ation either at His-119 or His-12. In this case, it is probable that the conformation in the crystal is the same, or is induced by bromoacetate to be the same, as that of the His-119-reactive form in solution.

The second difference is that raising the reaction temperature produces an increase in the proportion of 3-CMHis-12-RNase in solution, but in the crystal produces new products. This suggests that there is a conformation change in the crystal between 25 and 35°. We plan crystal structure analyses at several temperatures.

Yang and Hummel (1964) reported that CMHis-119-RNase does not bind anions. The fact that phosphate, at a phosphate to protein ratio of 2:1, stabilized CMHis-119-RNase crystals in form II suggests that phosphate is bound. X-Ray diffraction may resolve this question.

Acknowledgments

We are grateful to Mrs. T. Falzone for growing the crystals of the enzyme in this study and to Dr. G. Tritch for doing the amino acid analyses in the earlier part of this study. We are also grateful to Dr. Eric A. Barnard for permission to cite data in advance of publication, and to Dr. O. A. Roholt for helpful technical advice.

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Kinetic Studies of Yeast Nucleoside Diphosphate Kinase*

Edmundo Garces and W. W. Cleland

ABSTRACT: Initial velocity, product inhibition, and isotopic exchange studies have been carried out on crystalline nucleoside diphosphate kinase from yeast, with the magnesium complexes of adenine and uridine nucleotides as reactants.

The kinetic mechanism is Ping-Pong (the first product dissociates before the second substrate combines with the enzyme), and all reactants give competitive substrate inhibition by combining with the improper stable enzyme form. A stable phosphoenzyme was isolated which may contain a phosphohistidine linkage. All of the kinetic constants have been determined and shown to be consistent with the Haldane relationships. An analysis of the kinetic constants suggests that the turnover numbers are the maximum possible for the physiological conditions.

Nucleoside diphosphate kinase catalyzes the phosphorylation of nucleoside diphosphates by nucleoside triphosphates, and the substrate specificity is broad, with all nucleotides tested generally acting as substrates. With the availability of the crystalline yeast enzyme (Ratliff *et al.*, 1964), kinetic studies were undertaken to elucidate the mechanism of the reaction, employing the reaction between adenine and uridine nucleotides[1] (eq 1).

$$MgATP^{2-} + MgUDP^{-} \rightleftharpoons MgADP^{-} + MgUTP^{2-} \quad (1)$$

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The work reported here involves the determination of initial velocity, product inhibition, and isotopic exchange patterns, as well as chemical studies on the formation and stability of the phosphoenzyme intermediate. All of the kinetic constants have been determined with uridine and adenine nucleotides as the reactants, and shown to be internally consistent. The enzyme seems to be very similar to that from erythrocytes (Mourad and Parks, 1966a) in its kinetic properties, and the Ping-Pong mechanism with a stable phosphoenzyme intermediate is common to the enzymes from yeast, erythrocytes (Mourad and Parks, 1966b), and Jerusalem artichoke (Norman *et al.*, 1965).

Materials and Methods

Materials. The sodium salts of ADP, ATP, UDP, and UTP were obtained from P-L Biochemicals. Stock solutions were adjusted to pH 7.2 with NaOH and stored at −10°; their concentrations were determined spectrophotometrically. Triethanolamine (Eastman) was dissolved in water and adjusted to the proper pH with 5 N acetic acid. Crystalline bovine serum albumin was obtained from Armour, and EDTA and magnesium acetate from Fisher. [8-^{14}C]ATP, [8-^{14}C]ADP, [^{14}C]-UDP, and [^{14}C]UTP were purchased from Schwartz. DEAE-cellulose paper (DE-81) was purchased from Sargent and had a capacity of 0.4 mequiv/g and a basis weight of 85 g/m^2.

Crystalline nucleotide diphosphate kinase from yeast (Ratliff *et al.*, 1964) was kindly supplied by Dr. Henry A. Lardy. The stock enzyme solution contained 18 mg of protein/ml and was stored at −10°. Concentrations were determined from the absorption at 282 mμ, assuming a 1-mg/ml solution to give an optical density of 1.6 (Yue *et al.*, 1967). Dilutions were made in 1 mM triethanolamine acetate (pH 8.0) containing 0.01 mM EDTA and 0.1 mg/ml of bovine serum albumin. The presence of albumin stabilized the diluted enzyme for at least 5 hr.

Velocity Measurements. Kinetic studies were carried out at 30° in 0.1 M triethanolamine acetate (pH 8.0) in the presence of 0.01 mM EDTA and 1 mM free Mg^{2+}. Unless otherwise noted, the enzyme concentration was 3.2×10^{-3} μg/ml. The concentrations of total Mg^{2+} and nucleotides needed to generate the desired concentrations of the magnesium nucleotide complexes and leave 1 mM free Mg^{2+} were calculated by assuming the stability constants for the magnesium complexes of nucleotide tri- and diphosphates to be 70,000 and 4000 M^{-1}, respectively (O'Sullivan and Perrin, 1964). This procedure was adopted after preliminary studies showed that optimal activity was obtained only when the nucleotides were fully complexed with magnesium, but that free Mg^{2+} levels above 1 mM were somewhat inhibitory. Since chloride ions were also slightly inhibitory, acetate was used as the counterion of the buffer and magnesium acetate was used as the source of Mg^{2+}.

Initial velocities in the absence of products and initial velocities of isotopic exchange were determined by measuring the rate of formation of UTP from [^{14}C]UDP or of ATP from [^{14}C]ADP. For determination of product inhibition patterns one has to follow the rate of appearance of the product not present initially (otherwise isotopic exchange between the radioactive substrate and the pool of product increases the apparent velocities), and thus it was necessary in some cases to follow the appearance of UDP from [^{14}C]UTP and of ADP from [^{14}C]ATP.

Reactions were run in a 0.2-ml total volume. After all components except enzyme were added, the tubes were kept in ice until ready for use. The tubes were then incubated at 30° for 3 min before reaction was initiated by addition of enzyme. Samples (usually 30 μl) were taken shortly after mixing and at several accurately timed intervals thereafter (such as 1, 2, and 4 min after the first sample) and applied directly to 12 × 13 cm sheets of DEAE-cellulose paper on a line 2.5 cm from the bottom. An equal volume of absolute ethanol was immediately added at the same point (this procedure was shown to result in immediate stopping of the reaction, which otherwise continues for a time with considerable resulting error). Sufficient carrier nucleotides (0.1 μmole of each of ATP and ADP, or of UTP and UDP) were also added later to permit identification of the nucleotide spots under ultraviolet light after chromatography. A pad of four 2.5-cm wide strips of Whatman No. 3MM paper was stapled to the top of the paper sheet to act as a wick and permit overrunning of the chromatogram. The paper was folded into a cylinder around a support and placed in a 2-qt mason jar. After 30 min for vapor equilibration, the sheet was developed by ascending chromatography in freshly prepared 0.6 M ammonium formate (pH 3.1) containing 15 mM EDTA for 2.5 hr at 23°. Under these conditions, the ADP and ATP spots are well separated with no tailing by the faster moving ADP. When uridine nucleotides were being separated, the chromatograms were developed for 5 hr to improve resolution. After drying in air overnight, the chromatograms were heated for 10 min at 60° and the nucleotide spots were located under ultraviolet light and cut out. The sections of paper containing the nucleotides were fluted, placed in glass vials containing scintillation fluid (3 g of 2,5-diphenyloxazole and 100 mg of 1,4-[bis-2-(5-phenyloxazolyl)]benzene in 1 l. of toluene), and counted in a liquid scintillation counter. Velocities are expressed as millimicromoles per minute per milliliter of reaction mixture.

Data Analysis. The radioactivity appearing in product was plotted as a function of time, and the slope of the line was taken as the initial velocity (the reactions were usually linear over the full time period during which samples were taken; if not, the initial slope of the line was used). Data from each experiment were first plotted as $1/v$ *vs.* reciprocal substrate concentration. When these plots were linear, the data were first fitted to eq 2 using the least-squares method and assuming equal variance for the velocities (Wilkinson, 1961). All least-squares fits were performed by a digital computer using the FOR-

[1] Abbreviations used: MgATP, MgADP, MgUTP, and MgUDP, the magnesium complexes of the corresponding nucleotides.

$$v = \frac{VA}{K + A} \quad (2)$$

TRAN programs of Cleland (1963a, 1967). These programs provide values for the constants and various combinations of constants in a fitted equation together with standard errors of their estimates. Slopes, K/V, and intercepts, $1/V$, from fits to eq 2 were then plotted graphically against either the reciprocal of the other substrate concentration for initial velocity or isotopic exchange experiments, or against the inhibitor concentration for inhibition experiments to determine the form of the over-all rate equation. Final values for the kinetic constants were obtained by fitting all data points used in the first analysis to this over-all equation. Data conforming to a Ping-Pong initial velocity pattern were fitted to eq 3, or to eq 4 if competitive substrate inhibition by the second substrate was observed. Inhibition data were

$$v = \frac{VAB}{K_aB + K_bA + AB} \quad (3)$$

$$v = \frac{VAB}{K_aB\left(1 + \frac{B}{K_{Ib}}\right) + K_bA + AB} \quad (4)$$

fitted to eq 5 or 6 when the inhibition was competitive or noncompetitive, respectively. The points on figures

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (5)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (6)$$

are experimentally determined values. The lines are calculated from fits to eq 2 unless otherwise noted in the figure caption. The kinetic constants reported in Table I are weighted averages of values from replicate experiments derived by fitting the data to the appropriate over-all rate equation (eq 3–6).

Stability of Phosphoenzyme. Aliquots of chromatographically purified phosphoenzyme containing about 2000 cpm were exposed to 0.3 M trichloroacetic acid for 2 min at 100° or to 0.1 M borate (pH 9.2) for 5 min at 80° by adding the aliquot to the preheated solution. After the heating period the tubes were cooled by immersion in ice and the phosphomolybdate complex was formed and extracted rapidly into cold isobutyl alcohol–benzene (Lindberg and Ernster, 1956). The phases were separated and centrifuged, and clear aliquots of both phases were placed in aluminum planchets, dried first in air and then over P_2O_5 under vacuum, and counted in a windowless flow counter. Efficiency was determined by adding [γ-^{32}P]ATP to planchets containing fractions from a blank reaction mixture without phosphoenzyme. To determine the degree of hydrolysis caused by exposure to acid during the extraction process, an unheated sample was also carried through the procedure.

Results

Initial Velocity Patterns. When the concentrations of MgATP and MgUDP were varied, reciprocal plots of the resulting data showed a parallel pattern characteristic of a Ping-Pong mechanism where one product is released before the second substrate combines with the enzyme (Figure 1). An identical pattern was obtained for the reverse reaction involving MgUTP and MgADP. These data and data from similar replicate experiments were fitted to eq 3; weighted averages of the resulting Michaelis constants are given in Table I.

At higher substrate levels, competitive substrate inhibition by all substrates could be demonstrated. Inhibition by the nucleotide diphosphates was most prominent; that caused by high levels of MgUDP is shown in Figure 2. Such data were fitted either to eq 5 or 4, depending on whether the pattern of reciprocal plots crossed on the vertical axis or to the right of it, and weighted averages of the inhibition constants for MgUDP and MgADP from replicate experiments are given in Table I.

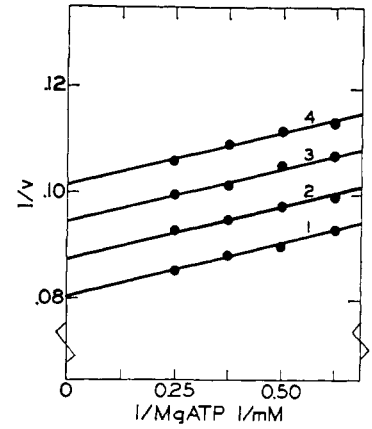


FIGURE 1: Initial velocity pattern for the forward reaction. Concentrations of [^{14}C]MgUDP were: 1, 0.2 mM; 2, 0.133 mM; 3, 0.10 mM; 4, 0.08 mM.

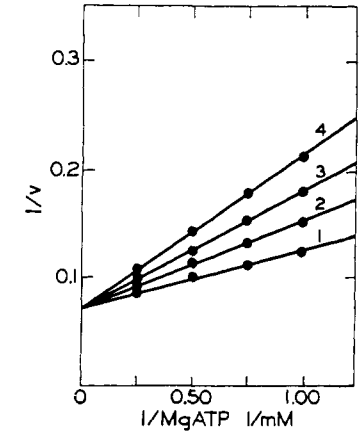


FIGURE 2: Competitive substrate inhibition by MgUDP. Concentrations of [^{14}C]MgUDP were: 1, 1.0 mM; 2, 2.0 mM; 3, 3.0 mM; 4, 4.0 mM.

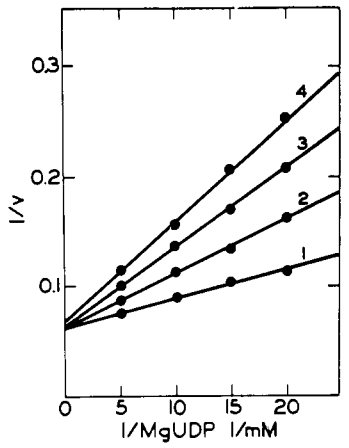


FIGURE 3: Product inhibition by MgADP with MgUDP as variable substrate. MgATP, 3.0 mM. MgADP concentrations were: 1, none, 2, 0.05 mM; 3, 0.10 mM; 4, 0.15 mM. [^{14}C]MgUDP was varied as shown.

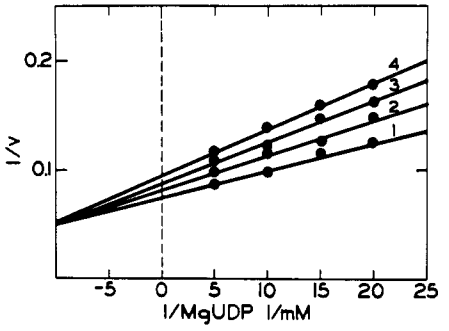


FIGURE 4: Product inhibition by MgUTP with MgUDP as variable substrate. [8-^{14}C]MgATP, 3.0 mM. Concentrations of MgUTP were: 1, none; 2, 0.40 mM; 3, 0.80 mM; 4, 1.20 mM.

The maximum velocities in forward and reverse directions were determined by varying both substrate concentrations together in constant ratio (tri-/diphosphate = 20). The resulting reciprocal plots were linear as required for a Ping-Pong mechanism, and the data were fitted to eq 2 to obtain the apparent maximum velocities. These apparent values were corrected for the competitive substrate inhibition by all four substrates (see Discussion) and the weighted averages of the corrected values from replicate experiments are given in Table I.

Product Inhibition Patterns. Each reactant was tested as a product inhibitor against the two possible substrates. Figure 3 shows linear competitive inhibition of MgUDP by MgADP and a similar pattern was obtained with MgATP as variable substrate and MgUTP as inhibitor. The reverse combinations (MgUDP *vs.* MgADP, and MgATP *vs.* MgUTP) also gave linear competitive inhibition and the data from these experiments were fitted to eq 5.

When the concentration of MgUDP was varied and MgUTP was the inhibitor, linear noncompetitive inhibition was observed (Figure 4). A similar pattern was obtained with MgATP as variable substrate and MgADP as inhibitor, and the reverse combinations (MgUDP *vs.* MgUTP, and MgATP *vs.* MgADP) also gave linear noncompetitive inhibition. These data were fitted to eq 6. Inhibition constants calculated from the product inhibition data (see Discussion) are given in Table I.

Isotopic Exchange Patterns. The initial velocity of isotopic exchange between MgADP and MgATP was measured at various levels of the two reactants in the absence of uridine nucleotides. The resulting pattern shown in Figure 5 clearly indicates competitive substrate inhibition by MgADP. High levels of MgATP also give competitive substrate inhibition of the exchange (not shown). These data were fitted to eq 4 and the kinetic constants from these fits are given in Table I. The MgUDP–MgUTP exchange was also investigated and Figure 6 shows that MgUTP gives competitive substrate

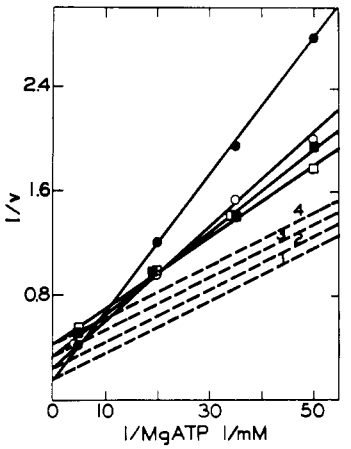


FIGURE 5: Isotopic exchange between MgADP and MgATP, showing competitive substrate inhibition by MgADP. The concentrations of [8-^{14}C]MgADP were: 1 (●), 0.05 mM; 2 (○), 0.025 mM; 3 (■), 0.0167 mM; 4 (□), 0.0125 mM. The solid lines are drawn from a fit to eq 4; the dashed lines indicate the expected velocities in the absence of the competitive substrate inhibition.

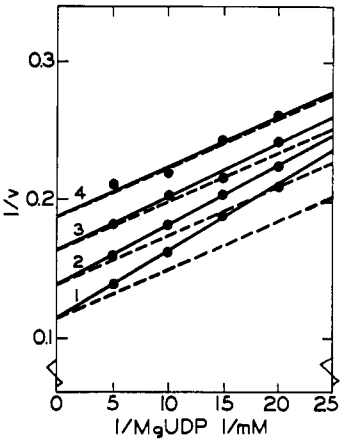


FIGURE 6: Isotopic exchange between MgUDP and MgUTP. MgUTP concentrations were: 1, 1.0 mM; 2, 0.5 mM; 3, 0.333 mM; 4, 0.25 mM. [^{14}C]MgUDP was varied as shown. The solid lines are drawn from a fit to eq 4; the dashed lines indicate the expected pattern in the absence of competitive substrate inhibition by MgUTP.

inhibition at high levels. These data were also fitted to eq 4 and the resulting constants are included in Table I.

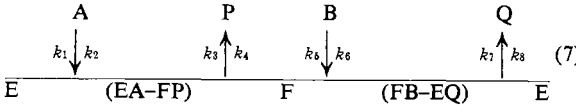
The basically parallel nature of these exchange patterns after allowance for the competitive substrate inhibition was shown by varying the concentrations of the two reactants together in constant ratio (tri-/diphosphate = 14). For both the MgADP–MgATP and MgUDP–MgUTP exchanges such experiments gave completely linear reciprocal plots, thus confirming the form of the rate equation (see Discussion).

Identification of a Phosphoenzyme. The Ping-Pong kinetic mechanism indicated by the kinetic studies suggests that a stable phosphoenzyme should be formed during the enzymatic reaction. The formation of such an intermediate was shown by incubating enzyme with [γ-^{32}P]MgATP and [8-^{14}C]MgATP and chromatographing the reaction mixture on Sephadex G-25. The elution pattern (Figure 7) showed a peak of active enzyme containing ^{32}P but not ^{14}C preceding the bulk of the radioactivity. Upon rechromatography, the ratio of ^{32}P to enzyme activity remained constant throughout the peak. Incubation of this phosphoenzyme with MgADP and MgATP removed the ^{32}P, however, as shown by chromatography again on Sephadex (Figure 8). The ^{32}P-containing fractions from this last column were pooled and a portion was chromatographed on DEAE-cellulose paper in the usual system (see Methods); the ^{32}P was found in the position of ATP. The product was also identified as ATP by descending chromatography for 20 hr on Whatman 1 paper with isobutyric acid–NH_4OH–water (66:1:33) as solvent.

The stability of the phosphoenzyme was tested by incubating portions of it at pH 9.2 at 80° for 5 min, or at pH 0.5 at 100° for 2 min, followed by formation and extraction at low temperature of phosphomolybdate into isobutyl alcohol–benzene. After low pH treatment, all ^{32}P was found as P_i, while at pH 9.2 the amount of ^{32}P found in P_i was the same as for an unheated sample of phosphoenzyme (a certain amount of hydrolysis is caused by the acid that must be added to permit formation of phosphomolybdate).

Discussion

The parallel initial velocity patterns seen in the present work suggest that the mechanism of the reaction catalyzed by nucleoside diphosphate kinase is Ping-Pong (that is, that the first product is released from the enzyme surface before the second substrate combines). If there is only one site for absorption of all reactants (or at least the combination of reactants is mutually exclusive) such a mechanism may be represented as de-



(7)

picted in eq 7, where E is free enzyme, F is phosphoenzyme, and in the present work A and P are MgATP and MgADP, and B and Q are MgUDP and MgUTP. The

TABLE I: Kinetic Constants for Yeast Nucleoside Diphosphate Kinase.

Type of Constant	Forward Reaction		Reverse Reaction	
	MgATP	MgUDP	MgADP	MgUTP
Michaelis (mM)	$K_a = 0.31 \pm 0.01$	$K_b = 0.043 \pm 0.001$	$K_p = 0.050 \pm 0.002$	$K_q = 0.25 \pm 0.01$
Inhibition (mM)				
From isotopic exchange	$K_{ia} = 0.28 \pm 0.05$	$K_{ib} = 0.045 \pm 0.002$	$K_{ip} = 0.054 \pm 0.010$	$K_{iq} = 0.03 \pm 0.02$
From product inhibition	$K_{ia} = 0.21 \pm 0.04$	$K_{ib} = 0.040 \pm 0.001$	$K_{ip} = 0.057 \pm 0.003$	$K_{iq} = 0.35 \pm 0.09$
Dead-end inhibition (mM)	$K_{Ia} = 3.1$	$K_{Ib} = 0.78 \pm 0.10$	$K_{Ip} = 0.14 \pm 0.02$	$K_{Iq} = 2.7$
Maximum velocity (μmoles/min/μg of enzyme)	$V_1 =$	5.9 ± 0.2	$V_2 =$	5.1 ± 0.1

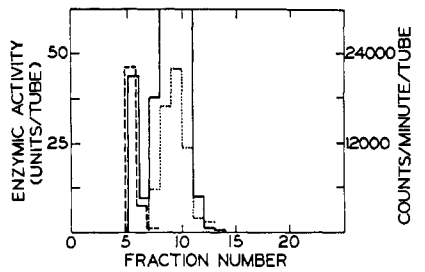


FIGURE 7: Separation of ^{32}P-labeled nucleoside diphosphate kinase from [^{14}C]- and [^{32}P]ATP on Sephadex. In 0.1-ml volume were placed 0.1 M triethanolamine acetate (pH 8.0), 1 mM [γ-^{32}P, 8-^{14}C]ATP (specific activities 2.9 and 1.1 mCi/mmole), 2 mM magnesium acetate, and 0.18 mg of enzyme. After incubation at 30° for 2 min, the reaction mixture was placed on a 26 × 1 cm column of Sephadex G-25 (fine) and eluted with 0.1 M triethanolamine acetate (pH 7.5) at 27 ml/hr at 4°. Fractions (2 ml) were collected. (———) ^{32}P, (· · · · · ·) ^{14}C, and (– – – – – –) enzymatic activity.

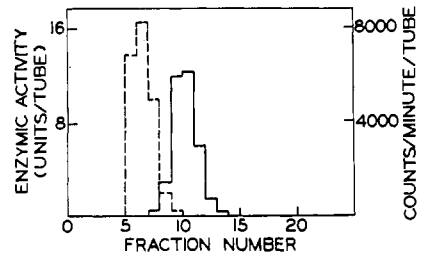


FIGURE 8: Chromatography of phosphoenzyme after incubation with MgADP and MgATP. Fraction 5 from the experiment shown in Figure 7 was incubated for 2 min at 30° with 0.3 mM MgADP, 1.0 mM MgATP, 1.0 mM free Mg^{2+}, and 0.1 M triethanolamine acetate (pH 8.0). The reaction mixture was then chromatographed on the original Sephadex column as described in Figure 7. Fractions (2 ml) were collected. (———) ^{32}P and (– – – – – –) enzymatic activity.

rate equation describing initial velocity in the absence of products in such a mechanism is eq 3.

The competitive substrate inhibition observed for all substrates is characteristic for such Ping-Pong mechanisms and results from combination of reactants in dead-end fashion with the wrong stable enzyme form (A or Q with F, or B or P with E). The effect is competitive since the combination in dead-end fashion is with the same enzyme form as the other substrate, and the characteristic observed result is an increase in the slopes (but not intercepts) of reciprocal plots at high levels of the nonvaried substrate. This is most clearly seen in Figure 6, where the parallel nature of the pattern without inhibition is clear. Equation 4 describes such a pattern and least-squares fits to this equation yield both of the Michaelis constants as well as the dissociation constant of the inhibitory substrate from its dead-end complex. When the substrate inhibition occurs only at levels sufficient to saturate the enzyme, as in Figure 2, a pattern resembling normal competitive inhibition is seen and from such an experiment it is possible to determine for the inhibitory substrate only the dissociation constant from its dead-end complex.

Apparent maximum velocities were determined by varying the concentrations of both substrates together in constant ratio. For Ping-Pong mechanisms reciprocal plots of such data are linear, and by determining both maximum velocities in the same experiment, one can derive an accurate estimate of the ratio between them for use in checking the Haldane relationship (see below). When competitive substrate inhibitions occur, however, one must correct the observed maximum velocities for this phenomenon. The rate equation predicted by mechanism 7 for initial velocities in the absence of products but allowing for competitive substrate inhibition by both substrates is

$$v = \frac{VAB}{K_aB(1 + B/K_{Ib}) + K_bA(1 + A/K_{Ia}) + AB}$$

where K_a and K_b are Michaelis constants for A and B, K_{Ia} and K_{Ib} are dissociation constants for A and B from dead-end complexes with F and E, and V is the maximum velocity. If $B = xA$, where x is a constant, this equation reduces to

$$v = \frac{[V/(1 + K_ax/K_{Ib} + K_b/(xK_{Ia}))]A}{(K_a + K_b/x)/(1 + K_ax/K_{Ib} + K_b/(xK_{Ia})) + A}$$

which has the form of eq 2 where

$$\text{apparent } V = \frac{V}{(1 + K_ax/K_{Ib} + K_b/(xK_{Ia}))}$$

A reciprocal plot should be still linear but the observed maximum velocity will be too small. The correction will be a minimum at a value of x given by

$$x = \sqrt{\frac{K_bK_{Ib}}{K_aK_{Ia}}}$$

but unless K_{Ia} is much greater than K_a and K_{Ib} is much greater than K_b the correction cannot be ignored; in the present case the correction factors calculated from the constants in Table I and the value of x used in the experiments were 1.30 and 1.46 for forward and reverse reactions. The corrected values for the maximum veocities are given in Table I.

Mechanism 7 predicts that nucleoside diphosphates will be mutually competitive in product inhibition experiments and that the same will be true for the triphosphates. On the other hand, any corresponding di- and triphosphate should give noncompetitive inhibition. These are precisely the patterns observed in Figures 3 and 4 and all of the possible patterns conform to these predictions. The product inhibition data can also be used to derive the numerical values of the dissociation (as opposed to Michaelis) constants for the reactants. These constants which are called inhibition constants by Cleland (1963b) do not appear in the rate equation for initial velocity in the absence of products for a Ping-Pong mechanism and must be calculated from product inhibition data or measured by isotopic exchange (see

below). The rate equation predicted by mechanism 7, ignoring substrate inhibition, is

$$v = \frac{V_1(AB - PQ/K_{eq})}{K_aB + K_bA + AB + \frac{K_{ia}K_bP}{K_{ip}} + \frac{K_aK_{ib}Q}{K_{iq}} + \frac{K_aK_{ib}PQ}{K_pK_{iq}} + \frac{K_bAP}{K_{ip}} + \frac{K_aBQ}{K_{iq}}} \quad (8)$$

where K_a, K_b, K_p, and K_q are Michaelis contants for A, B, P, and Q, K_{ia}, K_{ib}, K_{ip}, and K_{iq} are the inhibition (or dissociation) constants (equal to k_2/k_1, k_6/k_5, k_3/k_4, and k_7/k_8 in mechanism 7), V_1 is the maximum velocity in the forward direction (V_2 would be the maximum velocity in the reverse direction), and K_{eq} is the equilibrium constant for the reaction of A and B to give P and Q. When the concentration of Q is set equal to zero in eq 8 and the resulting equation rearranged to show product inhibition by P, we get eq 9 or 10 for the cases where A or B are varied

$$\frac{1}{v} = \frac{K_a}{V_1}\left[1 + \frac{P}{\left(\frac{K_aK_{ip}B}{K_{ia}K_b}\right)}\right]\frac{1}{A} + \frac{1}{V_1}\left[1 + \frac{K_b}{B}\right]\left[1 + \frac{P}{K_{ip}(1 + B/K_b)}\right] \quad (9)$$

$$1/v = \frac{K_b}{V_1}\left[1 + \frac{P}{K_{ip}/(1 + K_{ia}/A)}\right]\frac{1}{B} + \frac{1}{V_1}[1 + K_a/A] \quad (10)$$

When these equations are compared with eq 5 or 6 it is apparent that for the competitive inhibitions (eq 5 and 10)

$$\text{apparent } K_{is} = \frac{K_{ip}}{(1 + K_{ia}/A)}$$

while for the noncompetitive case (eq 6 and 9)

$$\text{apparent } K_{is} = K_{ip}\left[\frac{B}{\left(\frac{K_{ia}K_b}{K_a}\right)}\right]$$

$$\text{apparent } K_{ii} = K_{ip}\left(1 + \frac{B}{K_b}\right)$$

Determination of K_{ip} from such data requires a prior knowledge of other kinetic constants; the value is normally determined from apparent K_{ii} since the Michaelis constants are known, and the relationships involving apparent K_{is} are used to check internal consistency among the constants. Values of the inhibition constants derived in this fashion are given in Table I.

Mechanism 7 predicts that isotopic exchange should be observed between A and P in the absence of B and Q, or between B and Q in the absence of A and P. The rate equation describing the initial velocity of the A–P exchange is

$$v^* = \frac{\left(\frac{V_1K_{ia}}{K_a}\right)AP}{K_{ip}A + K_{ia}P + AP} \quad (11)$$

where the constants are the same as those in eq 8. For B–Q exchange, B, Q, K_{ib}, and K_{iq} replace A, P, K_{ia}, and K_{ip}, and the apparent maximum velocity is V_1K_{ib}/K_b (the apparent maximum velocities can also be written V_2K_{ip}/K_p and V_2K_{iq}/K_q for the two equations). This equation has the form of eq 3 except that it includes inhibition constants in the denominator instead of Michaelis constants. The inhibition constants are determined in this way more directly than from product inhibition data and the values derived in this manner are shown separately in Table I.

When competitive substrate inhibition is observed in initial velocity experiments, it is also expected in exchange studies, and at high levels of any substrate, eq 11 will have the form of eq 4 (that is, high levels of A multiply the A term by $(1 + A/K_{ia})$, and high levels of P multiply the P term by $(1 + P/K_{ip})$). The patterns shown in Figures 5 and 6 both show competitive substrate inhibition, and the values for the dissociation constants of MgATP and MgUTP from their dead-end complexes with phosphoenzyme given in Table I are derived from fits of the data in Figure 6 and others not shown to eq 4. There is no doubt that the patterns in Figures 5 and 6 do correspond to eq 4, however, since linear reciprocal plots were obtained for both exchanges when their reactant concentrations were varied in constant ratio. This can only happen when the original rate equation lacks a constant term in the denominator (as do eq 3 and 4), and substrate inhibition is strictly competetive.

The kinetic constants for any mechanism are not completely independent but must be related to the thermodynamic equilibrium constant by what are called Haldane relationships. The Haldanes for mechanism 7 are

$$K_{eq} = \frac{K_{ip}K_{iq}}{K_{ia}K_{ib}} = \frac{V_1K_{ip}K_q}{V_2K_{ia}K_b} = \frac{V_1K_pK_{iq}}{V_2K_aK_{ib}} = \left(\frac{V_1}{V_2}\right)^2\frac{K_pK_q}{K_aK_b}$$

1.28 1.28 1.31 1.26 1.28

Below each expression is the value calculated from the kinetic constants in Table I (the inhibition constants used were those from the isotopic exchange studies since they are determined directly); these are compared with the equilibrium constant of 1.28 at pH 8.0, 30°, measured in this laboratory. The excellent agreement confirms the postulated mechanism and justifies confidence in the values of the kinetic constants.

The kinetic constants can also be used to calculate values for the partial equilibria in the reaction. Thus, for the reaction

$$\text{MgATP} + \text{E} \rightleftharpoons \text{F} + \text{MgADP}$$

$$K_{eq\,1} = \frac{V_1K_p}{V_2K_a} = 0.188$$

and for the reaction

$$\text{MgUDP} + \text{F} \rightleftharpoons \text{E} + \text{MgUTP}$$

$$K_{eq\,2} = \frac{V_1K_q}{V_1K_b} = 6.76$$

These equilibria favor the free enzyme, suggesting that the form in which phosphate is bound in F has a free energy of hydrolysis about 1000 cal more negative than the bond in MgATP.

The kinetic mechanism described above suggests that form F is a phosphoenzyme which could be isolated. This is indeed the case as shown by the experiments illustrated in Figures 7 and 8. The phosphoenzyme retains its activity after chromatography on Sephadex and is capable of transferring the phosphate back to MgADP to give MgATP. The lability at low pH, but stability at high pH of the enzyme–phosphate bond, and its free energy of hydrolysis, suggest that the phosphate is bound as phosphohistidine rather than as phosphoserine or in some other form. Phosphohistidine has been identified as the active group in partially purified nucleoside diphosphate kinase from Jerusalem artichokes (Norman *et al.*, 1965), and has been isolated (along with some phospholysine, which may result from migration during hydrolysis) from nucleoside diphosphate kinases of erythrocytes and liver (Wälinder, 1968).

Some evidence concerning the number of active sites per molecule of enzyme can be obtained from these experiments. The molecular weight of yeast nucleoside diphosphate kinase has been determined to be 102,000 by Yue *et al.* (1967), and thus 1.75 mμmoles of enzyme was present in experiments similar to that shown in Figure 7. By taking into account the equilibrium between MgATP and free enzyme to form phosphoenzyme and MgADP (see above), one can calculate the millimicromoles of phosphoenzyme that should have been formed if the enzyme has a given number of active sites per molecule. These figures come out 4.3, 5.4, and 6.4 for 3, 4, and 5 active sites per molecule. The actual incorporation was in the range of 5 to 6 mμmoles of ^{32}P, suggesting that there may be 4 sites/molecule.

Since the molecular weight of yeast nucleoside diphosphate kinase is 102,000, the molecular activity of the enzyme for phosphorylation of MgUDP by MgATP is 600,000 min^{-1}. If there are four active sites per molecule the turnover number or catalytic center activity would be 2500 sec^{-1}. Since the dissociation constant of MgADP is 5.4×10^{-5} M and the unimolecular rate constant for dissociation of MgADP must exceed the turnover number, the bimolecular rate constant for combination of MgADP and enzyme has a minimum value of about 5×10^{7} M^{-1} sec^{-1}, which is close to the limit set by diffusion. Thus the turnover number could not be any higher unless the dissociation constant were also higher, since the turnover number cannot exceed the rate at which MgADP can dissociate from the enzyme. The Haldane relationships place certain constraints on the values of the kinetic constants, and in this case it is easy to see that one cannot raise the dissociation constant of MgADP (K_{ip}) and V_1 both without raising the dissociation or Michaelis constants of the substrates (K_{ia}, K_a, K_{ib}, or K_b) or lowering the Michaelis constant of MgUTP (K_q). (V_2 cannot be raised without increasing the dissociation constant of MgUDP (K_{ib}) for the same reasons which apply to V_1 and K_{ip}.) Raising the Michaelis or dissociation constants of the substrates is self-defeating, since the resulting decrease in degree of saturation under physiological conditions will cancel the value of any increase in maximum velocity, and lowering the constants for MgUTP will result in undesirable product inhibition which would also cancel the value of an increased maximum velocity. Thus, within the restraints imposed by the physiological concentrations of the reactants, the size of diffusion-limited bimolecular rate constants, and the Haldanes, yeast nucleoside diphosphate kinase appears to have the highest feasible turnover numbers.

Examination of the kinetic constants in Table I shows that the Michaelis constants for the triphosphates are about six times those of the diphosphates, so that the enzyme seems adapted to rephosphorylate other nucleoside diphosphates at the expense of MgATP. Under physiological conditions the enzyme will be largely saturated with MgATP, and in fact some substrate inhibition by MgATP may be present at very low nucleoside diphosphate levels (this should cause no problem physiologically, however, since the inhibition is competitive). It is interesting to note the order of magnitude differences between the Michaelis and dead-end inhibition constants for all reactants except MgADP. For MgADP the dead-end inhibition constant is only three times its Michaelis constant; this no doubt reflects the fact that MgADP is physiologically a product and never a substrate, so that there has never been any evolutionary pressure to produce specificity of MgADP binding to phospho- as opposed to free enzyme.

The kinetic mechanism deduced from the present work for the yeast enzyme seems similar in all respects to that shown for the erythrocyte enzyme by Mourad and Parks (1966a,b). The erythrocyte enzyme also involves a stable phosphoenzyme intermediate and was shown by several kinetic techniques to involve combination of all reactants at only one site. Presumably the active sites of these enzymes are specific only for a purine or pyrimidine base, ribose or 2-deoxyribose, and the proper number of phosphates. It would be intriguing to see what sort of substrates ribose or deoxyribose 5-di- or triphosphates would be!

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