{3-} concentration at the relatively low concentration of 0.1 mM while the concentration of $MgIDP^-$ was varied. Under these conditions, the concentration of free Mg^{2+} is equal to 2.5 times the concentration of $MgIDP^-$ and increases concomitantly with the concentration of the latter. Because of this and the ability of free Mg^{2+} to inhibit the reaction (Schramm and Morrison, 1968), true reaction velocities would not be measured at the higher concentrations of $MgIDP^-$. While it appears that the inhibitory action of free Mg^{2+} does not alter the form of eq 4, as judged by the results of Figure 1, it would influence the curved portion of double-reciprocal plots and the values of the parameters obtained by direct analysis of the data would be erroneous. Determination of the true values for these constants requires a knowledge of the inhibition constants associated with free Mg^{2+}, but because of the difficulty of carrying out experiments which would yield accurate values, a simpler approach was used to obtain approximate values. Thus the inhibition by different fixed concentrations of free Mg^{2+} was studied over a small range of relatively high substrate concentrations where double-reciprocal plots could be considered to approximate to straight lines. The results (Figure 2A) demonstrate that linear plots are obtained and that appreciable inhibition occurs at levels of free Mg^{2+} encountered with $MgIDP^-$ concentrations from 1.0 to 5.0 mM.

For comparative purposes, a similar experiment was done to determine the apparent inhibition constants for free Mg^{2+} when the modifier, $MgATP^{2-}$, was present and varied in constant ratio with the substrate. It is apparent (Figure 2B) that less inhibition is obtained under these conditions, but nevertheless, it is sufficient to require correction of the velocity values, especially at higher substrate concentrations. The kinetic constants obtained from analysis of the data in Figure 2 are listed in Table III, while the method used to calculate uninhibited reaction velocities at any given concentrations of free Mg^{2+} and $MgIDP^-$ is elaborated in Table II. In practice, all the directly determined velocities were corrected by multiplying by factors. These ranged from 1.94 to 1.04 and from 1.29 to 1.02 in the absence and presence of $MgATP^{2-}$, respectively, for concentrations of $MgIDP^-$ varying from 5.0 to 0.1 mM.

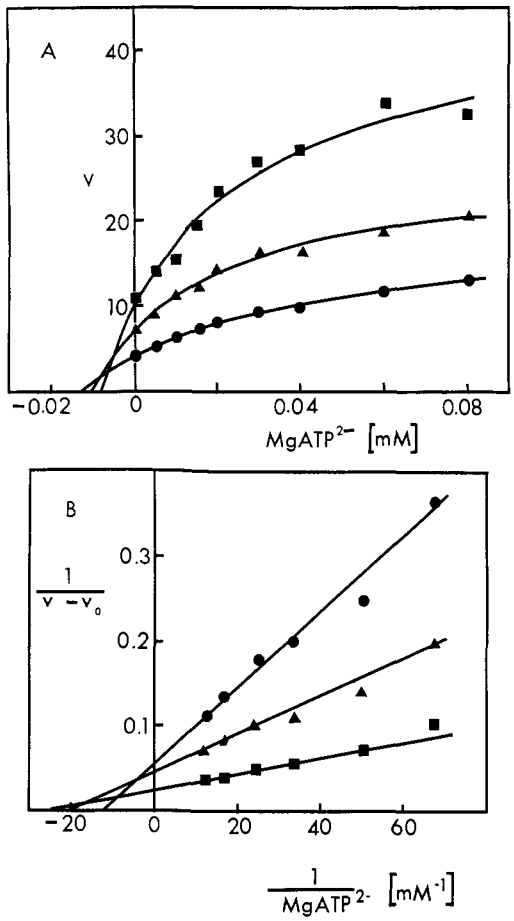


FIGURE 3: Effect of the concentration of $MgATP^{2-}$ on the initial velocity of the reaction at various fixed substrate concentrations. The concentrations of the substrate, $MgIDP^-$, were: 0.1(●), 0.125(▲), and 0.167 mM (■). The lines in parts A and B were drawn using the constants obtained by fitting the data to eq 3 and 1, respectively. In part B, v and v_0 represent initial velocities in the presence and absence of $MgATP^{2-}$, respectively. All velocities are expressed as millimicromoles per minute per microgram of protein.

TABLE III: Apparent Kinetic Constants[a] for the Inhibition of the Reaction by Free Mg^{2+} in the Absence and Presence of $MgATP^{2-}$.

App Kinetic Constant	Value	
	I[b]	II[c]
K	1.62 ± 0.29	0.51 ± 0.05
K_{is}	6.56 ± 1.25	11.9 ± 1.4
K_{ii}	21.1 ± 5.0	58.5 ± 8.9

[a] Values were obtained by fitting the data of Figure 2 to eq 2. [b] $MgATP^{2-}$ was absent. [c] Concentrations of $MgATP^{2-}$ and $MgIDP^-$ were varied in constant ratio.

TABLE IV: Values for K_N and K_D as Determined from the Results of the Activation of the Reaction by $MgATP^{2-}$.

Concn of $MgIDP^-$ (mM)	Value[a]	
	K_N (mM)	K_D (mM)
0.1	0.0124 ± 0.0005	0.038 ± 0.003
0.125	0.0093 ± 0.0009	0.038 ± 0.003
0.167	0.0123 ± 0.0016	0.051 ± 0.007

[a] Values are the weighted means of those obtained by fitting three sets of data, including that of Figure 3A to eq 3.

Reanalysis of the data of Figure 1A,B after correction for the inhibition by free Mg^{2+} gave the results illustrated in Figure 1C,D. Comparison of the two sets of results shows that the general shape of the plot with corrected data is unchanged, although the curvature at higher substrate concentrations is decreased. Further, the data can still be fitted well to eq 4 to give the true values for the parameters listed in Table II.

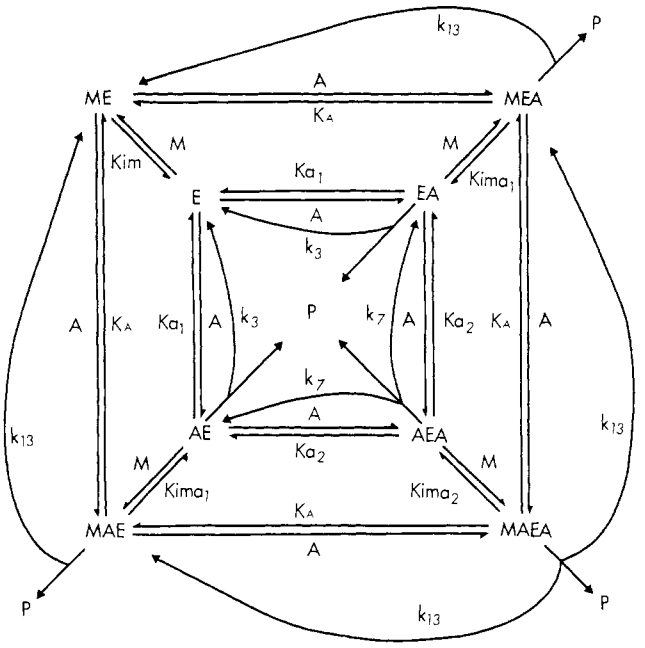
Kinetics of the Activation of the Reaction by $MgATP^{2-}$. It has been demonstrated previously (Yamazaki and Hayaishi, 1968; Schramm and Morrison, 1968) that there is activation of the reaction by $MgATP^{2-}$ and that in the presence of sufficiently high concentrations of this nucleotide complex, linear double-reciprocal plots of velocity as a function of substrate concentrations are obtained. More detailed investigations of the effect of $MgATP^{2-}$ on the initial velocity of the reaction at three different substrate concentrations gave the results illustrated in Figure 3. Those of Figure 3A show that each set of experimental data gives a good fit to eq 3, and thus it may be concluded that only one molecule of $MgATP^{2-}$ combines with the enzyme or that multiple molecules of the modifier react in an independent manner. Analysis of the data yielded values for K_N and K_D which are recorded in Table IV.

The data of Figure 3A were also analyzed by an alternative procedure which involves the plotting of $1/(v - v_0)$, where v and v_0 are initial velocities in the presence and absence of the modifiers, respectively, against the reciprocal of the modifier concentration (Figure 3B). The fact that linear plots are obtained confirms the above conclusions.

Kinetics of the Reaction in the Presence of High Concentrations of $MgATP^{2-}$. The Michaelis constant for the reaction of $MgIDP^-$ with the enzyme–modifier complex was determined by studying the initial velocity of the reaction as a function of the $MgIDP^-$ concentration at different fixed modifier concentrations. The concentration of substrate was varied over the range from 0.2 to 1.0 mM while $MgATP^{2-}$ was held constant at concentrations ranging from 0.6 to 3.0 mM. The resulting data gave linear double-reciprocal plots, and since the lines were virtually superimposble then, for all practical purposes, concentrations of $MgATP^{2-}$ at or above 0.6 mM, may be considered to be saturating. The weighted mean value for the Michaelis constant from five determinations was calculated to be 0.69 ± 0.03 mM.

Maximum Velocities of the Reaction in the Presence and Absence of $MgATP^{2-}$. The maximum velocity of the reaction in the absence of the modifier, $MgATP^{2-}$, was determined by using a range of high substrate concentrations such that plots of $1/v$ against $1/MgIDP^-$ would be linear. The maximum velocity in the presence of modifier was determined by using the

SCHEME I



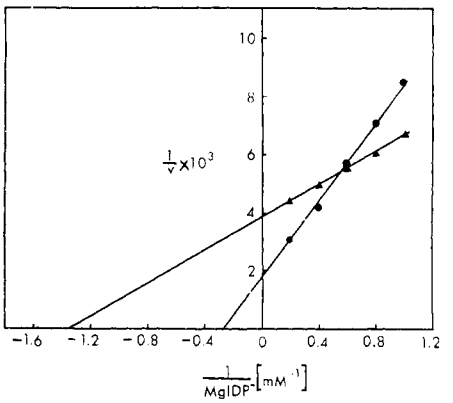


FIGURE 4: Effect of the concentration of $MgIDP^-$ on the initial velocity of the reaction in the presence and absence of $MgATP^{2-}$. For the control experiment (●), free IDP^{3-} was held constant at 0.1 mM while the effect of $MgATP^{2-}$ was determined by varying the concentrations of $MgATP^{2-}$ and $MgIDP^-$ in a constant ratio of 2 (▲). Before fitting to eq 1, all data sets were corrected for inhibition by free Mg^{2+} as described in the legend to Figure 1. v is expressed as millimicromoles per minute per microgram of protein.

same range of high substrate concentrations and holding the ratio of substrate:modifier constant. The initial velocity data illustrated in Figure 4 in the form of a double-reciprocal plot were corrected, when necessary, for the inhibition by free Mg^{2+}. The results indicate quite clearly that while the modifier increases the reaction velocity when substrate is present at lower concentrations, its presence on the enzyme reduces the maximum velocity of the reaction as compared with that obtained in its absence (260 ± 1 as compared with 558 ± 20 mμmoles/min per μg). It is also evident that in the presence of high concentrations of $MgATP^{2-}$, there is no tendency for the plots to curve up. Thus it would appear that the modifier cannot react at all substrate sites to form dead-end enzyme–modifier complexes. Further evidence for this conclusion comes from the finding that there is no inhibition of the reaction by high $MgATP^{2-}$ concentrations when substrate is present at a concentration of 0.1 mM. There was also an appreciable reduction in the maximum velocity of the reaction when the modifier was added at a relatively low concentration of 0.04 mM (Figure 5A).

Discussion

A reaction mechanism that is in accord with the experimental data for the nucleoside diphosphatase reaction is illustrated in Scheme I where E, A, and M represent enzyme, $MgIDP^-$, and $MgATP^{2-}$, respectively; K_{a1}, K_{a2}, and K_A represent dissociation constants for the reaction of A with E, EA, and AE, and ME, MEA, MAE, and MAEA, respectively; K_{im}, K_{ima1}, and K_{ima2} represent dissociation constants for the reaction of M with E, EA and AE, and AEA, respectively.

In accordance with what is known about allosteric enzymes, it is likely that nucleoside diphosphatase consists of identical subunits and in Scheme I, it is proposed that the enzyme is made up of two such subunits so that it possesses two equivalent catalytic sites. This allows for the formation of two identical enzyme–substrate complexes, EA, and AE, which can yield products at the same rate, k_3, and react in the same manner with a second molecule of A to form an AEA complex. It is envisaged that the addition of one substrate molecule to the enzyme can induce a conformational change and thus influence the combination of a second molecule of substrate, as well as the rate of product formation. The assumption is made that both substrate molecules of the AEA complex are converted into products at the same rate, k_7, which is not necessarily equal to k_3. Further, it is considered that a molecule of modifier (M) is capable of reacting at a highly specific site on the enzyme which is distinct and separate from the substrate sites and that the presence of M on the enzyme can affect both the reaction of substrate and rate of product formation. Indeed, it is supposed that the same kinetic constant, K_A, is associated with the reactions of A with ME, MEA, and MAE and that product is formed from MEA, MAE, and MAEA at a rate, k_{13}, which differs from the rates of product formation from EA and AE, k_3, and AEA, k_7. Allowance is also made for the

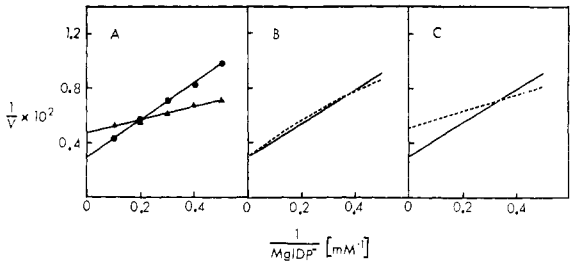


FIGURE 5: Effect of a fixed concentration of $MgATP^{2-}$ on the initial velocity of the reaction at higher substrate concentrations. (A) Experimental data corrected for the inhibition by Mg^{2+} as described in the legend to Figure 1. (●) No $MgATP^{2-}$; (▲) 0.04 mM $MgATP^{2-}$. (B and C) Theoretical curves expected on the basis of the reaction mechanisms illustrated in Schemes II and I, respectively. Solid line, no $MgATP^{2-}$; broken line, 0.04 mM $MgATP^{2-}$. v is expressed as millimicromoles per minute per microgram of protein.

reaction of M with free enzyme as well as with the EA, AE, and AEA complexes.

On the basis that rapid equilibrium conditions are applicable, the initial velocity equation for the above mechanism can be expressed as

$$v = \frac{V\left[A^2 + \frac{k_3}{k_7}K_{a2}A + \frac{k_{13}}{k_7}\frac{K_{a1}K_{a2}}{K_A K_{im}}\left(1 + \frac{A}{K_A}\right)MA\right]}{A^2 + 2K_{a2}A + K_{a1}K_{a2} + \frac{K_{a1}K_{a2}M}{K_{im}} + \frac{K_{a1}K_{a2}}{K_A K_{im}}\left(2 + \frac{A}{K_A}\right)MA} \quad (5)$$

where $V = 2k_7E_t$. When $M = 0$, eq 5 reduces to

$$v = \frac{V\left(A^2 + \frac{k_3}{k_7}K_{a2}A\right)}{A^2 + 2K_{a2}A + K_{a1}K_{a2}} \quad (6)$$

and when $M \rightarrow \infty$, eq 5 simplifies to

$$v = \frac{2k_{13}E_t}{K_A + A} \quad (7)$$

With M as the variable reactant, rearrangement of the same equation gives

$$v = V\left[\frac{A^2 + \frac{k_3}{k_7}K_{a2}A}{A^2 + 2K_{a2}A + K_{a1}K_{a2}}\right] \times \left[\frac{1 + \frac{M}{\left\{A + \frac{k_3}{k_7}K_{a2}\right\}\left\{\frac{k_7K_A^2K_{im}}{k_{13}K_{a1}K_{a2}(K_A + A)}\right\}}}{1 + \frac{M}{\frac{K_A^2K_{im}(A^2 + 2K_{a2}A + K_{a1}K_{a2})}{K_{a1}K_{a2}(K_A + A)^2}}}\right] \quad (8)$$

It will be noted that eq 6–8 have the same forms as eq 4, 1, and 3, respectively.

The results of initial velocity studies in the absence of modifier (Figure 1) are qualitatively in agreement with those predicted by eq 6 since plots of v against A and $1/v$ against $1/A$ yield sigmoidal curves and nonrectangular hyperbolas, respectively. Furthermore, since the experimental data gave good fits to eq 6, which describes the inner sequence of reactions of Scheme I under either steady-state or rapid equilibrium conditions, there is also quantitative agreement between theory and results. On the other hand, the data did not fit well to the equation for a mechanism in which the first molecule of A acts only as an essential modifier. Since double-reciprocal plots of v as a function of substrate concentration are linear in the presence of a relatively high concentration of M, the results are consistent with those expected for a reaction that occurs *via* the outer reaction sequence of Scheme I. This sequence is described by eq 7 which would be valid if either steady-state or rapid equilibrium conditions were applicable.

The kinetic data that were obtained with M as the variable reactant, in the presence of lower substrate concentrations (Figure 3) fitted well to eq 3 which is of the same general form as eq 8. Thus it may be concluded that only one molecule of modifier undergoes reaction with the enzyme. However, a similar result would be expected if multiple combinations of M occurred in an independent manner. These two possibilities cannot be distinguished by kinetic techniques and for the sake of simplicity, it has been assumed (Scheme I) that only one molecule of modifier is involved in the reaction. The results of Figure 3 are in accord with the idea that the mechanism for the over-all reaction (Scheme I) is of the rapid equilibrium type. If steady-state conditions prevailed and M reacted with the E, EA, AE, and AEA complexes then theoretically the initial velocity would be a complex, rather than a hyperbolic, function of the modifier concentration. However, because of the practical difficulty of distinguishing between steady-state and rapid equilibrium models, it cannot be argued that the steady-state model is eliminated by the experimental data. It should be noted that the results illustrated in Figure 3 would be expected under steady-state conditions if M reacted with only one of the above enzyme forms. But such a postulate would appear to be unwarranted when it is considered that the enzyme possesses separate sites for A and M. None of the results presented is inconsistent with the reaction obeying rapid equilibrium kinetics, although it is possible that this description is only an approximate one.

On the basis that all the reaction steps of Scheme I are rapid except those concerned with the interconversion of central complexes, values have been calculated for the various dissociation constants associated with the reactants and for the ratios of the rate constants. From the results of Table V, it may be concluded that the presence of one substrate molecule on the enzyme hinders to a small extent the combination of a second substrate molecule and that the nonlinearity of double-reciprocal plots of velocity as a function of substrate concentration is due largely to the fact that the rate of product formation from AEA is about six times higher than from the EA and AE complexes. The data of Table V also indicate that $MgATP^{2-}$ combines strongly with the enzyme, but has little or no effect in facilitating the reaction of substrate unless one substrate molecule is already present on the enzyme. However, the rate of product formation is higher from the MEA and MAE complexes than from the EA and AE complexes. By contrast, the AEA complex gives rise to product at a faster rate than does the MAEA complex. The relative rates of breakdown of the complexes are approximately AEA: (MEA, MAE, and MAEA): (EA and AE), 100: 45:17, and it is, therefore, primarily in terms of the rate of product formation that the activation and inhibition of the reaction by M, at, respectively, lower and higher substrate concentrations, can be explained. This finding is of interest because of suggestions that modifiers activate by enhancing the binding of substrate.

The ability of $MgATP^{2-}$ to inhibit the reaction is clearly demonstrated by the results of Figure 4, which also show that there is a particular concentration of substrate at which the modifier causes neither activation nor inhibition. The good agreement between the two estimates for the k_{13}/k_7 ratio (Table V), as determined from the data of Figures 3 and 4, is

TABLE V: Summary of the Values for the Kinetic Constants Relating to the Reaction of $MgIDP^-$ and $MgATP^{2-}$ with Nucleoside Diphosphatase.

Kinetic Constant[a]	Value[c] (mM)	Kinetic Constant[b]	Value[c]
K_{a1}	1.10 ± 0.38	V	637 ± 38 mμ-moles/min per μg
K_{a2}	1.98 ± 0.16	k_3/k_7	0.17 ± 0.07
K_m	3.80 ± 0.40		
K_A	0.69 ± 0.03	k_{13}/k_7	0.42 ± 0.14[d] 0.47 ± 0.02[e]
K_{im}	0.039 ± 0.010		
K_{ima1}	0.024 ± 0.011		
K_{ima2}	0.0084 ± 0.0039		

[a] With the exception of K_m, the kinetic constants are dissociation constants as defined in Scheme I. K_m is defined as the concentration of substrate that gives half-maximum velocity and is equal to

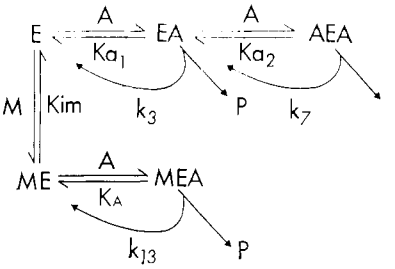
$$K_{a2}\left(1-\frac{k_3}{k_7}\right)+\sqrt{K_{a2}^2\left(1-\frac{k_3}{k_7}\right)^2+K_{a1}K_{a2}}$$

[b] V is equal to $2k_7E_t$, where E_t represents total enzyme concentration. k_3, k_7, and k_{13} have the meanings ascribed to them in Scheme I. [c] Values for V, k_3/k_7, K_{a1}, and K_{a2} were calculated from the data of Table II (column II) in conjunction with eq 6. The value for K_A was determined as described in the text. The value for K_{im} is the weighted mean of three values which were calculated from the values for K_D (Table IV) in conjunction with eq 8 and the values for K_{a1}, K_{a2}, and K_A, as well as, the concentration of substrate. Values for K_{ima1} and K_{ima2} were calculated from the equilibrium relationships: $K_{a1}K_{ima1} = K_AK_{im}$ and $K_{a2}K_{ima2} = K_AK_{ima1}$. [d] The k_{13}/k_7 ratio is the weighted means of three values which were calculated from the values for K_N (Table IV) in conjunction with eq 8, values for k_3/k_7, K_{a1}, K_{a2}, K_A, K_{im}, and the appropriate substrate concentration. [e] This value represents the ratio of the maximum velocities as obtained in the presence and absence of modifier (Figure 4).

worthy of note. Since it is unlikely that concentrations of $MgIDP^-$ above 5 mM are ever attained *in vivo*, the inhibition by $MgATP^{2-}$ probably has little physiological significance. Nevertheless, the results obtained with this modifier raise the question of the meaning of the term, allosteric activator, and draw attention to the need for a more rigid classification of modifiers.

While the aforementioned results are consistent with the mechanism proposed in Scheme I, they are also in accord with that shown in Scheme II and previously suggested by Schramm and Morrison (1968). In the latter scheme, it is considered that two molecules of substrate react with the enzyme in an ordered sequence to give nonlinear double-reciprocal plots of velocity against substrate concentration and that the plots become linear in the presence of high concentrations of modifier because one molecule of the modifier reacts at one of the two substrate

SCHEME II



sites. The finding that the maximum velocity of the reaction in the presence of saturating concentrations of modifier is reduced by about 50% could be interpreted as evidence in favor of the hypothesis. To distinguish between the two mechanisms, the kinetics of the reaction were studied over a range of high substrate concentrations, using $MgATP^{2-}$ at a concentration approximately equal to its K_{im} value (Table V), and the results compared with the theoretical curves for the two mechanisms. The theoretical curves for the mechanism shown in Scheme I were drawn using eq 5 and values for the appropriate kinetic constants (Table V). A similar procedure was used to draw the theoretical curves for the alternative mechanism after derivation of the initial velocity equation as eq 9 and analysis of the data in terms of this equation. Since the same form of equation is obtained for steady-state and rapid

$$v=\frac{V\left[A^2+\frac{k_3}{k_7}K_{a2}A+\frac{k_{13}}{k_7}\frac{K_{a1}K_{a2}}{K_AK_{im}}MA\right]}{A^2+K_{a2}A+K_{a1}K_{a2}+\frac{K_{a1}K_{a2}}{K_{im}}M+\frac{K_{a1}K_{a2}}{K_AK_{im}}MA} \quad (9)$$

equilibrium conditions, the kinetic constants of eq 9 can be considered to be dissociation constants as defined in Scheme II. It is apparent (Figure 5) that the theoretical curves for the two mechanisms, in the presence and absence of modifier, are quite different. Further, it is clear that the experimental results are consistent with the mechanism illustrated in Scheme I and inconsistent with that shown in Scheme II.

Consideration has been given to the interpretation of the initial velocity data (Figure 1) in terms of the hypotheses of Monod *et al.* (1965) and Koshland *et al.* (1966) which, when $n = 2$, are described by binding equations of the same general form as eq 4. In both these hypotheses, it is assumed that all enzyme–substrate complexes give rise to products at equal rates and thus a requirement of the hypotheses is that in terms of eq 4 $b = 2d$. Since the present study has shown that the values for these parameters differ by a factor of 12, it can be concluded that neither proposal accounts for the kinetic data of the nucleoside diphosphatase reaction. Furthermore, it is not possible to explain these results in terms of the polymerization model of Nichol *et al.* (1967), since gel filtration studies have indicated that the molecular weight of the enzyme is about 100,000 both in the presence and absence of $MgATP^{2-}$ and at concentrations comparable with those used for the kinetic investigations.

The relatively low molecular weight of nucleoside diphosphatase makes it unlikely that the enzyme contains a large number of subunits. But it is conceivable that the native en-

zyme could be formed, for example, from four subunits, each possessing a catalytic site, and the fact that the kinetic data are consistent with two molecules of substrate being involved in the reaction does not exclude this possibility. If these four subunits were arranged as two equivalent pairs, kinetic techniques would give evidence for the reaction of only two molecules of substrate. Thermodynamic experiments would be needed to determine the number of moles of substrate combining per mole of enzyme. It should also be mentioned that kinetic methods might give only a minimum value for the number of substrate molecules reacting. Determination of the total number requires that the magnitude of the kinetic constants be such as to make kinetically significant the steady-state concentration of each enzyme–substrate complex.

It must be emphasized that agreement between experimental results and those predicted on the basis of the present hypothesis does not constitute proof of the correctness of the hypothesis. The investigation has been confined to initial velocity studies and these have limitations with respect to the elucidation of reaction mechanisms (Cleland, 1963b). There is a need for the further development of the kinetic theory for allosteric enzyme reactions which takes into account the order of product release and which can be utilized for the interpretation of product inhibition and isotope-exchange studies. It is also apparent that the elucidation of mechanisms for allosteric enzymes would be facilitated by performing binding studies, with modifiers and substrate analogs, similar to those reported by Changeux *et al.* (1968). But this type of experiment has been precluded by the limited amounts of nucleoside diphosphatase that are available (Schramm and Morrison, 1968). A quantitative approach to kinetic studies on allosteric enzymes is made difficult by virtue of the fact that the initial velocity equations are represented by equations that are the ratios of polynomials. The velocity equation used in the present work is the simplest of this type, being the ratio of quadratic polynomials, and it has been found that large amounts of highly accurate data are essential for satisfactory mathematical analysis. At the present time, it is not possible to fit data to this type of function which contains cubic or higher power terms in substrate concentration, as would be obtained if three or more molecules of substrate underwent interdependent reactions with an enzyme. Thus the possibility that the nucleoside diphosphatase reaction involves the interdependent combination of more than two molecules of $MgIDP^-$ cannot be excluded. In any event, it will probably be difficult by curve-fitting techniques to determine the exact form of the initial velocity equation that describes a mechanism, especially when double-reciprocal plots of velocity as a function of substrate concentration exhibit only a small curvature.

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