# Kinetic Mechanisms of Polyphosphate Glucokinase from *Mycobacterium tuberculosis*†

Pei-Chung Hsieh,‡ Tomasz H. Kowalczyk,‡ and Nelson F. B. Phillips*,‡,§

*Departments of Biochemistry and Medicine, Case Western Reserve University, Cleveland, Ohio 44106*

*Received December 4, 1995; Revised Manuscript Received May 13, 1996*⊗

ABSTRACT: Polyphosphate glucokinase from *Mycobacterium tuberculosis* catalyzes the phosphorylation of glucose using inorganic polyphosphates [poly(P)] or ATP. The steady-state kinetic mechanisms of the poly(P)- and ATP-dependent glucokinase reactions were investigated using initial velocity, product inhibition, and dead-end inhibition analyses. In the poly(P)-dependent reaction, the enzyme follows an Ordered Bi Bi sequential mechanism with poly(P) binding to the enzyme first and glucose 6-phosphate dissociating last. Polyphosphate is utilized nonprocessively with a preference for longer chains due to higher $k_{cat}/K_m$ values. The lack of inhibition at high poly(P) concentrations suggests that binding of poly(P) as a product is not favorable. In the ATP-dependent glucokinase reaction, the data are also consistent with an Ordered Bi Bi sequential mechanism, with ATP binding to the enzyme first and glucose 6-phosphate leaving last. At high concentrations, ATP displays competitive substrate inhibition with respect to glucose, which is consistent with the formation of an enzyme·ATP·ATP nonproductive complex. The overall catalytic efficiencies ($k_{cat}/K_{ia}K_b$) of the poly(P)- and ATP-dependent reactions are $\sim 10^{11}$ $M^{-2}$ $s^{-1}$ and $\sim 10^8$ $M^{-2}$ $s^{-1}$, respectively. The higher catalytic efficiency, high value of the substrate specificity constant ($k_{cat}/K_a$) approaching a diffusion-controlled limit, and the absence of substrate inhibition in the poly(P)-dependent reaction suggest that poly(P), rather than ATP, is the major phosphate donor for poly(P)-glucokinase in *M. tuberculosis*.

Inorganic polyphosphates, poly(P),[1] are linear polymers of orthophosphate ($P_i$) residues linked by phosphoanhydride bonds whose free energy of hydrolysis is thermodynamically comparable to that of ATP. They have been found in almost all representatives of living cells including mammals (Kulaev, 1979) and human (Pisoni & Lindley, 1992). The *in vivo* role of these polymers is uncertain, although numerous functions have been proposed (Kulaev, 1979; Kornberg, 1995). One of them is substitution for ATP in certain kinase-catalyzed reactions (Kulaev & Vagabov, 1983; Wood & Clark, 1988). Polyphosphate glucokinase [poly(P)-glucokinase, EC 2.7.1.63], one of the few known enzymes that utilize poly(P) (Szymona, 1957, 1962), has only been found in *Mycobacterium tuberculosis* (Szymona & Widomski, 1974), *Nocardia minima* (Szymona & Szymona, 1979), *Propionibacterium shermanii* (Pepin & Wood, 1986), and some other bacteria (Szymona, 1957; Kulaev, 1979; González et al., 1990; Bobyk et al., 1980). This poly(P)-glucokinase utilizes poly(P) by transferring the terminal phosphate to glucose to generate glucose 6-phosphate:

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

Studies with the purified poly(P)-glucokinase from *M. tuberculosis* showed that this enzyme is also able to utilize ATP as the source of phosphate residues to generate glucose 6-phosphate without the involvement of poly(P) (Szymona et al., 1977; Hsieh et al., 1993b), suggesting a double function of this enzyme (reaction II). A similar observation was also reported with poly(P)-glucokinase isolated from *P. shermanii* (Pepin & Wood, 1986), although in a few cases these two activities were fractionated separately during purification of the enzyme (Kulaev, 1979; Szymona & Ostrowski, 1964; González et al., 1990).

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

We have demonstrated previously that the poly(P)- and ATP-dependent glucokinase activities of the enzyme from *P. shermanii* (Phillips et al., 1993) and *M. tuberculosis* (Hsieh et al., 1993b) are the catalytic properties of a single protein. Moreover, kinetic studies suggest that, although the binding site of the adenosine moiety of ATP is probably separate from the binding site of poly(P), the glucose binding site and the glucose phosphorylating center may be common for both poly(P) and ATP (Phillips et al., 1993; Hsieh et al., 1993b). Further studies provided evidence that one or two tryptophans may be located at a catalytic center common for both activities (Hsieh et al., 1993a). Despite the vast number of data available from the studies on poly(P)-glucokinase (Wood & Clark, 1988), neither systematic kinetic studies have been performed, nor was the kinetic mechanism of the enzyme determined. The lack of mechanistic information has hindered a detailed understanding of the bifunctionality of this enzyme, i.e., its ability to utilize both inorganic and organic phosphoryl donors.

† Supported by Grant GM 29569 from the National Institutes of Health.

* Corresponding author: Department of Medicine W-127, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4983. (216) 368-4816, Fax: (216) 368-4825.

‡ Department of Biochemistry.

§ Department of Medicine.

⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1996.

[1] Abbreviations: poly(P), inorganic polyphosphate; $poly(P)_n$, polyphosphate containing *n* phosphate residues; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; PAGE, polyacrylamide gel electrophoresis.

S0006-2960(95)02865-0 CCC: $12.00 

It is estimated that one-third of the world's population is infected with *M. tuberculosis* and that it will kill 30 million people in this decade (WWW: http://action.org). Although tuberculosis was thought to be eradicated in the United States, an increased number of reports indicates that acquired immunodeficiency syndrome is often associated with mycobacterial infection (Perriens et al., 1991). The recent resurgence of drug-resistant strains of *M. tuberculosis* (Alland et al., 1994; Snider & Roper, 1992) has triggered a renewed interest in the development of antituberculosis therapy, including the search for new targets for chemotherapeutic intervention. The poly(P)-glucokinase from *M. tuberculosis*, which is absent in the host, offers the potential to be such a target. However, an understanding of its kinetic properties is a prerequisite in the development of drugs targeted toward this mycobacterial enzyme.

In this paper, we analyze the steady-state kinetics of the poly(P)- and ATP-dependent glucokinase reactions. Based on initial velocity, product inhibition, and dead-end inhibitor studies, we conclude that both the reactions follow an Ordered Bi Bi mechanism, with glucose being the second substrate to bind and glucose 6-phosphate being released last. Our results also indicate that poly(P), rather than ATP, is a better substrate in terms of the substrate specificity and overall catalytic efficiency. To our knowledge, this is the first report elucidating the kinetic mechanism of two reactions of glucose phosphorylation with two different phosphoryl donors catalyzed by a single poly(P)-glucokinase.

## EXPERIMENTAL PROCEDURES

*Materials*. Acrylamide, *N*,*N*′-Methylenebis(acrylamide), and urea were from Boehringer; xylene cyanole FF was from Eastman Kodak; bromophenol blue was from Fisher; toluidine blue O, G6P dehydrogenase (type XXIII from *Leuconostoc mesenteroides*), L-lactate dehydrogenase (type XXXIX from rabbit muscle), poly(P) (phosphate glass type 35 with average chain length of 31), sodium salts of G6P and F6P, pyruvate, glucose, xylose, ADP, ATP, AMP, and NAD were all from Sigma. F6P supplied by Sigma contains ~1 mol % G6P which is a source of high background in spectrophotometric assays of poly(P)-glucokinase performed during inhibition studies with F6P. The level of this contaminating G6P was reduced by incubating 1.5 M F6P with 0.3 M NAD, 0.3 M pyruvate, 10 units of G6P dehydrogenase, and 10 units of lactate dehydrogenase. After completion of the reaction, the solution was deproteinized using Ultrafree-CL Filter Unit (10 000 NMWL) from Millipore. *M. tuberculosis* $H_{37}Ra$ was a gift from Dr. O. Szymona (Department of Biochemistry, Medical School of Lublin, Lublin, Poland) and was maintained as described previously (Hsieh et al., 1993b).

*Preparation of Poly(P)-Glucokinase*. The enzyme from *M. tuberculosis* $H_{37}Ra$ was purified to homogeneity as was described previously (Hsieh et al., 1993b). The purity of the protein was normally examined by SDS−PAGE (Laemmli, 1970), and 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.

*Assay of the Poly(P)-Dependent Glucokinase Activity*. For kinetic experiments, we used a coupled assay with G6P dehydrogenase as the coupling enzyme (Szymona et al., 1977; Hsieh et al., 1993b). The formation of G6P was followed indirectly by monitoring at 340 nm the amount of NADH formed ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$), using a Shimadzu-UV160U spectrophotometer thermostated at 30 °C with a CPS 240A temperature controller. One unit of poly(P)-glucokinase activity is defined as the amount of enzyme which catalyzes the formation of 1 $\mu$mol of G6P/min. The catalytic constant ($k_{cat}$) is calculated in terms of active site concentration assuming one active site per subunit ($M_r$ = 33 000) of the dimeric enzyme (Hsieh et al., 1993b). The assays in a total volume of 250 $\mu$L contained the following: 100 mM Tris-HCl (pH 7.5 or 8.6), variable concentration of glucose and poly(P) (Sigma phosphate glass type 35), 200 mM NaCl, 6 mM $MgCl_2$, 0.58 mM NAD, and 1 unit of G6P dehydrogenase. The stock solution of poly(P) was additionally supplemented with $MgCl_2$, so that the $P_i$/Mg ratio was 2 in that solution. Reactions were initiated with poly(P)-glucokinase, and all velocity measurements were obtained from linear portions of the progress curves. In product inhibition studies with G6P, the activity of poly(P)-glucokinase was measured by monitoring the disappearance of substrate, acid-labile polyphosphate, according to the method of Szymona (1962). In this case one unit of activity refers to the transfer of one orthophosphate residue from poly(P) to glucose per minute. The concentration of poly(P) (acid-labile phosphate) was determined by the method of Szymona (1962) with minor modifications as described previously (Kowalczyk & Phillips, 1993). Throughout the paper, the poly(P) concentration is expressed in terms of polymer, assuming average chain length of 31 phosphate residues, unless otherwise stated.

*Assay of the ATP-Dependent Glucokinase Activity*. The ATP-dependent glucokinase activity was measured spectrophotometrically as described above for the poly(P)-dependent activity. Stock solutions of ATP and ADP were prepared with $MgCl_2$ at a 1:1 molar ratios, and pH was adjusted to 7 with 1 M NaOH. Assay mixtures contained additional 6 mM $MgCl_2$ to maintain a constant amount of MgATP or MgADP (Storer & Cornish-Bowden, 1976). Excess amount of $Mg^{2+}$, up to 12 mM, does not inhibit the enzymatic activity (data not shown). The assay contained 100 mM Tris-HCl (pH 7.5 or 8.6), varied glucose and MgATP, 200 mM NaCl, 6 mM $MgCl_2$, 0.6 mM NAD, and 1 unit of G6P dehydrogenase (Hsieh et al., 1993b). One unit of ATP-dependent poly(P)-glucokinase activity is defined as that amount of enzyme which catalyzes the formation of 1 $\mu$mol of G6P/min. The catalytic constant ($k_{cat}$) is calculated as described above.

*Polyacrylamide Gel Electrophoresis of Poly(P)*. The procedure and the apparatus used were similar to those already described (Clark & Wood, 1987; Robinson et al., 1984; Pepin & Wood, 1986). Fractions of poly(P) having a narrow range of sizes were prepared and sized by the electrophoretic method of Clark and Wood (1987).

*Kinetic 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 value. Slopes and intercepts obtained from primary double-reciprocal plots were then plotted against either the reciprocal of the nonvaried substrate concentration (for initial velocity experi-

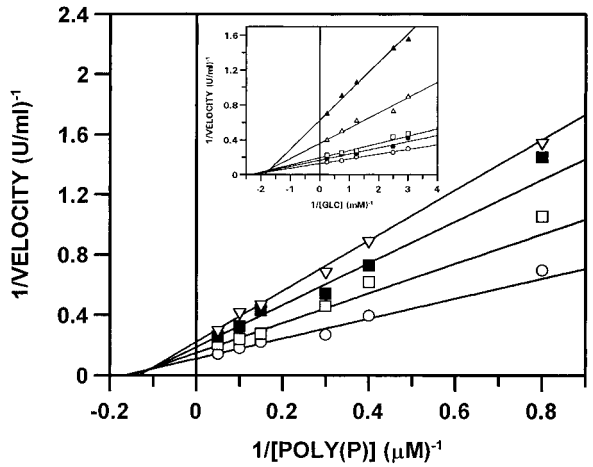



FIGURE 1: Initial velocity patterns in the poly(P)-dependent reaction at pH 8.6. Primary double-reciprocal plot with poly(P) as the variable substrate. The concentrations of the fixed substrate glucose in mM are (from top to bottom): 0.33, 0.4, 0.8, and 4.0. Inset: Primary double-reciprocal plot with glucose as the variable substrate. The concentrations of the fixed substrate poly(P) in $\mu$M are (from top to bottom): 1.25, 2.5, 6.67, 10, and 20.

ments) 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 III. Data from product and dead-end inhibition experiments were fitted to eqs IV, V, or VI for linear competitive, noncompetitive, and uncompetitive inhibitions, respectively, unless otherwise stated [Cleland's definitions and notations are used throughout (1963a,b)].

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

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

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

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

The choice of an appropriate inhibition model was primarily based on inspection of double-reciprocal plots (primary and secondary). In some cases, however, it was difficult to distinguish a competitive or uncompetitive model from a noncompetitive one based on a plot alone, in which case 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 VII, where $K_I$ is the apparent substrate inhibition constant and $V$ and $K$ are the apparent $V_1$ and $K_m$ (respectively), or to eq VIII, where $K_i^s$ is the true substrate inhibition constant. The rationale for using eq VIII is explained in Results.

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

$$v = \frac{V_1AB}{K_{ia}K_b + K_aB + K_bA(1 + A/K_i^s) + AB} \quad \text{(VIII)}$$

$K_I$ and $K_i^s$ are related to each other by:

$$K_I = K_i^s(1 + B/K_b) \quad \text{(IX)}$$

Values of $K_{is}$ and $K_{ii}$ for noncompetitive, competitive, or uncompetitive inhibition occurring simultaneously with substrate inhibition were calculated by fitting all data points obtained at various fixed inhibitor concentrations to eq X, XI, or XII, respectively. In these cases, the $K_I$ value was treated as a constant and calculated from eq IX.

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

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

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

For visualization of quality of fitting, the experimental data points are presented in plots along with theoretical lines fitted by "GraFit" program.

## RESULTS

### *Kinetic Mechanism of the Poly(P)-Dependent Glucokinase Reaction*

*Initial Velocity Studies*. When the initial velocity was determined at varying poly(P) concentrations (1.25−20 $\mu$M) and several fixed levels of glucose, a linear double-reciprocal plot with an intersecting pattern was obtained (Figure 1). The linearity of the graph was confirmed within a wide range of poly(P) concentrations (2.5−320 $\mu$M) at 29 mM glucose (not shown). The Michaelis constant for poly(P) at 29 mM glucose and pH 8.6 was found to be 7.0 ± 0.5 $\mu$M, which agrees very well with the value calculated from results obtained at nonsaturating glucose concentrations (Table 1). When glucose was the variable substrate (0.33−4 mM), a similar linear double-reciprocal plot was observed with all lines intersecting (Figure 1, inset). The slope and intercept replots of these primary plots were also linear (not shown). Similar results were obtained at pH 7.5 (not shown) which is closer to physiological conditions. These symmetrical initial velocity patterns with either substrate varied rule out a Rapid-Equilibrium Ordered mechanism, while linearity and intersecting patterns rule out a Ping Pong Bi Bi mechanism, as will be discussed in detail under Discussion. Moreover, when the velocity of the reaction was plotted against poly(P) and glucose concentrations, which were varied together at a constant ratio, a parabolic double-reciprocal plot was obtained (not shown), indicating a reversible connection of the enzyme forms between the points of addition of poly(P) and glucose (Segel, 1993a). In principle, linearity of reciprocal plots in Figure 1 also excludes a Steady-State

Table 1: Kinetic Parameters for the Poly(P)-Dependent Reaction at 30 °C

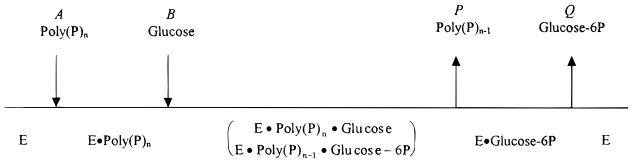
| | kinetic constant ± SE[a] | |
|---|---|---|
| | pH 7.5 | pH 8.6 |
| $k_{cat}$ ($s^{-1}$)[b] | 199 ± 10 | 208 ± 12 |
| $K_{Poly(P)}$ ($\mu$M)[c] | 13.9 ± 1.4 | 6.1 ± 0.9 |
| $K_{Glc}$ (mM) | 0.28 ± 0.04 | 0.37 ± 0.08 |
| $K_{i,Poly(P)}$ ($\mu$M)[c] | 3.4 ± 1.6 | 9.5 ± 3.1 |
| $k_{cat}/K_{Poly(P)}$ ($M^{-1}$ $s^{-1}$)[d,e] | 1.4 (±0.1) × $10^7$ | 3.5 (±0.3) × $10^7$ |
| $k_{cat}/K_{Glc}$ ($M^{-1}$ $s^{-1}$)[e,f] | 7.1 (±0.7) × $10^5$ | 5.6 (±0.9) × $10^5$ |
| $k_{cat}/K_{i,Poly(P)}K_{Glc}$ ($M^{-2}$ $s^{-1}$)[e,g] | 2.1 (±0.7) $10^{11}$ | 6 (±1) × $10^{10}$ |
| $K_{i,Poly(P)} \times k_{cat}/K_{Poly(P)}$ ($s^{-1}$)[h] | 48 | 333 |

[a] Kinetic constants were calculated assuming a Steady-State Ordered Bi Bi mechanism with poly(P) binding to the enzyme first and followed by glucose (eq III). SE = standard error. [b] Calculated from limiting maximum velocity expressed in terms of G6P concentration. [c] Expressed in terms of poly(P) concentration as polymer. [d] True second-order rate constant for productive binding (it applies to the first substrate). [e] Value of this parameter was obtained directly by the fitting procedure after reparametrizing eq III (Northrop, 1983) and replacing $V_1$ with $1.818k_{cat}E_t$, where $E_t$ = 0.048 mg/mL (pH 7.5) or 0.026 mg/mL (pH 8.6). [f] Lower limit of the second-order rate constant for productive binding (it applies to the second substrate). [g] Overall catalytic efficiency. [h] Calculated dissociation rate constant for an enzyme·poly(P) complex.

Random Bi Bi mechanism which is described by the velocity equation containing square terms of substrates concentrations (Segel, 1993b). However, if both routes to the ternary complex [i.e., enzyme·poly(P)·glucose] are about equally favorable, the departure from linearity would be impossible to detect and the pattern for a Steady-State Random Bi Bi mechanism would be similar to that shown in Figure 1. All these initial velocity patterns are consistent with a Rapid-Equilibrium Random Bi Bi mechanism or a Steady-State Ordered Bi Bi mechanism. The kinetic parameters are listed in Table 1. It is apparent from the table that changing pH from 7.5 to 8.6 influences only parameters associated with the binding of poly(P) to the enzyme.

*Product and Product Analog Inhibition Studies*. In order to distinguish between ordered and random mechanisms, product inhibition experiments at pH 8.6 were performed. When G6P was used as a product inhibitor, the rate of the reaction was determined by measuring the rate of decrease in concentration of acid-labile phosphorus, i.e., poly(P). With poly(P) as the variable substrate and glucose at a fixed concentration (20 mM), G6P was found to be a competitive inhibitor (not shown). Likewise, F6P, the product analog of G6P, also displayed linear competitive inhibition against poly(P). The use of F6P in this study allowed monitoring the rate of G6P formation, which provides a more accurate and convenient method than determining disappearance of poly(P). With glucose as the variable substrate and poly(P) fixed at 8 $\mu$M, the inhibition by F6P was noncompetitive. These results rule out random mechanisms and are consistent with an Ordered Bi Bi mechanism with poly(P) being the first substrate to add and G6P the last product to leave the enzyme as shown in Scheme 1. The release of shortened poly(P) before G6P is a surprising consequence of these results, because it suggests very different binding of poly(P) as substrate and product, as will be discussed below. All kinetic parameters and inhibition patterns are listed in Table 2. For this mechanism, the dissociation constant for enzyme·G6P complex can be estimated, since $K_{is} = K_{iq}$ when the first substrate is the variable one.

Scheme 1: A Proposed Kinetic Mechanism for Poly(P)-Glucokinase in the Poly(P)-Dependent Reaction




*Dead-End Inhibition Studies*. In order to confirm the order of substrate addition, dead-end analogs of the substrates were used as inhibitors (Chao et al., 1969). In the poly(P)-dependent reaction, xylose and AMP were found to be linearly competitive with glucose and poly(P), respectively (not shown). Xylose was an uncompetitive inhibitor with reference to poly(P) at a fixed nonsaturating glucose concentration (0.8 mM). AMP, on the other hand, displayed noncompetitive inhibition with glucose at a fixed nonsaturating concentration of poly(P) (6.7 $\mu$M). Again, random mechanisms for substrate addition are excluded because they would have resulted in symmetrical inhibition patterns for both inhibitors. In agreement with the initial velocity patterns, the Rapid-Equilibrium Ordered mechanism is also excluded, since neither of the inhibitors was competitive with respect to both substrates. These results are consistent with an Ordered Bi Bi mechanism with poly(P) binding first. All kinetic parameters and dead-end inhibition patterns are listed in Table 2. The inhibition constant ($K_i$) for F6P, as well as for xylose and AMP, was calculated independently from different data as described in Table 2, according to the established mechanism and the order of substrate binding. Close similarity of the obtained values provides an internal check for self-consistency of these data.

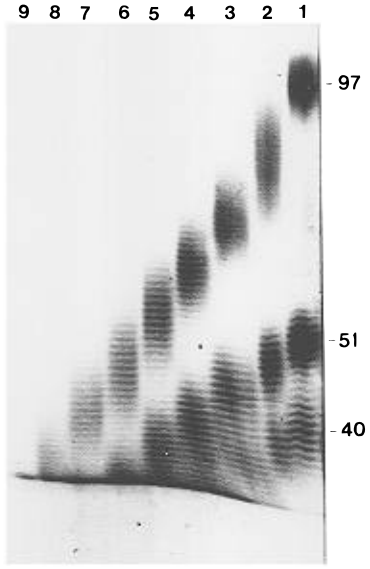



FIGURE 2: Simultaneous nonprocessive utilization of poly(P) of average chain lengths 40, 51, and 97. The reaction mixture contained 13 mM glucose, 7 mM $MgCl_2$, 200 mM NaCl, 100 mM Tris-HCl, pH 8.6, 0.03 unit of poly(P) glucokinase from *M. tuberculosis*, and about 1.3 $\mu$M poly(P) of each size [or in terms of phosphorus: 0.05 mM $poly(P)_{40}$, 0.07 mM $poly(P)_{51}$, and 0.12 mM $poly(P)_{97}$]. At different time intervals, aliquots (200 $\mu$L) were removed, the poly(P) was isolated, and the samples were electrophoresed at 10 mA into a 15% polyacrylamide gel containing 7 M urea. Lanes 1−9 are the poly(P) isolated after 0, 2, 4, 6, 8, 10, 12, 15, and 18 min, respectively. At 18, 21, 30, and 40 min, the poly(P) is at the salt line and, therefore, is not visible.

Table 2: Product and Dead-End Inhibition Patterns in the Poly(P)-Dependent Reaction at pH 8.6

| inhibitor[a] | variable substrate | fixed substrate | pattern[b] | $K_{is} \pm$ SE (mM)[c] | $K_{ii} \pm$ SE (mM)[c] |
|---|---|---|---|---|---|
| G6P (0−20) | poly(P) | glucose (20 mM) | C | $12.1 \pm 2.7$ ($=K_{iq}$) | |
| F6P (0−29) | poly(P) | glucose (12 mM) | C | $26 \pm 2$ ($=K_i$)[e] | |
| F6P (0−82) | glucose | poly(P) (8 $\mu$M) | NC[d] | $78 \pm 34$ ($K_i = 42$)[e] | $109 \pm 22$ ($K_i = 47$)[e] |
| xylose (0−10) | glucose | poly(P) (20 $\mu$M) | C | $2.5 \pm 0.4$ ($K_i = 1.7$)[f] | |
| xylose (0−10) | poly(P) | glucose (0.8 mM) | UC | | $9.0 \pm 0.6$ ($K_i = 2.8$)[f] |
| AMP (0−50) | poly(P) | glucose (1.3 mM) | C | $15.0 \pm 0.8$ ($=K_i$)[e] | |
| AMP (0−50) | glucose | poly(P) (6.7 $\mu$M) | NC | $33 \pm 11$ ($K_i = 20$)[e] | $26 \pm 3$ ($K_i = 12$)[e] |

[a] Values in parentheses give the range of inhibitor concentrations in mM used in these studies. [b] The abbreviations for the inhibition patterns are as follows: C, competitive; UC, uncompetitive; NC, noncompetitive. SE = standard error. [c] $K_{is}$ and $K_{ii}$, apparent inhibition constants, represent the values of inhibitor concentration that double the slope or intercept in the double-reciprocal plot, respectively. [d] Values of slope and intercept for the line obtained at 82 mM F6P are omitted from the calculations due to the departure from linearity in the secondary plots, probably due to inhibition of G6P dehydrogenase by F6P. [e] True inhibition constant ($K_i$, in mM) for an inhibitor competitive with substrate *A* [i.e., poly(P)] was calculated by dividing $K_{is}$ by $(1 + A/K_{ia})$ or $K_{ii}$ by $(1 + A/K_a)$ (Fromm, 1995); parameter values were taken from Table 1. Also, $K_i = K_{is}$ when *A* is the variable substrate and *B* is the fixed one. [f] True inhibition constant ($K_i$, in mM) for an inhibitor competitive with substrate *B* (i.e., glucose) was calculated by dividing $K_{is}$ by $(1 + K_{ia}/A)$ or $K_{ii}$ by $(1 + B/K_b)$ (Viola et al., 1982); parameter values were taken from Table 1.

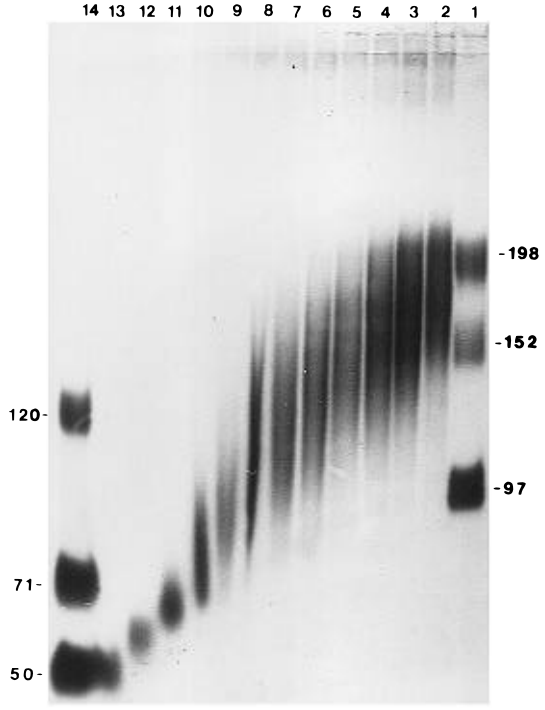



FIGURE 3: Nonprocessive utilization of poly(P) of average chain length 170. The reaction mixture was as in Figure 2, except that it contained 0.33 mM $poly(P)_{170}$ in terms of phosphate residues. The samples were electrophoresed at 13 mA into a 10% polyacrylamide gel without urea. The linearity of poly(P) migrations is between chain lengths 60 and 210 (Clark & Wood, 1987). Lanes 1 and 14 contain standards; lanes 2−13 are the poly(P) isolated after 0, 2, 4, 6, 8, 10, 12, 15, 18, 21, 24, and 30 min, respectively.

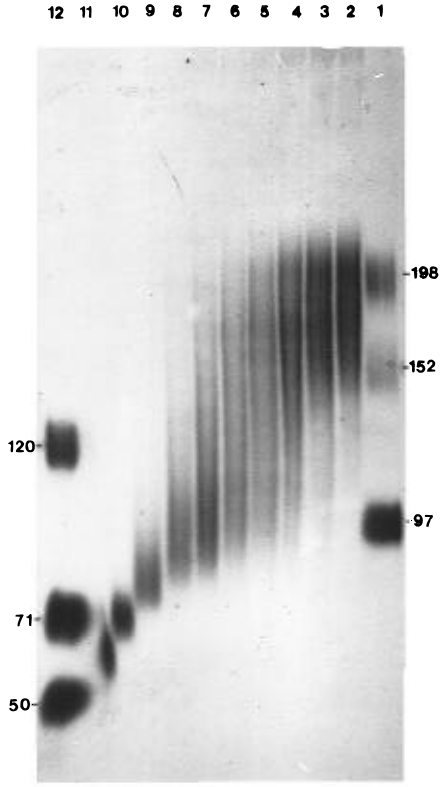



FIGURE 4: Utilization of poly(P) of average chain length 170 by poly(P) glucokinase from *P. shermanii*. All conditions were as in Figure 3, except that 0.04 unit of poly(P) glucokinase from *P. shermanii* and pH 7.5 were used. Lanes 1 and 12 contain standards; lanes 2−11 are the poly(P) isolated after 0, 2, 4, 6, 8, 10, 12, 15, 18, and 21 min, respectively.

*Mechanism of Poly(P) Utilization—Processivity vs Nonprocessivity*. The processivity of poly(P)-glucokinase was qualitatively investigated by the product analysis method employed by Pepin and Wood (1986, 1987). For comparison, some experiments were also repeated with poly(P) glucokinase from *P. shermanii*. Figures 2 and 3 show that, regardless of the original substrate size, poly(P) is progressively shortened as it is utilized by the mycobacterial enzyme, which results in the accumulation of poly(P) of intermediate sizes. Moreover, the difference in length between the longest and the shortest poly(P) present during the reaction remains constant or decreases, contrary to what is observed for very-long-chain poly(P) utilized by the enzyme from *P. shermanii*. In the latter case, at the very early stage of the reaction, the range of poly(P) sizes becomes at least 2 times broader when poly(P) of average chain length of 170 phosphate residues (range ~130-210) is used as a substrate (Figure 4, lanes 2−6). Only below ~100 residues, the range of poly(P) sizes becomes more narrow. The most striking difference in poly-

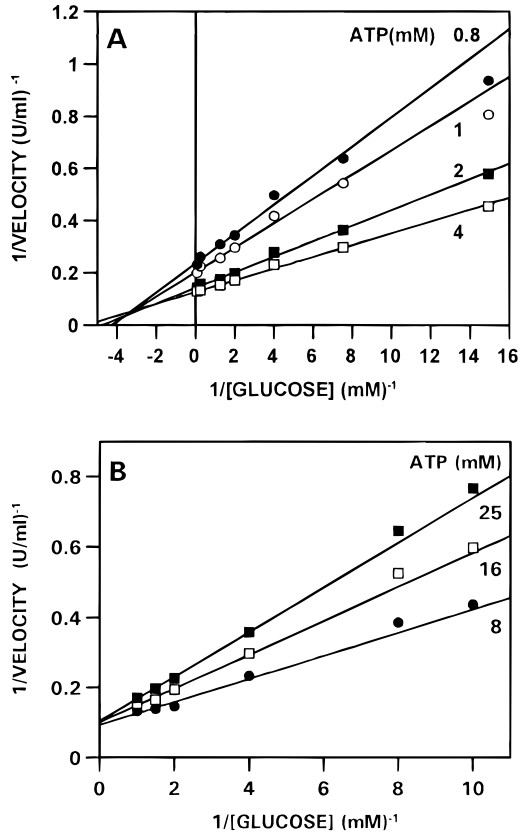



FIGURE 5: Initial velocity patterns in the ATP-dependent reaction with glucose as the variable substrate at pH 7.5. Double-reciprocal plots at low (A) and high (B) fixed ATP concentrations are shown. Glucose concentration in panel A varied from 0.07 to 12 mM, and in panel B from 0.1 to 1 mM.

(P) size distribution between the two enzymes was seen after 6 min of reaction (lane 5 in Figures 3 and 4). Interestingly, after 21 min (lane 11), the distribution of poly(P) sizes was almost identical in both cases, in spite of initially different mechanisms of poly(P) utilization. These results rule out a strictly processive mechanism and are most consistent with a nonprocessive mechanism for the mycobacterial enzyme and a quasiprocessive mechanism [switching to nonprocessive for short poly(P)] for the propionibacterial enzyme, as will be discussed later. Figure 2 also shows that poly(P) of different sizes are utilized simultaneously, with slight preference for longer chains.

### *Kinetic Mechanism of the ATP-Dependent Glucokinase Reaction*

*Initial Velocity Studies*. The initial velocity was first determined at varied glucose concentration and several fixed levels of ATP at pH 7.5. The intersecting pattern obtained at low ATP concentrations (Figure 5A) rules out a Ping Pong mechanism. No substrate inhibition by glucose is observed even at saturating concentrations. On the other hand, ATP at high concentrations displays competitive inhibition against glucose (Figures 4B and 5). Secondary plots of slopes and intercepts obtained from Figure 5A were linear. Similar results were obtained at pH 8.6 (not shown).

*Substrate Inhibition by ATP*. The pattern of inhibition shown in Figure 5B suggests that ATP acting as an inhibitor binds to the same enzyme form as glucose. It could be either free enzyme or enzyme·ATP complex (or both), depending on the sequence of binding of substrates. Results of product and dead-end inhibition studies described below imply

Table 3: Kinetic Parameters for the ATP-Dependent Reaction at 30 °C

| | kinetic constant ± SE[a] | |
|---|---|---|
| | pH 7.5 | pH 8.6 |
| $k_{cat}$ ($s^{-1}$) | 108 ± 2 | 116 ± 22 |
| $K_{ATP}$ (mM) | 0.88 ± 0.07 | 1.4 ± 0.8 |
| $K_{Glc}$ (mM) | 0.06 ± 0.03 | 0.22 ± 0.11 |
| $K_{i,ATP}$ (mM) | 6.6 ± 2.9 | 7.4 ± 3.2 |
| $k_{cat}/K_{ATP}$ ($M^{-1}$ $s^{-1}$)[b,c] | 1.2 (±0.1) × $10^5$ | 0.8 (±0.2) × $10^5$ |
| $k_{cat}/K_{Glc}$ ($M^{-1}$ $s^{-1}$)[c,d] | 1.7 (±0.8) × $10^6$ | 5.3 (±1.7) × $10^5$ |
| $k_{cat}/K_{i,ATP}K_{Glc}$ ($M^{-2}$ $s^{-1}$)[c,e] | 2.6 (±0.3) × $10^8$ | 7.2 (±0.9) × $10^7$ |
| $K_i^s$ (mM)[f] | 1.82 ± 0.95 | —[g] |
| $K_{i,ATP} \times k_{cat}/K_{ATP}$ ($s^{-1}$)[h] | 792 | 592 |

[a] Kinetic constants were calculated assuming a Steady-State Ordered Bi Bi mechanism with ATP binding to the enzyme first (eq III). At pH 7.5, substrate inhibition by ATP was also assumed (eq VIII). SE = standard error. [b] True second-order rate constant for productive binding (it applies to the first substrate). [c] Value of this parameter was obtained directly by the fitting procedure after reparametrizing (Northrop, 1983) eq VIII or eq III and replacing $V_1$ with $1.818k_{cat}E_t$, where $E_t$ = 0.060 mg/mL (pH 7.5) or 0.048 mg/mL (pH 8.6). [d] Lower limit of the second-order rate constant for productive binding (it applies to the second substrate). [e] Overall catalytic efficiency. [f] Substrate inhibition constant, i.e., the dissociation constant of an enzyme·ATP·ATP complex. [g] Substrate inhibition by ATP was not assumed in the fitting procedure. [h] Calculated dissociation rate constant for an enzyme·ATP complex.

binding of glucose and the second ATP molecule to the enzyme·ATP complex, which is represented by the $K_bA$ term in the denominator of the velocity equation for a Steady-State Ordered Bi Bi mechanism (eq III). In order to obtain values of kinetic parameters, eq III has been modified to give eq VIII, which accounts for this substrate inhibition. Results of several initial velocity experiments (44 data points) were fitted to eq VIII, and the kinetic parameters were calculated (Table 3). Figure 6 shows that eq VIII and the calculated parameters accurately describe experimental data. In addition, velocity values predicted by this equation and the parameters from Table 3 are in excellent agreement with the experimental values obtained at different glucose concentrations (not shown). The data obtained at pH 8.6 (25 points) were fitted to eq III since no substrate inhibition by ATP was observed up to 4 mM. Table 3 shows that increasing pH from 7.5 to 8.6 causes very small changes in values of the kinetic parameters for ATP.

*Product and Product Analog Inhibition Studies*. These studies were performed at pH 7.5. When ADP was the inhibitor and ATP was the variable substrate at fixed glucose concentration (0.25 mM), linear noncompetitive inhibition was observed. Since at low glucose concentrations substrate inhibition by ATP is not negligible (see Figure 6), the data for ADP inhibition were analyzed in a different way than it is routinely done. First, they were fitted separately to a general equation for substrate inhibition without any assumptions made regarding the inhibition pattern (eq VII), and apparent maximum velocities and Michaelis constants were calculated. Then, these parameters were used to plot asymptotes shown in Figure 7 to determine the inhibition pattern. Finally, $K_{is}$ and $K_{ii}$ values were calculated by fitting all data points to eq X. When glucose was the variable substrate at fixed ATP concentration (3 mM), linear noncompetitive inhibition pattern was observed.

Inhibition by F6P (i.e., product analog of G6P) was found to be competitive *versus* ATP and noncompetitive *versus*

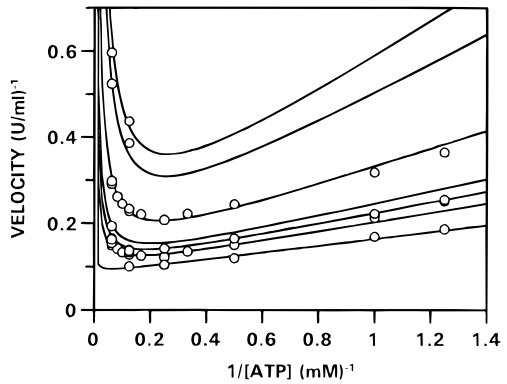



FIGURE 6: Initial velocity pattern in the ATP-dependent reaction with ATP as the variable substrate at pH 7.5. The glucose concentrations in mM are (from bottom to top): 10, 1, 0.67, 0.5, 0.25, 0.125, and 0.1. All data were fitted simultaneously to eq VIII. All lines were calculated using parameter values given in Table 3. Enzyme concentration, 0.06 mg/mL.

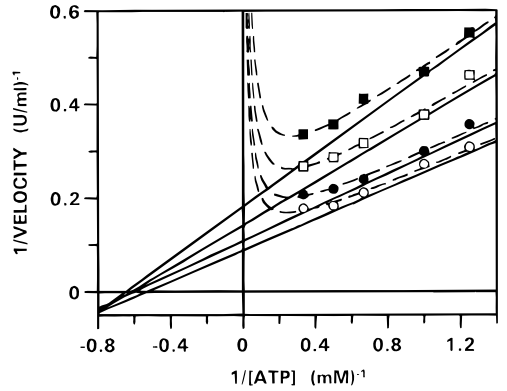
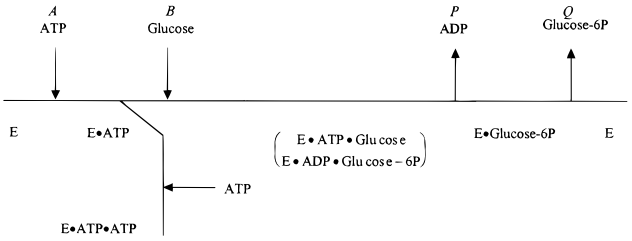



FIGURE 7: Product inhibition by ADP with ATP as the varied substrate at pH 7.5. Glucose concentration, 0.25 mM. ADP concentrations in mM are (from bottom to top): 0, 5, 10, and 15. The data were fitted to eq VII assuming $K_I = 9.12$ mM (calculated from eq IX using parameter values from Table 3). The results of the fitting procedure are shown as dashed lines. The asymptotes (solid lines) represent predicted lines which would be obtained in the absence of substrate inhibition.

Scheme 2: A Proposed Kinetic Mechanism for Poly(P)-Glucokinase in the ATP-Dependent Reaction




glucose. These results are consistent with a Steady-State Ordered Bi Bi mechanism with ATP being the first substrate to add and G6P being the last product to leave the enzyme as shown in Scheme 2. All kinetic parameters and inhibition patterns are listed in Table 4. Due to substrate inhibition by ATP, apparent inhibition constant ($K_{is}$) for F6P was calculated by fitting data points to eq XI when ATP was the variable substrate. This constant refers to slopes of asymptotes constructed as described above in the case of ADP inhibition. The values of true inhibition constant ($K_i$) for F6P calculated from different data as explained in Table 4 are in a good agreement with those calculated previously for the poly(P)-dependent reaction (Table 2), which is an expected result, if this parameter is really the dissociation constant for enzyme·F6P complex.

*Dead-End Inhibition Studies.* AMP is a linear competitive inhibitor *versus* ATP and a linear noncompetitive inhibitor *versus* glucose. Inhibition constants for AMP were calculated as in the case of F6P. Xylose was found to be a linear competitive inhibitor with respect to glucose and a linear uncompetitive inhibitor against ATP. When ATP was the variable substrate, the data were fitted to eq XII in order to account for substrate inhibition by ATP. These results confirm the order of substrate addition with ATP binding first and followed by glucose. All results from dead-end inhibition studies, along with the kinetic constants, are summarized in Table 4. The values of inhibition constant ($K_i$) for AMP agree well with the corresponding values for the poly(P)-dependent reaction, as in the case of F6P.

Table 4: Product and Dead-End Inhibition Patterns in the ATP-Dependent Reaction at pH 7.5

| inhibitor[a] | variable substrate | fixed substrate | pattern[b] | $K_{is} \pm$ SE (mM)[b] | $K_{ii} \pm$ SE (mM)[b] |
|---|---|---|---|---|---|
| ADP (0−15) | MgATP | glucose (0.25 mM) | NC | $24 \pm 9$ | $16 \pm 4$ |
| ADP (0−15) | glucose | MgATP (3 mM) | NC | $19 \pm 3$ | $12 \pm 1$ |
| F6P (0−60) | MgATP | glucose (4 mM) | C | $35 \pm 4$ ($=K_i$)[d] | |
| F6P (0−60) | MgATP | glucose (0.8 mM) | C | $33 \pm 2$ ($=K_i$)[d] | |
| F6P (0−90) | glucose | MgATP (2 mM) | NC[c] | $58 \pm 12$ ($K_i = 35$)[d] | $101 \pm 20$ ($K_i = 31$)[d] |
| xylose (0−10) | glucose | MgATP (4 mM) | C | $0.40 \pm 0.08$ ($K_i = 0.26$)[e] | |
| xylose (0−5) | MgATP | glucose (0.5 mM) | UC | | $5.9 \pm 0.5$ ($K_i = 0.63$)[e] |
| AMP (0−40) | MgATP | glucose (10 mM) | C | $34 \pm 3$ ($=K_i$)[d] | |
| AMP (0−50) | glucose | MgATP (2.67 mM) | NC | $42 \pm 14$ ($K_i = 21$)[d] | $48 \pm 10$ ($K_i = 12$)[d] |

[a] Values in parentheses give the range of inhibitor concentrations in mM used in these studies. [b] The abbreviations and symbols used are as in Table 2. [c] Values of slope and intercept for the line obtained at 90 mM F6P are omitted from the calculations as they depart from linearity in the secondary plots, probably due to inhibition of G6P dehydrogenase by F6P. [d] True inhibition constant ($K_i$, in mM) for an inhibitor competitive with substrate *A* (i.e., ATP) was calculated by dividing $K_{is}$ by $[1 + A(1 + A/K_i^s)/K_{ia}]$ or $K_{ii}$ by $(1 + A/K_a)$; parameter values were taken from Table 3. Also, $K_i = K_{is}$ when *A* is the variable substrate and *B* is the fixed one. [e] True inhibition constant ($K_i$, in mM) for an inhibitor competitive with substrate *B* (i.e., glucose) was calculated by dividing $K_{is}$ by $[1 + K_{ia}/A(1 + A/K_i^s)]$ or $K_{ii}$ by $(1 + B/K_b)$; parameter values were taken from Table 3.

## DISCUSSION

In previous studies, we have shown that both poly(P)- and ATP-dependent glucokinase reactions are catalyzed by a single enzyme (Hsieh et al., 1993b). Further studies were consistent with a common catalytic center for both poly(P) and ATP, even though these two substrates might have different binding sites (Hsieh et al., 1993a). In this paper, we determine the kinetic mechanisms of both poly(P)- and ATP-dependent reactions in order to understand how these substrates are utilized by a single bifunctional poly(P)-glucokinase. Data from initial velocity, product inhibition, and dead-end inhibition studies indicate that poly(P)-glucokinase follows a Steady-State Ordered Bi Bi mechanism in both poly(P)- and ATP-dependent reactions. The established sequential mechanism allows for calculation of overall catalytic efficiencies for poly(P)-glucokinase in both reactions (Avis & Fersht, 1993). The comparison of these efficiencies, along with other observations (see below), suggests that poly(P) is favored over ATP as the phosphoryl donor for poly(P)-glucokinase in *M. tuberculosis*.

*Kinetic Mechanism of the Poly(P)-Dependent Reaction*. The polymeric nature of poly(P) and the mechanism of its utilization affect the interpretation of the kinetic parameters, as was discussed in detail by McClure and Chow (1980). One possibility is that this mechanism is strictly processive, in which case poly(P) would not dissociate from the enzyme until it is completely utilized. If this were the case, poly(P) would be the last product to leave, as was suggested for DNA polymerases (Majumdar et al., 1988). Consequently, G6P would be a noncompetitive inhibitor *versus* glucose and poly(P). This prediction does not agree with our results (Table 2). Moreover, accumulation of intermediate-size poly(P) during the course of the reaction (Figures 2 and 3) directly shows that the mechanism of poly(P) utilization is not strictly processive and is most likely nonprocessive, where there is dissociation of poly(P) prior to complete utilization. Although these results cannot completely rule out a slight degree of processivity, the probability of phosphorylating more than one glucose prior to the release of the poly(P) from the enzyme appears to be very small. Any significant processivity would result in noticeable broadening of poly(P) range with time, according to the analysis of product distribution patterns (McClure & Chow, 1980). For very-long-chain poly(P), this broadening would result in the formation of smears (rather than spots with sharply defined edges) because of limited resolution of the polyacrylamide gels. This effect was clearly observed for poly(P) glucokinase from *P. shermanii* (Figure 4), in agreement with previously published results (Pepin & Wood, 1987), but not for the enzyme from *M. tuberculosis* (Figure 3).

The nonprocessive mechanism implies the presence of poly(P) as substrate and product simultaneously. Consequently, initial velocities are always determined in the presence of one of the products, which makes the rate equation different from that of the usual two-substrate systems (Chao et al., 1969; Majumdar et al., 1988; McClure & Jovin, 1975). Product and dead-end inhibition studies suggest that poly(P) binds to the free enzyme as the first substrate (A) and is released as the first product (P), after the terminal phosphate is transferred to glucose, in the Ordered Bi Bi mechanism. Intersecting initial velocity patterns with both substrates as variables (Figure 1) rule out the usual Bi Bi Ping Pong mechanism. However, if interaction of the enzyme with poly(P)$_{n-1}$ as product is also considered, the predicted patterns will be distinctly different from the usual Bi Bi Ping Pong mechanism (Chao et al., 1969). With respect to glucose, the plots will show converging patterns at different fixed poly(P) concentrations, similar to what is observed for a sequential mechanism; however, with respect to poly(P) as the variable substrate, the double-reciprocal plots would be nonlinear. Since all the plots are linear within a wide range of poly(P) concentrations, a modified Ping Pong mechanism is also excluded. The complete general velocity equation for the Steady-State Ordered Bi Bi mechanism including the presence of the first product, which is considered to be identical with the first substrate, is given below (Cleland, 1963a):

$$\frac{v}{V_1} = \frac{AB}{K_{ia}K_b + K_bA + K_aB + AB + \frac{K_qK_bK_{ia}A}{K_{iq}K_p} + \frac{K_qK_bA^2}{K_{iq}K_p} + \frac{A^2B}{K_{ip}}} \quad \text{(XIII)}$$

*A* represents poly(P) and *B* represents glucose. The square terms in the denominator of the equation indicate that the double-reciprocal plots should be nonlinear with respect to poly(P) at fixed glucose concentrations, especially at high poly(P) levels. The discrepancy between this prediction and the observed linearity of all graphs (Figure 1) would be eliminated if those square terms were negligible compared to other terms in the denominator. This condition is satisfied if both $K_{ip}$ and $K_p$ are relatively large. In other words, if the binding of poly(P) as product is unfavorable, eq XIII reduces to the usual initial velocity equation in the absence of products (eq III). Therefore, all kinetic parameters listed in Tables 1 and 2 were calculated assuming negligible poly(P) binding to the product site on the enzyme. This assumption is consistent with lack of substrate inhibition by poly(P) at concentrations up to ~50 times the $K_m$ value. The mechanistic basis of this differential binding of poly(P) as substrate (to free enzyme) and as product (to an enzyme·G6P complex) remains to be determined.

The phosphate glass, type 35, used in these studies contains a broad range of poly(P) sizes from about 15 to 150 phosphate residues (Clark & Wood, 1987). The use of heterogeneous poly(P) substrate was intended to mimic physiological conditions, since in living cells poly(P) is always present as mixtures of different sizes (Kulaev, 1979). Polyphosphate isolated from *M. tuberculosis* is acid-insoluble, which means that it consists of highly polymerized species, presumably longer than ~20 phosphate residues (Kulaev, 1979). Purification of homogeneous long-chain poly(P) is technically very difficult; even poly(Ps) separated by PAGE consist of several sizes, as can be seen from Figures 2 and 3. We have used those separated fractions of poly(P), purified from phosphate glass 35, in initial velocity studies at pH 8.6, and found that the size of poly(P) does not influence the catalytic constant, while the second-order rate constant increases with increasing chain length from 4 $\times$ $10^7$ $M^{-1}$ $s^{-1}$ for average size 30 to 5 $\times$ $10^8$ $M^{-1}$ $s^{-1}$ for average size 148 (results to be published elsewhere). Thus, all kinetic parameters from Tables 1 and 2, involving poly-

(P) binding to the enzyme, are apparent ones. This, however, does not change the overall conclusion regarding inhibition patterns and the mechanism of the reaction, because in all experiments poly(P) behaved kinetically as a homogeneous population: all primary and secondary graphs were linear over a wide range of poly(P) concentrations, and all intersecting points were well-defined. This conclusion is supported by a good agreement between values of inhibition constants for F6P and AMP obtained separately for the poly(P)- and ATP-dependent reactions.

*Kinetic Mechanism of the ATP-Dependent Reaction*. Product and dead-end inhibition of this reaction was analyzed assuming substrate inhibition by ATP. The value of the substrate inhibition constant ($K_i^S$) used in this analysis was calculated by fitting initial velocity data to an Ordered Bi Bi mechanism (eq VIII) that was chosen based on inhibition patterns shown in Table 4. The choice of an appropriate mechanism, however, was independent of the substrate inhibition assumption. When product and dead-end inhibition data were analyzed by fitting them to a general velocity equation $v = VA/(K + A)$, a set of inhibition patterns identical to that in Table 4 was obtained, since at low ATP concentrations (up to 4 mM) the departure of the double-reciprocal plots from linearity (due to substrate inhibition) is relatively small, especially at higher glucose concentrations (0.8−10 mM). However, inclusion of the substrate inhibition into the analysis resulted in slightly better overall fit (i.e., variance lower by 1−5%) and more consistent values of the true inhibition constants for F6P, xylose, and AMP calculated from different sets of data.

Competitive substrate inhibition of the type discussed in this paper has been demonstrated with other enzymes. Byrnes et al., (1994) have shown that ATP nonallosterically inhibits phosphofructokinase from *Bacillus stearothermophilus* by formation of a dead-end enzyme·$(ATP)_2$ complex, where ATP as an inhibitor binds in the F6P site *via* its $\gamma$-phosphate group. Similarly, Gruys et al., (1992) have shown that phosphoenolpyruvate (PEP) inhibits 5-enolpyruvoylshikimate-3-phosphate synthase from *Escherichia coli* by binding at the shikimate 3-phosphate site to form an enzyme·$(PEP)_2$ complex. In the case of poly(P)-glucokinase, the possibility of binding of the second ATP molecule at the glucose site seems unlikely, although it cannot be completely rejected. Another possibility assumes binding of the second ATP molecule at a different site, overlapping with the site for glucose to account for the competitive inhibition. A similar mechanism was suggested for *Vibrio harveyi* luciferase inhibited by formation of an inactive enzyme·$(aldehyde)_2$ ternary complex (Lei et al., 1994). It is possible that binding of the first ATP molecule changes the structure of the enzyme (see below) in such a way that poly(P) binding site, which is separate from ATP site (Hsieh et al., 1993b), can now accept the second ATP molecule. Further studies are necessary to elucidate the mechanism of ATP inhibition.

The proposed order of substrate addition and product release (Scheme 2) is unusual in the sense that G6P, as a structural counterpart of glucose being the second substrate, was expected to be released before ADP. Alternatively, if G6P was assumed to be the last product, glucose was expected to bind before ATP, as in the case of rat liver glucokinase (Gregoriou et al., 1981). Nevertheless, the reaction sequence shown in Scheme 2 accommodates results of all product and dead-end inhibition studies, and any change of this sequence would be inconsistent with one or more sets of these results.

*Interpretation of the Kinetic Parameters*. For an Ordered Bi kinetic mechanism, $k_{cat}/K_a$ is the true second-order rate constant for the binding of the first substrate, since at saturating concentration of the second substrate the binding of the first substrate is completely rate determining (Gawlita et al., 1995). Consequently, the true dissociation rate constant for the binary enzyme·substrate complex can be calculated by multiplying $K_{ia}$ by $k_{cat}/K_a$. The comparison of dissociation rate constants from Tables 1 and 3 reveals that at pH 7.5 the binding of poly(P) is ∼17-fold tighter than ATP. However, at pH 8.6 the poly(P) binding is less than 2-fold tighter than that of ATP. Thus, the greater overall catalytic efficiency of poly(P) utilization over ATP can be largely due to faster association of this substrate and not due to its tighter binding. Strong chain-length dependence of the association rate constant over a broad range of poly(P) sizes of 30−400 phosphate residues (to be shown elsewhere) suggests that the mechanism of poly(P) recognition is similar to that described for site-specific associations of proteins and DNA (Lohman, 1986), at least for very-long-chain poly(P). This interpretation, however, is correct only if the binding of poly(P) occurs in a single step. It is conceivable that the association actually consists of two kinetically distinct steps, a bimolecular collision followed by a unimolecular isomerization (conformational change) to form the Michaelis complex (Johnson, 1992). If this is the case, the second-order rate constant is a function of the collision (association) rate constant as well as the rate constant for the dissociation of the enzyme·poly(P) complex and the rate constant for its isomerization.

The rate constant for ATP binding ($\sim 10^5$ $M^{-1}$ $s^{-1}$) is far below the expected diffusion-controlled limit, which implies that only a small fraction of collisions is productive (Simopoulos & Jencks, 1994). This result is consistent with a two-step association process and the isomerization step being rate-limiting, as was suggested for phosphoenolpyruvate carboxylase and pyruvate kinase (Gawlita et al., 1995).

*Which Reaction Is Favorable in M. tuberculosis?* Several observations suggest that ATP is not preferred by poly(P)-glucokinase, although the actual preference for poly(P) in the bacterial cell cannot be evaluated without knowledge of cellular poly(P) concentration. (i) Poly(P)-glucokinase has broad nucleotide specificity, i.e., besides ATP, it can efficiently utilize GTP, UTP, TTP, XTP, and CTP as the phosphoryl donor (Hsieh et al., 1993b). (ii) In the Sequential Bi reaction, the overall catalytic efficiency can be expressed by the term $k_{cat}/K_{ia}K_b$, which describes the termolecular reaction of the enzyme with both substrates at low concentrations, and is proportional to the free energy of activation of the overall reaction (Avis & Fersht, 1993). Regardless of the phosphoryl donor, the catalytic efficiency of this enzyme is slightly better at pH 7.5 than at pH 8.6 (Tables 1 and 3); however, the efficiency of poly(P) utilization is 1000-fold better than that of ATP at both pH values. (iii) The poly(P)-glucokinase is well adapted to utilize poly(P), since the second-order rate constant for the reaction of free enzyme with the mixture of poly(P) ($\sim 10^7$ $M^{-1}$ $s^{-1}$) is close to the diffusion-controlled limit (Fersht, 1985), which means that the fraction of productive collisions is near unity. On the

other hand, the rate constant for ATP association is far below the diffusion-controlled limit (see above). (iv) ATP causes substrate inhibition at physiological pH and concentration [ATP available to mycobacteria in the host cells is present at 5.6 mM concentration (Wheeler & Ratledge, 1994)].

## ACKNOWLEDGMENT

We are grateful to Dr. Olga Szymona (Medical School of Lublin, Lublin, Poland) for the generous gift of *Mycobacterium tuberculosis* $H_{37}Ra$. We also thank Dr. Vernon Anderson for valuable discussions.

## REFERENCES

Alland, D., Kalkut, G. E., Moss, A. R., McAdam, R. A., Hahn, J. A., Bosworth, W., Drucker, E., & Bloom, B. R. (1994) *N. Engl. J. Med. 330*, 1710−1716.
Avis, J. M., & Fersht, A. R. (1993) *Biochemistry 32*, 5321−5326.
Bobyk, M. A., Afinogenova, A. V., Dubinskaya, M. V., Lambina, V. A., & Kulaev, I. S. (1980) *Zentralbl. Bakteriol., Parasitenkd., Infektionskrankh. Hyg. 135*, 461−466.
Bradford, M. (1976) *Anal. Biochem. 72*, 248−254.
Byrnes, M., Zhu, X., Younathan, E. S., & Chang, S. H. (1994) *Biochemistry 33*, 3424−3431.
Chao, J., Johnson, G. F., & Graves, D. J. (1969) *Biochemistry 8*, 1459−1466.
Clark, J. E., & Wood, H. G. (1987) *Anal. Biochem. 161*, 280−290.
Cleland, W. W. (1963a) *Biochim. Biophys. Acta 67*, 104−137.
Cleland, W. W. (1963b) *Biochim. Biophys. Acta. 67*, 173−187.
Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., pp 147−153, W. H. Freeman and Co., New York.
Fromm, H. J. (1995) *Methods Enzymol. 249*, 123−143.
Gawlita, E., Caldwell, W. S., O'Leary, M. H., Paneth, P., & Anderson, V. E. (1995) *Biochemistry 34*, 2577−2583.
González, F., Fernández-Vivas, A., Arias, J. M., & Montoya, E. (1990) *Arch. Microbiol. 154*, 438−442.
Gregoriou, M., Trayer, I. P., & Cornish-Bowden, A. (1981) *Biochemistry 20*, 499−506.
Gruys, K. J., Walker, M. C., & Sikorski, J. A. (1992) *Biochemistry 31*, 5534−5544.
Hsieh, P.-C., Shenoy, B. C., Haase, F. C., Jentoft, J. E., & Phillips, N. F. B. (1993a) *Biochemistry 32*, 6243−6249.
Hsieh, P.-C., Shenoy, B. C., Jentoft, J. E., & Phillips, N. F. B. (1993b) *Protein Exp. Purif. 4*, 76−84.
Johnson, K. A. (1992) in *The Enzymes* (Sigman, D. S., Ed.) Vol. 20, pp 1−61, Academic Press, New York.
Kornberg, A. (1995) *J. Bacteriol. 177*, 491−496.
Kowalczyk, T. H., & Phillips, N. F. B. (1993) *Anal. Biochem. 212*, 194−205.
Kulaev, I. S. (1979) *The Biochemistry of Inorganic Polyphosphates*, Wiley, New York.
Kulaev, I. S., & Vagabov, V. M. (1983) *Adv. Microb. Physiol. 24*, 83−171.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lei, B., Cho, K. W., & Tu, S. (1994) *J. Biol. Chem. 269*, 5612−5618.
Lohman, T. M. (1986) *CRC Crit. Rev. Biochem. 19*, 191−245.
Majumdar, C., Abbotts, J., Broder, S., & Wilson, S. H. (1988) *J. Biol. Chem. 263*, 15657−15665.
Mannervik, B. (1982) *Methods Enzymol. 87*, 370−390.
McClure, W. R., & Jovin, T. M. (1975) *J. Biol. Chem. 250*, 4073−4080.
McClure, W. R., & Chow, Y. (1980) *Methods Enzymol. 64*, 277−297.
Northrop, D. B. (1983) *Anal. Biochem. 132*, 457−461.
Pepin, C. A., & Wood, H. G. (1986) *J. Biol. Chem. 261*, 4476−4480.
Pepin, C. A., & Wood, H. G. (1987) *J. Biol. Chem. 262*, 5223−5226.
Perriens, J. H., Colebunders, R. L., Karahunga, C., Willame, J. C., Jeugmans, J., Kaboto, M., Mukadi, Y., Pauwels, P., Ryder, R. W., Prignot, J., et al. (1991) *Am. Rev. Respir. Dis. 144*, 750−755.
Phillips, N. F. B., Horn, P. J., & Wood, H. G. (1993) *Arch. Biochem. Biophys. 300*, 309−319.
Pisoni, R. L., & Lindley, E. R. (1992) *J. Biol. Chem. 267*, 3626−3631.
Robinson, N. A., Goss, N. H., & Wood, H. G. (1984) *Biochem. Int. 8*, 757−769.
Segel, I. H. (1993a) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 590 and 609−612, Wiley, New York.
Segel, I. H. (1993b) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 646−649, Wiley, New York.
Simopoulos, T. T., & Jencks, W. P. (1994) *Biochemistry 33*, 10375−10380.
Snider, D. E., Jr., & Roper, W. L. (1992) *N. Engl. J. Med. 326*, 703−705.
Storer, A. C., & Cornish-Bowden, A. (1976) *Biochem. J. 159*, 1−5.
Szymona, M. (1957) *Bull. Acad. Pol. Sci. Ser. Sci. Biol. 5*, 379−381.
Szymona, M. (1962) *Acta Biochim. Polon. 9*, 165−181.
Szymona, M., & Ostrowski, W. (1964) *Biochim. Biophys. Acta 85*, 283−295.
Szymona, M., & Widomski, J. (1974) *Physiol. Chem. Phys. 6*, 393−404.
Szymona, M., Kowalska, H., & Pastuszak, I. (1977) *Acta Biochim. Polon. 24*, 133−142.
Szymona, O., & Szymona, M. (1979) *Acta Microbiol. Polon. 28*, 153−160.
Viola, R. E., Raushel, F. M., Rendina, A. R., & Cleland, W. W. (1982) *Biochemistry 21*, 1295−1302.
Wheeler, P. R., & Ratledge, C. (1994) in *Tuberculosis: Pathogenesis, Protection, and Control* (Bloom, B. R., Ed.) pp 353−385, ASM Press, Washington, DC.
Wood, H. G., & Clark, J. E. (1988) *Annu. Rev. Biochem. 57*, 235−260.

BI9528659