Initial Rate and Equilibrium Isotope Exchange Studies on the ATP-Dependent Activity of Polyphosphate Glucokinase from *Propionibacterium shermanii*†

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ABSTRACT: Polyphosphate glucokinase [EC 2.7.1.63] catalyzes the phosphorylation of glucose using either inorganic polyphosphate [poly(P)] or ATP as the phosphoryl donor. Both activities purified from *Propionibacterium shermanii* are the functional properties of a single enzyme with separate binding sites for the two phosphoryl donor substrates. The enzyme was found to utilize poly(P) much more efficiently than it does ATP, with a $k_{cat}/K_{poly(P)}$ to k_{cat}/K_{ATP} ratio of 2800. The catalytic constant for poly(P) is about 2-fold higher than for ATP. Other nucleotides like GTP and dATP also served as substrates with good efficiencies. The ATP-dependent reaction was analyzed using steady-state kinetics and isotopic exchange kinetics at chemical equilibrium. Intersecting initial velocity patterns for both glucose and ATP indicate sequential addition of substrates. Product inhibition studies resulted in two competitive and two noncompetitive patterns, which is characteristic of a Theorell−Chance mechanism or a random mechanism with two dead-end complexes. Results of isotope exchange experiments, however, rule out a Theorell−Chance mechanism, as well as a truly random mechanism. They are most consistent with a partially random mechanism (although a kinetically compulsory order of substrate binding is not excluded), where glucose is preferentially bound to free enzyme before ATP, and ADP is preferentially released as the first product, followed by glucose 6-phosphate. Dead-end inhibition analysis confirms this order of substrate binding. Competitive inhibition of ADP *vs* ATP is explained as resulting primarily from binding as a dead-end inhibitor (E·Glc·ADP) and not as a product. Another weaker abortive complex, E·ATP·G6P, is also formed. The chemical transformation or the release of ADP is the rate-limiting step in ATP utilization.

Polyphosphate glucokinase [EC 2.7.1.63] was first observed in *Mycobacterium phlei* by Szymona (1957, 1962), who reported phosphorylation of glucose with inorganic polyphosphate [poly(P),[1] reaction 1] and also with ATP (reaction 2).

$$\text{glucose} + \text{poly(P)}_n \rightarrow \text{glucose 6-phosphate} + \text{poly(P)}_{n-1} \quad (1)$$

$$\text{glucose} + \text{ATP} \rightarrow \text{glucose 6-phosphate} + \text{ADP} \quad (2)$$

Inorganic polyphosphates are linear polymers of orthophosphate residues linked by phosphoanhydride bonds, whose free energy of hydrolysis is thermodynamically comparable to that of ATP. These polymers have been found in almost all representatives of living cells (Kulaev, 1979).

Glucokinases that utilize inorganic poly(P) to phosphorylate glucose, on the other hand, are relatively rare and have been reported to be present mostly in the bacteria belonging to the order Actinomycetales, according to the classification of Krasil'nikov (Kulaev, 1979). Besides its presence in the Actinomycetales, poly(P) glucokinase has also been found in *Myxococcus coralloides* (González et al. 1990), in bacterial parasite *Bdellovibrio bacteriovorus* (Bobyk et al. 1980), and in the oligotrophic bacteria *Renobacter vacuolatum* [Nikitin et al. (1983), and referred to by Kulaev and Vagabov (1983)]. The enzyme was found in the propionibacteria by Uryson and Kulaev (1968), and its purification and characterization have been reported from our laboratory (Phillips et al., 1993). It was concluded from those studies that a single enzyme was responsible for both the poly(P)- and ATP-dependent activities of the propionibacterial glucokinase. Except for the studies on the mode of utilization of poly(P) by the propionibacterial enzyme (Pepin & Wood, 1986, 1987), there has been virtually no mechanistic information on this type of glucokinase from any source. The lack of this information has hindered a detailed understanding of the evolutionary development of the bifunctionality of this enzyme.

In this study, we have utilized steady-state analysis and measurements of isotope exchange at chemical equilibrium to determine the kinetic mechanism of the ATP-dependent glucokinase reaction of *Propionibacterium shermanii*. The results are consistent with a sequential mechanism in which glucose is preferentially (or even obligatorily) bound to free enzyme before ATP, and ADP is preferentially released as the first product, followed by G6P. The system apparently approaches rapid equilibrium in the sense that glucose and

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[1] Abbreviations used: poly(P), inorganic polyphosphate; poly(P)_n, polyphosphate with *n* phosphate residues; G6P, glucose 6-phosphate; PEI, polyethyleneimine; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography.

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G6P equilibrate rapidly between free and bound forms. The catalytic center of poly(P) glucokinase is flexible, as suggested by the ability of this enzyme to utilize various phosphoryl donors and to form unreactive mixed complexes with substrates and products.

EXPERIMENTAL PROCEDURES

Materials. G6P dehydrogenase (Type XXIII from *Leuconostoc mesenteroides*, or recombinant, expressed in *Escherichia coli*), poly(P) (phosphate glass Type 35 with average chain length of 31), sodium salts of G6P, ATP, ADP, NAD^+, and $NADP^+$ were all from Sigma. [^{32}P]ATP (3000 Ci/mmol, 10 mCi/mL) and D-[^{14}C(U)]Glc (251 mCi/mmol, 0.1 mCi/mL) were from Du Pont-New England Nuclear. Thin-layer plates of PEI-impregnated cellulose with fluorescence indicator (POLYGRAM CEL 300 PEI/UV_{254}) were purchased from Macherey-Nagel (Germany) several years ago and stored at 4 °C; currently available plates from the same manufacturer have different physical properties and do not give good separations of nucleotides. CytoScint ES liquid scintillation cocktail was from ICN Biomedicals. DEAE-5PW TSK-Gel column (0.75 × 7.5 cm, particle size 10 μm) was from TosoHaas. Protein Assay Kit II was from Bio-Rad.

Purification and Assay of Poly(P) Glucokinase. The enzyme was purified by Method 1 as described (Phillips et al., 1993). Both the ATP- and poly(P)-dependent glucokinase activities were assayed spectrophotometrically by measuring the rate of G6P formation by coupling with G6P dehydrogenase and monitoring the reduction of NAD^+ or $NADP^+$ (Pepin & Wood, 1986). However, for kinetic assays involving product inhibition by G6P, the ATP-dependent activity was assayed by measuring the rate of ADP formation by coupling to pyruvate kinase and lactate dehydrogenase as described previously (Phillips et al. 1993). Both assays were carried out on a Shimadzu-UV160U spectrophotometer thermostated at 30 °C with a CPS 240A temperature controller. The assay mixture contained, in a final volume of 250 μL, 200 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM NAD^+ or $NADP^+$, 1 unit of G6P dehydrogenase, and various concentrations of glucose and phosphoryl donor. One unit of poly(P) glucokinase activity is defined as that amount of enzyme that catalyzes the formation of 1 μmol of G6P or ADP per min. The catalytic constant (k_{cat}) is calculated in terms of active site concentration assuming one active site per subunit (M_r = 30 000) of the dimeric enzyme (Phillips et al. 1993). The enzyme concentration was determined by the Bradford dye-binding procedure (Bradford, 1976) as described in the Bio-Rad protein assay protocol using bovine serum albumin as a standard.

Initial Velocity Data Analysis. Initial velocity data were first analyzed for linearity of double-reciprocal plots and quality of fitting to theoretical hyperbolas using nonlinear regression analysis ("GraFit" program by Leatherbarrow from Erithacus Software). Simple weighting was chosen since the residual error was found to be independent of velocity values. Slopes and intercepts obtained from primary double-reciprocal plots were then plotted against either the reciprocal of the nonvaried substrate concentration (for initial velocity experiments) or the inhibitor concentration (for inhibition experiments) to determine the form of the overall rate equation. Final values for the kinetic constants were obtained by fitting all data points used in the first analysis to this overall equation. Data conforming to a Steady-State Ordered Bi Bi mechanism were fitted to eq 3. Data from product and dead-end inhibition experiments were fitted to eq 4, 5, or 6 for linear competitive, noncompetitive, or uncompetitive inhibition, respectively, unless otherwise stated [Cleland's definitions and notations are used throughout (1963a,b)].

$$v = \frac{V_1AB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (3)$$

$$v = \frac{V_1A}{K(1 + I/K_{is}) + A} \quad (4)$$

$$v = \frac{V_1A}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (5)$$

$$v = \frac{V_1A}{K + A(1 + I/K_{ii})} \quad (6)$$

The choice of an appropriate inhibition model was primarily based on inspection of double-reciprocal plots (primary and secondary). In the cases where distinguishing a competitive or uncompetitive model from a noncompetitive one based on a plot alone was difficult, the data were fitted to two different equations to evaluate the slope and intercept effects of an inhibitor. A noncompetitive model was considered inappropriate, if fitting to its equation resulted in very high (>70%) standard error of one of the inhibition constants (i.e., K_{ii} or K_{is}) and/or very high (>10) or very low (<0.1) K_{ii}/K_{is} ratio. Usually these criteria for rejecting the noncompetitive model agreed with lower variance for the competitive or uncompetitive models (Mannervik, 1982). Data showing substrate inhibition were fitted to eq 7 (Gulbinsky & Cleland, 1968), where K_I is the apparent substrate inhibition constant.

$$v = \frac{VA}{K + A + A^2/K_I} \quad (7)$$

Isotope Exchange at Equilibrium. Equilibrium isotope exchange experiments were carried out according to established procedures (Hsuanyu & Wedler, 1987; Wedler, 1995). Exchange rates were determined at 30 °C in the presence of 100 mM Tris/HCl, pH 7.5, and $MgCl_2$ in 8−10 mM excess[2] over the total concentrations of ATP and ADP, in the final volume of 101 μL. The concentration of poly(P) glucokinase in the stock solution was 0.53 mg/mL as determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. The concentrations of substrates[3] were varied from below to about 10 times their K_m values. Stock solutions of ATP, ADP (~0.5 M), and G6P (~0.6 M) were adjusted to pH 7 with 1 M NaOH and stored at −20 °C. No $MgCl_2$ was added to the stock solutions of the

[2] A constant excess of >5 mM $MgCl_2$ is sufficient to ensure that the nucleotides exist in a large and nearly constant proportion as MgATP and MgADP (Storer & Cornish-Bowden, 1976; Gregoriou et al., 1981).

[3] In this paper, the term "substrates" refers to ATP and glucose while "products" refers to ADP and G6P. The term "reactants" refers to all reacting species, i.e., substrates and products.

nucleotides since at this pH and concentration MgATP and MgADP precipitate. The ATP and ADP concentrations were determined by measurement of the absorption at 260 nm assuming $\epsilon_{260,pH7} = 15.4\ mM^{-1}\ cm^{-1}$ (Bock et al., 1956). The concentration of G6P was determined enzymatically using G6P dehydrogenase and NAD^+ (Michal, 1984; Viola, 1984) in the presence of 80 mM Hepes buffer, pH 7.8, and 6 mM $MgCl_2$. The concentration of glucose was also determined enzymatically (Kunst et al., 1984) at pH 7.8 in a coupled assay with poly(P) glucokinase from *P. shermanii* and G6P dehydrogenase, using poly(P) as a source of phosphate residues (9.4 mM in terms of P_i, supplemented with 12 mM $MgCl_2$).

For experiments with one substrate-product pair varied, two stock solutions, A and B, were prepared. Solution A (containing all reaction components at maximal concentrations and at ratios consistent with the anticipated equilibrium constant) was mixed with solution B (containing identical levels of components except those being varied) in different proportions to the same final volume (95 μL). Then 5 or 4 μL of poly(P) glucokinase (0.06−0.28 units) was added, and the reactions were incubated for 30 min at 30 °C to allow exact enzymic adjustment to chemical equilibrium. The enzyme was stable under these conditions. Isotopic exchange was initiated by adding 1 μL of [^{32}P]ATP (0.64−0.93 pmol, 1.22−3.05 μCi) or 2 μL of [^{14}C]Glc (0.8 nmol, 0.2 μCi). Labeled Glc is supplied in 90% ethanol so that the final concentration of ethanol in the reaction mixtures was 1.8%; this, however, did not influence the enzyme activity. The amount of added labeled ATP was less than 1/1000th that of the unlabeled ATP. The amount of labeled Glc did not exceed 1.3% of the pool size of unlabeled Glc. The reactions were then incubated for a specific time period, usually 30 min or less. Three time samples (25 μL) were taken after each 5−10 min and transferred to 5 μL of ice cold 24 M formic acid to quench the reaction. Substrates and products in each sample were then separated, and the fraction of isotopic equilibrium *(F)* attained at any given time was calculated as described below.

To determine the equilibrium exchange when all four reactants were varied in an equilibrium ratio, a stock solution was prepared containing maximal levels of all reactants and diluted to obtain the appropriate range of concentration values. Other conditions were the same as described above. To evaluate the possible influence of large changes in ionic strength caused by high concentrations of reagents on enzyme-rate behavior, initial rate experiments were performed in the presence of 200 mM Tris/HCl and 200 mM NaCl. No change in velocities was observed when the results were compared with determinations performed in 100 mM buffer lacking NaCl.

ATP and ADP were separated by applying 2−5 μL of the samples taken from the reactions at different time intervals on a PEI cellulose thin-layer plate, followed by development of the plate with freshly prepared 0.8 M ammonium bicarbonate in an open tank. The nucleotide-containing spots were visualized under UV light, cut from the thin-layer plate, and immersed in 2 mL of CytoScint scintillation cocktail, and their radioactive content was determined in a Beckman LS 9000 liquid scintillation counter.

Glc and G6P were separated by HPLC using a Shimadzu SCL-6A liquid chromatograph. Samples of 25 μL taken from the reactions were loaded on DEAE-5PW TSK-Gel column at room temperature. Glc and G6P were eluted at a flow rate of 1 mL/min with water (for Glc), followed by 0.5 M NaCl (for G6P), and 1-mL fractions were collected. Under these conditions, baseline separation of both compounds was achieved within 15 min. Then 200 μL of each fraction was mixed with 3 mL of CytoScint, and the radioactivity was determined in a Beckman scintillation counter.

Table 1: Substrate Specifity Constants of Poly(P) Glucokinase

substrate[a]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
$poly(P)_{31}$	56	1.2×10^{-3}	4.7×10^7
ATP	25	1.5	1.7×10^4
GTP	11	0.8	1.4×10^4
dATP	14	1.2	1.2×10^4
UTP	7.0	2.6	2.7×10^3
CTP	7.4	5.9	1.3×10^3

[a] Nucleotide concentrations were varied up to 4 mM, the $poly(P)_{31}$ concentration was varied from 4 to 32 μM (in terms of polymer), and the concentration of glucose was 10 mM in all cases.

In all isotope exchange experiments, controls were performed at the highest and lowest substrate concentrations to evaluate the stability of the enzyme and reactants throughout all steps of the experimental protocol. The proportionality of the isotopic exchange rates to the enzyme concentration was also checked.

Isotope Exchange Data Processing. First, the fraction of isotopic equilibrium between substrate A and P attained at three time points was calculated from the relationship $F = p([A] + [P])/(a + p)[P]$ (Wedler & Ley, 1993), where [A] and [P] are chemical equilibrium concentrations expressed in mM while *a* and *p* are the disintegrations/min measured in the respective pools at these time points. Then $\log(1 - F)$ was plotted *versus* incubation time, and a straight line (fitted using linear least-squares analysis) crossing the *y*-axis close to zero was obtained, which confirmed that the exchange process was first order (Segel, 1993). From this plot, the half-time ($t_{1/2}$), after which 50% of isotopic equilibrium has been achieved, was determined and used to calculate the initial exchange velocity (v^*) expressed in μmol $mL^{-1}\ min^{-1}$, according to eq 8 (Segel, 1993).

$$v^* = \left(\frac{[A][P]}{[A] + [P]}\right)\left(\frac{0.693}{t_{1/2}}\right) \quad (8)$$

RESULTS

Substrate Specificity of Poly(P) Glucokinase. The efficiency of utilization of different phosphoryl donors was assessed by determining the specificity constants, k_{cat}/K_m, for alternative substrates at saturating glucose level. It is obvious from the results displayed in Table 1 that the enzyme utilizes poly(P) much more efficiently than it does ATP, with a $k_{cat}/K_{poly(P)}$ to k_{cat}/K_{ATP} ratio of 2800. Since there is only a 2-fold difference in the k_{cat} while the K_m differs by 3 orders of magnitude, the much greater substrate specificity for poly(P) is due to the rate constants that determine substrate binding rather than those responsible for catalysis or product release. Other nucleotides, especially GTP and dATP, are also utilized with good efficiencies.

Initial Velocity Kinetics. At lower ATP concentrations up to 4 mM, initial velocity double-reciprocal plots with both ATP and glucose as the varied substrate were intersecting above the *x*-axis (not shown). At higher ATP levels,

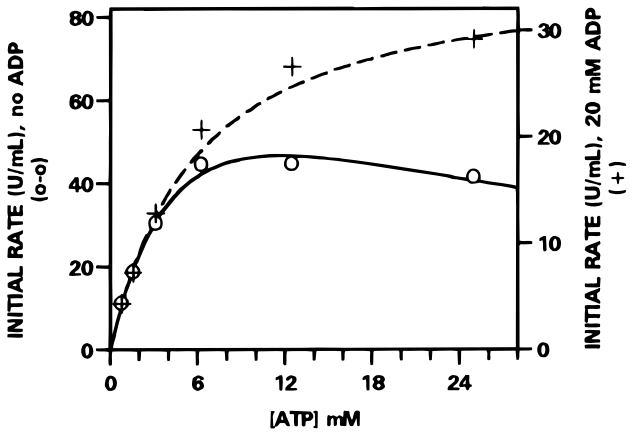

FIGURE 1: Substrate inhibition by ATP under initial velocity conditions. Glucose was 1 mM and $MgCl_2$ was in 6 mM excess over the total ATP and ADP concentrations. Data obtained in the absence of ADP (circles, left scale) were fitted to eq 7. Fitting parameters for the solid line are $V = 93 \pm 18$ (standard error) units/mL, $K = 5.8 \pm 1.7$ mM, and $K_I = 24 \pm 10$ mM. The dashed line represents velocity values that would be obtained in the absence of substrate inhibition assuming the same values for V and K. Data obtained in the presence of 20 mM ADP (crosses, right scale) were not fitted.

Table 2: Kinetic Parameters for the ATP-Dependent Reaction from Initial Velocity Studies

	kinetic constant ± SE[a]
k_{cat} (s^{-1})	38 ± 2
K_{Glc} (mM)	0.47 ± 0.07
K_{ATP} (mM)	1.35 ± 0.17
$K_{i,Glc}$ (mM)	2.05 ± 0.26
k_{cat}/K_{Glc} (M^{-1} s^{-1})[b,c]	8.1 (±0.8) × 10^4
k_{cat}/K_{ATP} (M^{-1} s^{-1})[b,c]	2.8 (±0.2) × 10^4
$k_{cat}/K_{i,Glc}K_{ATP}$ (M^{-2} s^{-1})[c,d]	1.38 (±0.07) × 10^7

[a] Kinetic constants were calculated assuming a Steady-State Ordered Bi Bi mechanism with glucose binding to the enzyme first followed by ATP (eq 3). SE, standard error. [b] Lower limit of the second-order rate constant for productive substrate binding. [c] Value of this parameter was obtained directly by the fitting procedure after reparameterizing eq 3 (Northrop, 1983) and replacing V_1 with $2k_{cat}E_t$, where $E_t = 0.16$ mg/mL. [d] Overall catalytic efficiency.

however, substrate inhibition was apparent (Figure 1), which limits the useful range of ATP concentrations that can be used in initial velocity studies. This inhibition was found to be uncompetitive with respect to glucose (not shown) and influenced by the concentration of ADP. Figure 1 shows that in the presence of 20 mM ADP, the regular hyperbolic dependence of the initial velocity on ATP concentration was obtained (note, however, the different scales used in Figure 1: lower velocities in the presence of ADP are due to product and dead-end inhibition by ADP). A similar effect was also observed at 30 mM ADP, while at lower ADP concentrations substrate inhibition was still apparent (not shown). The kinetic constants calculated from 30 data points obtained at noninhibitory ATP levels are shown in Table 2. The highest glucose concentration used in initial velocity experiments was 2 mM.

Product and Dead-End Inhibition Patterns. The inhibition patterns together with inhibition constants for both products and competitive substrate analogs are shown in Table 3. A noticeable characteristic of the results is competitive inhibition displayed by both products against structurally related substrates (i.e., ADP *vs* ATP and G6P *vs* Glc) as well as noncompetitive inhibition against structurally unrelated substrates. Moreover, the inhibition by ADP *vs* glucose appears almost uncompetitive. The interpretation of these results is addressed under Discussion.

Initial Velocities of Isotopic Exchange at Chemical Equilibrium. The equilibrium constant at pH 7.5, expressed in terms of total nucleotide concentrations, was assumed to be around 234 (Gregoriou et al., 1981), since under conditions used in these experiments the G6P concentration was not altered when all four reactants present at this ratio were incubated with poly(P) glucokinase for up to 2 h. The terms "saturating" and "unsaturating" refer only to ATP and glucose concentrations since K_m values for ADP and G6P are not known for this enzyme.

The initial rates of the ATP↔ADP and Glc↔G6P exchanges were first measured as functions of all four reactant concentrations which were increased in a constant ratio to minimize effects of possible dead-end complex formation and competition between the exchanging pair and the pair whose concentration is raised (Wedler & Boyer, 1972; Wedler, 1995). Figure 2 shows that the ATP↔ADP exchange rate increased with increasing concentrations of all reactants without reaching a maximum, although the enzyme should be almost completely saturated by the substrates at the highest concentrations used in this experiment (i.e., [ATP] = 14.6 mM and [Glc] = 6.93 mM), as can be concluded from initial velocity measurements performed under steady-state conditions in the absence of products (Figure 3). It is likely, however, that the ATP↔ADP exchange rate actually approached its maximum value, but this is not obvious from Figure 2 due to a slight inhibitory or discontinuous behavior of the increase in the ATP↔ADP exchange above the relative concentration of 10. On the other hand, the Glc↔G6P exchange was first stimulated and then inhibited moderately. Because of limited solubility of reactants and relatively high value of the equilibrium constant, it was technically difficult to determine exchange rates at reactant concentrations higher than those of Figure 2. Figure 2 also reveals sigmoidicity of the ATP↔ADP and Glc↔G6P exchanges at low reactant concentrations. This effect, as well as the discontinuous behavior of the ATP↔ADP curve, was reproducible in repeated experiments.

In the next step, structurally related reactant pairs (i.e., ATP/ADP[4] or Glc/G6P) were varied in constant ratio, with coreactants held constant near half-saturation.

As shown in Figure 4, increasing the ATP/ADP concentration from about half-saturating to saturating levels (in terms of ATP) at constant unsaturating concentrations of glucose and G6P resulted in a smooth increase in the ATP↔ADP exchange rate to a maximum, whereas the Glc↔G6P exchange exhibited strong inhibition effects. Interestingly, at low ATP/ADP concentrations, the rate of Glc↔G6P exchange rose more steeply than that for ATP↔ADP, so that the ratio of the two rates was 2 at 10 mM ATP. At saturating ATP concentration, however, the ATP↔ADP rate became almost 5 times faster than that for Glc↔G6P.

Upon varying Glc/G6P with fixed unsaturating levels of ATP and ADP (Figure 5), both ATP↔ADP and Glc↔G6P exchanges were weakly and proportionally inhibited. The

[4] ATP/ADP indicates ATP and ADP at constant ratio. This designation is used for other reactant concentrations as well.

Table 3: Product and Dead-End Inhibition Patterns from Initial Velocity Studies

inhibitor[a]	variable substrate	fixed substrate[b]	pattern[c]	$K_{is} \pm$ SE[d]	$K_{ii} \pm$ SE[d]
ADP (0−30)	MgATP	glucose (1)	C	6.8 ± 0.6	
ADP (0−20)	glucose	MgATP (1)	NC[e]	105 ± 46	12.0 ± 0.9
G6P (0−30)	MgATP	glucose (0.5)	NC	22 ± 7	30 ± 10
G6P (0−30)	glucose	MgATP (1)	C	18 ± 2	
xylose (0−80)	glucose	MgATP (5)	C	41 ± 2	
xylose (0−30)	MgATP	glucose (0.5)	NC	31 ± 5	27 ± 5
poly(P)$_4$ (0−56)	MgATP	glucose (10)	C	21 ± 2	
poly(P)$_4$ (0−28)	glucose	MgATP (4)	UC		25.9 ± 0.7

[a] Values in parenthesis give the range of inhibitor concentrations in mM [except for poly(P)$_4$ which is given in μM] used in these studies. [b] In mM. [c] Abbreviations for the inhibition patterns are C, competitive, NC, noncompetitive, and UC, uncompetitive. SE = standard error. [d] K_{is} and K_{ii}, apparent inhibition constants (in mM), represent the values of inhibitor concentration that double the slope or intercept in the double-reciprocal plot, respectively. [e] Fitting these data to an uncompetitive model gives $K_{ii} = 10.4 \pm 0.5$ mM with a slightly higher value of variance than for a noncompetitive model. *F*-test analysis shows that the probability that the two models are equally appropriate is 0.62.

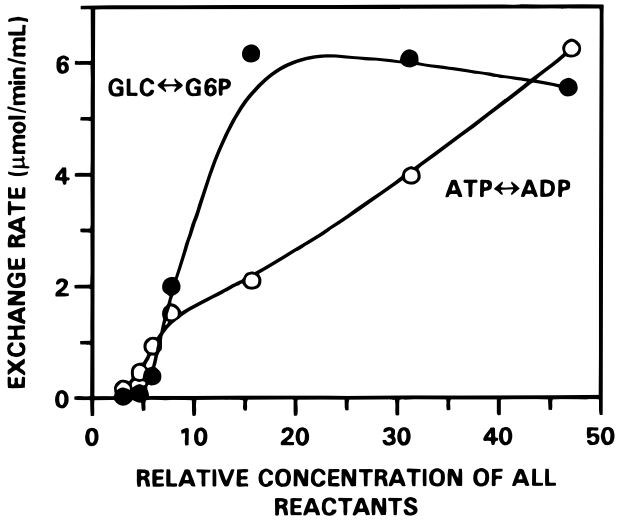

FIGURE 2: Initial rates of isotopic exchange for the ATP↔ADP and Glc↔G6P reactions. The concentrations of all four reactants were raised in constant equilibrium ratio from 0.97 mM ATP, 0.46 mM Glc, 10.1 mM ADP, and 10.3 mM G6P to 14.6 mM ATP, 6.93 mM Glc, 152 mM ADP, and 155 mM G6P.

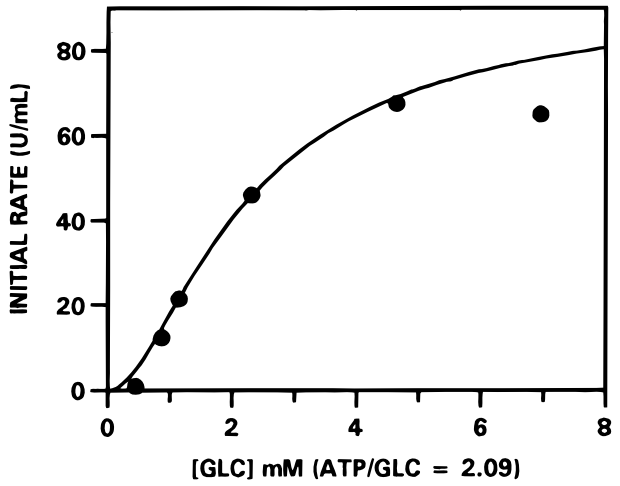

FIGURE 3: Initial velocity under steady-state conditions as a function of glucose and ATP concentrations varied in constant ratio. The line is described by eq 3, where B is replaced by $2.09A$. The parameters are $V_1 = 96$ units/mL, $K_a = 0.47$ mM, $K_b = 1.35$ mM, and $K_{ia} = 5$ mM. Substrate inhibition, apparent at the highest ATP concentration (14.6 mM), is not taken into account by this equation.

Glc↔G6P exchange rate was slightly faster than that for ATP↔ADP at low Glc/G6P concentration, while at saturating glucose levels the ATP↔ADP exchange rate was slightly faster than that for Glc↔G6P. Unlike the situation with ATP/ADP as variable, there was relatively little disparity between both exchange rates in this case.

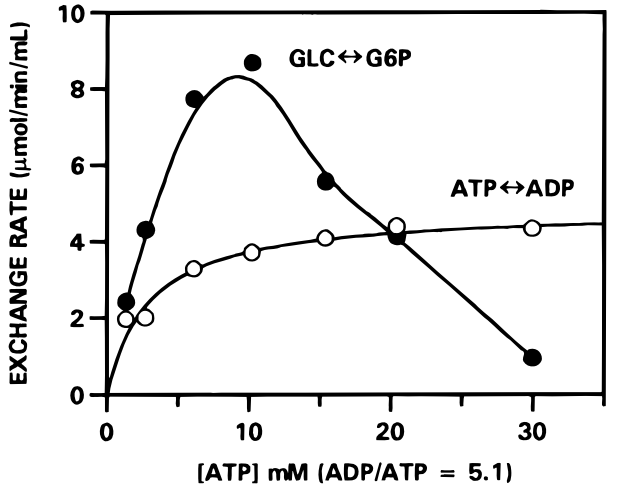

FIGURE 4: Effect of ATP/ADP concentration on the initial rates of the ATP↔ADP and Glc↔G6P isotopic exchanges at chemical equilibrium. Reaction mixtures contained 0.62 mM Glc, 28.3 mM G6P, and ATP/ADP as indicated in the figure. Data points for the ATP↔ADP exchange were fitted to the equation $v = VA/(K + A)$ for a rectangular hyperbola ($V = 4.8$ units/mL, $K = 2.9$ mM). The line drawn through the data for the Glc↔G6P exchange is intended to illustrate the shape of the plot and is not fitted to any data analysis program.

Varying the concentration of structurally dissimilar pairs (i.e., ATP/G6P or Glc/ADP) yielded results that were in many respects similar to those described above for structurally related pairs.

When ATP/G6P was increased in the presence of constant unsaturating concentrations of glucose and ADP (Figure 6), both exchanges initially rose, and then gradually decreased, with the Glc↔G6P exchange being inhibited much more strongly than the ATP↔ADP exchange. The Glc↔G6P exchange was faster than the ATP↔ADP exchange at all ATP/G6P concentrations; however, if the ATP↔ADP exchange were not inhibited by this reactant pair, a pattern similar to that shown in Figure 4 would be obtained.

Increasing concentration of Glc/ADP at constant ATP and G6P (Figure 7) resulted in almost parallel increase and decrease of both exchanges, with 2 times stronger inhibition of the Glc↔G6P exchange. As with variable Glc/G6P (Figure 5), relatively little disparity between both exchange rates was observed in this case; however, the Glc/ADP pair

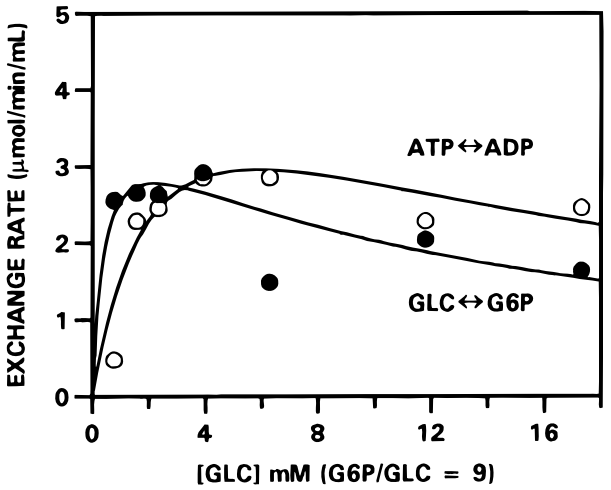

FIGURE 5: Effect of Glc/G6P concentration on the initial rates of the ATP↔ADP and Glc↔G6P isotopic exchanges at chemical equilibrium. Reaction mixtures contained 1.5 mM ATP, 39.1 mM ADP, and Glc/G6P as indicated in the figure. The data fitted to eq 7. The fitting parameters are $V = 5.5$ units/mL, $K = 2.5$ mM, $K_I = 13.6$ mM in terms of Glc (or 122.4 mM in terms of G6P) for the ATP↔ADP exchange, and $V = 3.8$ units/mL, $K = 0.4$ mM, $K_I = 12$ mM in terms of Glc (or 108 mM in terms of G6P) for the Glc↔G6P exchange.

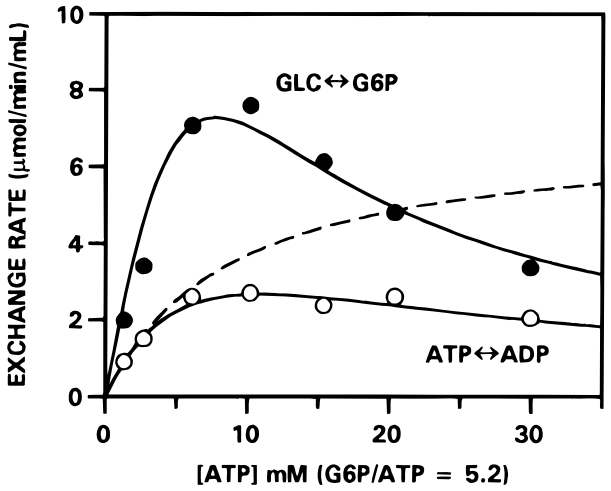

FIGURE 6: Effect of ATP/G6P concentration on the initial rates of the ATP↔ADP and Glc↔G6P isotopic exchanges at chemical equilibrium. Reaction mixtures contained 0.62 mM Glc, 28 mM ADP, and ATP/G6P as indicated in the figure. The data were fitted to eq 7. The fitting parameters are $V = 6.9$ units/mL, $K = 8.8$ mM, $K_I = 13.6$ mM in terms of ATP (or 70.7 mM in terms of G6P) for the ATP↔ADP exchange, and $V = 120$ units/mL, $K = 60$ mM, $K_I = 1$ mM in terms of ATP (or 5.2 mM in terms of G6P) for the Glc↔G6P exchange. The dashed line represents velocity values for the ATP↔ADP exchange that would be obtained in the absence of substrate inhibition assuming the same values for V and K.

inhibited both exchanges much more strongly (10−20 times, on the basis of K_I values) than the Glc/G6P pair.

DISCUSSION

Substrate Specificity of Poly(P) Glucokinase. The broad substrate specificity displayed by poly(P) glucokinase from *P. shermanii* is very similar to that observed for this enzyme from the phylogenetically younger bacterium, *Mycobacterium tuberculosis* (Hsieh et al., 1993). The k_{cat}/K_m values for various phosphoryl donors and the enzyme from the latter source were similar to those shown in Table 1, except for

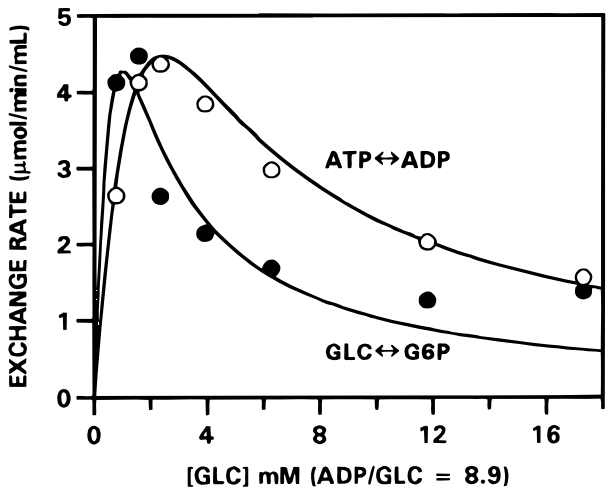

FIGURE 7: Effect of Glc/ADP concentration on the initial rates of the ATP↔ADP and Glc↔G6P isotopic exchanges at chemical equilibrium. Reaction mixtures contained 1.5 mM ATP, 39.5 mM G6P, and Glc/ADP as indicated in the figure. The data were fitted to eq 7. The fitting parameters are $V = 19$ units/mL, $K = 4.18$ mM, $K_I = 1.39$ mM in terms of Glc (or 12.4 mM in terms of ADP) for the ATP↔ADP exchange, and $V = 18.4$ units/mL, $K = 1.64$ mM, $K_I = 0.61$ mM in terms of Glc (or 5.43 mM in terms of ADP) for the Glc↔G6P exchange.

GTP (which was 2.0×10^5 M^{-1} s^{-1}) and poly(P) (which was 8.1×10^6 M^{-1} s^{-1}). However, the $k_{cat}/K_{poly(P)}$ to k_{cat}/K_{ATP} for the mycobacterial enzyme was ~200, which is 14 times less than that for the propionibacterial enzyme. This finding is consistent with the hypothesis that the poly(P)-dependent activity is evolutionary more ancient than the ATP-dependent activity of poly(P) glucokinase (Phillips et al. 1993).

Kinetic Mechanism of the ATP-Dependent Reaction

Theorell−Chance and Random Mechanisms Are Ruled Out. The intersecting initial velocity patterns of the double-reciprocal plots indicate that the mechanism is sequential, i.e., both substrates must add to the enzyme before either product is released. The symmetrical product inhibition patterns with respect to both products (Table 3) are characteristic of an ordered Theorell−Chance mechanism (Segel, 1975) or a random mechanism with two dead-end complexes (E·ATP·G6P and E·ADP·Glc) (Gulbinsky & Cleland, 1968; Raushel & Cleland, 1977). Additionally, uncompetitive inhibition *vs* glucose displayed by $poly(P)_4$ which is competitive *vs* ATP (Table 3) suggests that glucose binds to the enzyme before ATP. However, a Theorell−Chance mechanism is ruled out by the observation that the intersection points in the initial velocity double-reciprocal plots are above the horizontal axis. In this mechanism, the vertical coordinate of the crossover point of the initial velocity pattern for the forward reaction is given by $(1/V_1 - 1/V_2)$, and since for glucokinase V_1 (maximum velocity of the forward reaction) is presumably much larger than V_2 (maximum velocity of the reverse reaction), the intersecting point should be far below the horizontal axis and look nearly parallel (Raushel & Cleland, 1977), as was observed in the case of liver alcohol dehydrogenase (Theorell & McKinley-McKee, 1961). Results of isotope exchange experiments also exclude a Theorell−Chance mechanism. Since in this mechanism central complexes are kinetically insignificant and release of reactants from the binary complexes is solely rate-limiting

(Janson & Cleland, 1974), the extrapolated maximum exchange velocity between the inner pair of reactants (presumably ATP↔ADP) should be infinite or at least much faster than the maximum velocity for the chemical reaction when the concentration of this pair is varied (Ainslie, Jr., & Cleland, 1972; Purich & Allison, 1980). Moreover, the linear dependence of this exchange *vs* the absolute concentration of the varied pair should be observed (Purich & Allison, 1980). Figure 4 shows that the ATP↔ADP exchange is finite, hyperbolically dependent on the ATP/ADP concentration, and ~20 times slower than the rate of ATP utilization (Figure 3). Note, however, that the apparent V is lower than the true maximum rate of the ATP↔ADP exchange since not all reactants were saturating in this experiment.

A truly random mechanism,[5] on the other hand, is also excluded on the basis of the following results. (i) The inhibition patterns for the dead-end inhibitors xylose and poly(P)$_4$ (Table 3) are indicative of an ordered mechanism of substrate addition with glucose binding first followed by ATP; if the mechanism were random, then these inhibition patterns would have been symmetrical [i.e., two competitive and two noncompetitive (Fromm, 1995)]. However, these patterns are also consistent with random but highly synergistic binding of sugars and nucleotides (Viola et al., 1982) leading to a preferred order mechanism. (ii) Although the inhibition patterns for alike reactants (i.e., ADP *vs* ATP and G6P *vs* Glc) do converge on the vertical axis in a truly random mechanism, they will normally look noncompetitive at low concentrations of the variable substrate; moreover, the double-reciprocal plots should be nonlinear (Viola et al., 1982). In our experiments, the linearly competitive patterns were obtained, even when the substrate concentrations did not exceed four times their respective K_m values. (iii) For a random mechanism, both isotopic exchanges (i.e., ATP↔ADP and Glc↔G6P) should increase smoothly to a maximum without any inhibitory effects when the concentrations of all reactants are increased simultaneously in a constant ratio (Wedler & Boyer, 1972; Purich & Allison, 1980). Figure 2 shows that this is not the case. The pattern is consistent with compulsory order (or preferred order random) binding, where glucose and G6P either bind to the enzyme prior to (or faster than) nucleotides or dissociate after (or more slowly than) nucleotides from the central complexes or both may be true (Wedler & Ley, 1993). Noncompetitive inhibition due to binding nucleotides to the enzyme at noncatalytic sites [e.g., at poly(P) binding site] could also account for the observed inhibition of the Glc↔G6P exchange. This is, however, unlikely since in such a case both exchanges should be inhibited proportionally upon varying all substrates simultaneously, unless differential effect of an inhibitor on both exchanges is present (Wedler, 1974; Wedler & Gasser, 1974). The present data do not disprove (or support) the latter possibility, though. The sigmoidal effect observed in Figure 2 for both exchanges is most likely due to multiorder kinetics at low concentrations of substrates (Purich & Allison, 1980).

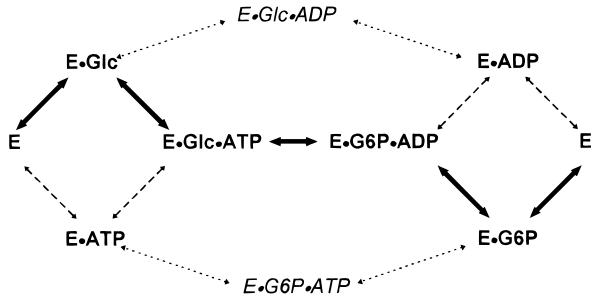

FIGURE 8: Postulated kinetic scheme for the ATP-dependent poly(P) glucokinase reaction. Glucose phosphorylation occurs in catalytically effective and interconvertible ternary complexes. Catalytically effective steps are indicated by solid (preferred order) and dashed (alternative order) arrows, and catalytically ineffective steps are indicated by dotted arrows leading to unreactive ternary complexes (in italics). The steps shown by dashed lines appear to make a negligible contribution to the reaction under steady-state conditions.

Mechanism Is Compulsory Ordered or Preferred Order Random. Other results (besides those discussed above) also support the concept of highly ordered mechanism of the ATP-dependent reaction catalyzed by poly(P) glucokinase. The observed strong inhibition of the Glc↔G6P exchange by increasing ATP/ADP concentration (Figure 4) indicates that glucose and G6P must preferentially bind to the enzyme before nucleotides. It was expected for a strictly compulsory mechanism that the Glc↔G6P exchange rate would have been lower than the ATP↔ADP exchange rate at all ATP/ADP concentrations. The unpredicted pattern of Figure 4 can be explained by dissociation of glucose and G6P from reactive ternary complexes (dashed lines in Figure 8), although at a much slower rate than from binary complexes. In this case, when certain relationships between rate constants for binding and dissociation hold (i.e., faster *association* of glucose and G6P with the ternary complexes and faster *dissociation* of ATP and ADP from those complexes), the Glc↔G6P exchange can be faster than the ATP↔ADP exchange at low ATP/ADP concentrations and slower upon saturation (Boyer & Silverstein, 1963), since association steps kinetically dominate equilibrium exchange rates at subsaturating substrate levels whereas dissociation steps determine these rates at near-saturating substrate levels (Wedler & Ley, 1993). A similar effect was reported for bovine-liver glutamate dehydrogenase with $NADP^+$ as coenzyme (Silverstein & Sulebele, 1973). It does not necessarily mean, however, that the mechanism must be partially random. An alternative possibility is that the binary complexes formed after dissociation of glucose and G6P (i.e., E·ATP and E·ADP) are dead-end and cannot further dissociate to release free enzyme (Purich & Allison, 1980). This would provide a second route of exchange between glucose and G6P even in a kinetically compulsory order of substrate binding. Nevertheless, the possibility of random dissociation of ternary complexes formed in ordered systems seems unlikely (Fromm et al., 1964).

A compulsory or preferred order of reactant binding is further confirmed by the fact that both isotopic exchanges are not proportionally inhibited by increasing concentration of ATP/G6P (Figure 6). The stronger inhibition of the

[5] In a *random order* of substrate (or product) addition, the addition of substrates to an enzyme or their dissociation from enzyme-substrate complexes occurs at equal rate whether or not another substrate is bound to the enzyme. In a *preferred order random* mechanism (also called *partially compulsory* or *partially random*), the rate constant for binding or dissociation of a substrate is affected by the presence of another substrate on the enzyme, yet complexes of free enzyme with either substrate may be formed. In a *compulsory* order mechanism, the addition of one specific substrate must occur before another substrate can be bound (Boyer & Silverstein, 1963; Wedler & Ley, 1993).

Glc↔G6P exchange compared to the ATP↔ADP exchange cannot be entirely due to the formation of an abortive E·G6P·ATP complex (see below) (Wedler & Ley, 1993) and implies an additional effect, most likely arising from ATP binding after glucose and from ADP being released prior to G6P.

Abortive Complexes. Several observations indicate that various unreactive ternary complexes are formed (Boyer & Silverstein, 1963; Silverstein & Sulebele, 1973), which is consistent with the apparently flexible nature of the catalytic center of poly(P) glucokinase (suggested by the broad substrate specificity as discussed above). (i) Moderate inhibition of the ATP↔ADP exchange seen in Figure 6 is compatible with the formation of the weak abortive E·G6P·ATP complex. Substrate inhibition by ATP, uncompetitive *vs* glucose and competitive *vs* ADP, observed in initial velocity experiments (Figure 1) is also consistent with the formation of this complex. (ii) The strong and proportional inhibition of both isotopic exchanges by increasing concentration of Glc/ADP (Figure 7) indicates substantial formation of the abortive E·Glc·ADP complex. The inhibition of the Glc↔G6P exchange, possibly arising from ordered dissociation and binding of ADP before G6P, does not seem to contribute significantly to the pattern observed in Figure 7. In agreement with this observation, the unusual (for an ordered mechanism) competitive inhibition displayed by the first product (i.e., ADP) *vs* the second substrate (i.e., ATP) under initial velocity conditions (Table 3) can be explained by much stronger (or kinetically favorable) binding of ADP as a dead-end inhibitor to the E·Glc complex than as a product to the E·G6P complex. The observation that the noncompetitive inhibition displayed by ADP *vs* the first substrate (i.e., glucose) appears almost uncompetitive also supports the above explanation. Figure 7 additionally shows that even 154 mM ADP does not inhibit the Glc↔G6P exchange completely. This observation suggests that glucose can slowly dissociate not only from the reactive ternary complex as discussed above but also from the abortive E·Glc·ADP complex, which provides an additional route to exchange between glucose and G6P (Fromm et al., 1964; Purich & Allison, 1980). (iii) The formation of a third abortive complex, E·Glc·G6P, suggested by a weak inhibition of both isotopic exchanges by increasing concentration of Glc/G6P (Figure 5), is not unequivocal and thus is not considered in the postulated kinetic scheme in Figure 8. The presence of this abortive complex was not detected in initial velocity and product inhibition studies under steady-state conditions (Table 3): its formation should have resulted in substrate inhibition by glucose and/or noncompetitive inhibition by G6P *vs* glucose and parabolic slope effect with ATP as the variable substrate. It is possible that an apparent inhibition of the exchange reactions observed in Figure 5 is a nonspecific effect caused by high concentration of G6P (up to 155 mM). Even though a G6P·Mg complex has a small association constant, at this level of G6P the concentration of free Mg^{2+} might be smaller than expected.

Rate-Limiting Steps. There was relatively little disparity between the ATP↔ADP and Glc↔G6P exchanges, as in the case of bovine-liver glutamate dehydrogenase (Silverstein & Sulebele, 1973), in contrast to remarkable differences noted with rat-liver glucokinase (Gregoriou et al., 1981). This finding suggests that chemical transformation significantly contributes to the equilibrium reaction rates or that the substrates that add to the enzyme first (glucose and G6P), equilibrate very rapidly between free and bound forms. In other words, the rates of dissociation of sugars from the binary complexes are probably much faster than the rates of binding nucleotides to those complexes (Boyer & Silverstein, 1963). The resulting very low steady-state level of the E·G6P complex can explain the unusual kinetic behavior of ADP acting primarily as a dead-end inhibitor and not as a product (see above). The system, however, cannot be described as truly rapid-equilibrium since in that limiting case (where the level of E·G6P is zero) the substrate inhibition by ATP would not have been observed; also, inhibition of the Glc↔G6P exchange would have been difficult to obtain at moderate ATP/ADP concentrations. Alternatively, it is possible that rate-limiting dissociation rates for both exchanging reactant paris are fortuitously similar at certain reactant concentrations.

It is concluded from all the above results that the mechanism of ATP utilization by poly(P) glucokinase is predominantly an ordered process with glucose binding first and G6P released last, modified by the presence of various abortive complexes formed by the reacting species. Slow dissociation of glucose and G6P from the reactive ternary complexes is also postulated. The chemical transformation or the release of ADP significantly contributes to the observed rate of ATP utilization (catalytic constant).

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