Perturbation of Yeast 3-Phosphoglycerate Kinase Reaction Mixtures with ADP: Transient Kinetics of Formation of ATP from Bound 1,3-Bisphosphoglycerate†

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ABSTRACT: 3-Phosphoglycerate kinase (PGK) is the first ATP-producing enzyme in glycolysis: ADP + 1,3-bisphosphoglycerate (bPG) ↔ ATP + 3-phosphoglycerate (PG). Whereas extensive studies have been carried out on its structure, there is less information about its reaction pathway, which is usually studied in the reverse direction because of the instability of bPG. We studied the transients of the PGK reaction by chemical sampling in a rapid quench flow apparatus, using [γ-^{32}P]ATP, in 30% methanol at 4 °C to decrease k_{cat}. There were two types of experiment, both at low PG concentrations to prevent bPG release. In the first, reaction mixtures were quenched in acid at different times (from 4 ms) and the bPG concentrations were determined. This type gave information about the ATP binding and phospho-transfer steps. In the second, PGK reaction mixtures at equilibrium were perturbed by the injection of ADP, the new mixtures aged for different times and quenched in acid, and the bPG concentrations were determined. This gave information about the kinetics of the binding of ADP to a PGK intermediate. The data from the two types of experiments were fitted to simple schemes and then treated together by a global fitting procedure using a five-step pathway, deduced from previous structural studies. Under our conditions, it appears that (1) a binary PGK·bPG complex is an important intermediate on the reaction pathway, i.e., that ADP is released before bPG, (2) ADP binds to a "closed" conformation in the PGK·bPG complex, and (3) the PGK reaction can be studied in the physiologically important direction without having to handle bPG.

3-Phosphoglycerate kinase (PGK,[1] EC 2.7.2.3) catalyzes the reversible transfer of phosphate between 1,3-bisphosphoglycerate (bPG) and ADP:

$$\text{bPG} + \text{ADP} \leftrightarrow \text{PG} + \text{ATP}$$

Although in vivo PGK catalyzes the first ATP-generating reaction in anaerobic glycolysis, in the laboratory the reaction is usually studied in the reverse direction, because of the instability of bPG. Extensive structural studies have been carried out on PGK (*1* and references therein), but there are relatively few works on its reaction pathway (*2*).

An aim of our work is to construct a reaction pathway for PGK, i.e., to identify the reaction intermediates and to obtain the rate constants for their interconversions. This knowledge is of interest not simply for mechanistic reasons; it could also aid in the development of disease-controlling drugs.

First, consider the reaction pathway of PGK, in particular the conformational transitions that are involved. In the apoenzyme, the substrate binding sites are too far apart to permit phospho-transfer: PGK is made up of two domains with the PG site on the N-domain and ATP site on the C-domain. For transfer to occur, it is thought that the two domains undergo an extensive hinge bending motion that allows the approximation of the sites. Each substrate on its own causes a partial closing of the hinge, but both are needed for the complete closing (*3*−*5*). These substrate-induced movements are an excellent example of the induced fit process of Koshland (*6*). They are in accord with the idea that enzymes are inherently flexible molecules and, in particular, that different conformations can have important functional consequences (*7*). Therefore, during its catalytic cycle, an enzyme may undergo several conformational changes (*8*), and it is important to connect these changes with the individual steps on the catalytic cycle.

Second, PGK could play an important role in disease control. PGK is a potential drug target for combatting protozoan parasites that cause diseases such as sleeping sickness and leishmaniases (*9*, *10*). Compounds that are uncompetitive inhibitors (i.e., that react with an enzyme intermediate and not the apoenzyme) may be particularly effective drugs (*11*, *12*). The design of such drugs requires a detailed knowledge of the chemical properties of the intermediates that compose the enzyme reaction pathway.

PGK may be involved in the activation of nucleoside analogue prodrugs. Thus, whereas nucleotides do not pass the cytoplasmic membrane, nucleosides do. Upon entry into the cell, the nucleoside prodrug is phosphorylated to the nucleoside triphosphate drug that acts as terminator of

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[1] Abbreviations: PGK, 3-phosphoglycerate kinase; bPG, 1,3-bisphosphoglycerate; PG, 3-phosphoglycerate; Ap5A, P^1,P^5-di(adenosine-5′) pentaphosphate; P_i, inorganic orthophosphate.

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transcription in the control of antiviral diseases such as HIV and hepatitis B and C (*13*). Therefore, in addition to its possible role in modulating the cellular NTP pools, the cascade of cellular kinases that activates nucleosides to the mono-, di-, and finally triphosphates may be responsible for the activation of prodrugs (*14*, *15*). It is thought that the last step of the cascade involves kinases such as nucleoside diphosphate kinase, pyruvate kinase, and PGK, all of which have low specificity for the nucleoside diphosphate substrate (*16*).

L-Nucleoside analogue prodrugs are of particular interest as a new class of antiviral and anticancer agents. With certain prodrugs, the last step of the kinase cascade appears to be inefficient because the L-nucleoside diphosphate accumulates, yet PGK phosphorylates L-nucleoside diphosphate analogues more efficiently than the corresponding D-isomers (*17*, *18*). A more complete understanding of the PGK reaction pathway could provide an explanation for these results.

Because of its high turnover rate and low substrate affinities, it is difficult to obtain transient kinetic information about PGK, even at 4 °C (*19*). In a previous study (*2*), we added methanol (30%, v/v) to our buffer, and by this means we at once decreased the turnover rate and increased the affinity of PGK for PG. In this way, we obtained kinetic data on the putative hinge bending motion of PGK. Further, we proposed a reaction pathway on which a complex of the enzyme and products (E·bPG·ADP) accumulates in the steady state from which the products are released simultaneously.

Here, we extend our previous studies by perturbing PGK reaction mixtures at equilibrium with ADP. Under our conditions (low PG concentration, 4 °C, 30% methanol), little bPG is released and the experiments are essentially single turnovers. From our results, we conclude that (1) ADP is released before bPG and a binary E·bPG complex is an important component of the equilibrium reaction mixture, (2) in the E·bPG complex the enzyme may be in a "closed" conformation, and (3) the transient kinetics of ATP formation can be studied without having to handle the unstable bPG.

Finally, our transient kinetic study, together with previous structural works, allowed us to elaborate a reaction pathway for PGK.

MATERIALS AND METHODS

Proteins and Reagents. 3-Phosphoglycerate kinase (PGK) was prepared as described by Minard et al. (*20*) and myosin (used to assay the total radioactivities; see below) as described by Weeds and Taylor (*21*). 3-Phospho-D-glycerate (PG) was from Boehringer Mannheim, [γ-^{32}P]ATP from Amersham International, and P^1,P^5-di(adenosine-5′) pentaphosphate (Ap5A) from Sigma Chemical Co.

The PGK was tested for bound PG (*22*) by incubating the enzyme with [γ-^{32}P]ATP in the experimental buffer and the amount of [^{32}P]P_i determined (see below). The reaction mixture contained 10 μM PGK and 100 μM ATP and was incubated at 4 °C for up to 2 h. The time course consisted of a small burst phase ($\leq$0.01 mol of P_i/mol of PGK) that was followed by a slow, linear increase in the level of P_i at a rate of $\sim 10^{-5}$ s^{-1}. Similar time courses were obtained with PGK that had been incubated with glyceraldehyde 3-phosphate dehydrogenase and NADH (results not illustrated). These low activities are almost certainly due to the ATPase activity of PGK (*23*). We conclude that the PGK contained little, if any, bound PG.

Assay Methods. PGK concentrations were estimated at 280 nm with an $E^{1\%,1cm}$ of 4.9 (*24*). The PGK reaction was followed in the direction of bPG formation by chemical sampling. Reactions were quenched in acid (22% trichloroacetic acid with 1 mM KH_2PO_4), and the quenched reaction mixtures kept on ice for less than 1 h before being analyzed. Under these conditions, ATP and PG are stable but any [1-^{32}P]bPG decomposes quantitatively to PG and [^{32}P]P_i (*19*). The amount of [^{32}P]P_i was determined by the filter paper method of Reimann and Umfleet (*25*).

Zero-time points (blanks) were obtained by mixing the PGK (plus PG) with acid before adding the [γ-^{32}P]ATP (plus PG). The total radioactivities (counts per minute) in the [γ-^{32}P]ATP solutions were obtained by completely hydrolyzing portions of the solution with myosin (in the absence of methanol). Zero-time points and total radioactivities were carried out in triplicate. The results are expressed as [[1-^{32}P]-bPG]/[PGK], where [PGK] represents the protein concentration (from optical density measurements at 280 nm). Active site concentrations were estimated from global fits (see Results and Discussion).

In ADP perturbation experiments, PGK reaction mixtures at equilibrium were perturbed by the addition of ADP, and the decrease in the amount of bPG was followed, also by chemical sampling.

Transient Kinetic Experiments. These were carried out in a home-built, thermostatically controlled rapid quench flow apparatus (*26*). The procedure consisted of mixing in the apparatus PGK (preincubated with PG) with [γ-^{32}P]ATP and PG, allowing the mixtures to age ($\geq$4 ms; see the figures), and quenching the reactions in 22% trichloroacetic acid and 1 mM KH_2PO_4, and the amount of [^{32}P]P_i was determined as described above. We assume that this amount of [^{32}P]P_i is equal to the total amount of [1-^{32}P]bPG produced by PGK, whether free or enzyme-bound (*19*). The concentrations of PGK, PG, and [γ-^{32}P]ATP used in the different experiments are given in the figure legends or in the text.

Fluorescence Spectroscopy Studies. These were carried out in a SLM-Amicon Bowman Series 2 luminescence spectrophotometer as described previously (*2*).

Experimental Conditions. The buffer was 20 mM triethanolamine (pH 7.5), 0.1 M potassium acetate, 1 mM free Mg^{2+}, and 30% (v/v) methanol. Thus, in all our experiments, the ATP and ADP were MgATP and MgADP, respectively. In ADP perturbation experiments, the myokinase inhibitor Ap5A (25 μM) was included with the PGK reaction mixture. Ap5A had no detectable effect on the kinetics of the perturbations. Unless otherwise stated, the temperature was 4 °C.

Treatment of Data. In the time courses for bPG formation under multiturnover conditions (MTO), the data were interpreted according to the simple scheme in Geerlof et al. (*2*) but without the release of products (Scheme 1). Each time course fitted well to a single exponential of kinetics k_{MTO} and amplitude B_{MTO} (Figure 1). In the ADP perturbation experiments, the data were interpreted according to a scheme including an ADP release step (Scheme 2). Each time course fitted well to a single exponential of kinetics k_{PER} and amplitude B_{PER} (Figure 2). The data from the two types of

experiments were fitted with simple weighting using Grafit (version 3.03, Erithacus Software Ltd., Staines, U.K.). These "simple" fittings allowed the determination of the observed kinetic constants, k_{MTO}, B_{MTO}, k_{PER}, and B_{PER}, and their dependence on the ATP (MTO) or ADP (perturbation) concentrations, using simplified mathematical expressions (exponential time courses and linear or hyperbolic concentration dependences for k and B, respectively) that reasonably described the data. From these, constants (e.g., k_2/K_1 and k_{-2} from the k_{MTO} dependence; see Table 2) of the proposed reaction pathway (Scheme 2) were estimated.

Global fitting was used to estimate the rate constants of the proposed reaction pathway from (1) the two sets of experiments carried out with different conditions at the start of the reaction ([ATP] in the bPG formation experiments or [ADP] in the perturbation experiments) and (2) the complete description of the equations used to describe the reaction (here, differential equations; see the Supporting Information). Parameters were estimated via a least-squares fitting using Scientist (version 2.0, MicroMath Research, St. Louis, MO). k_2/K_1 and k_{-4}/K_5 were held constant; unknown parameters were adjusted in two steps to fit the two experimental data sets, and the best values obtained are given in Table 2. Constraints imposed on the parameters are described in the text, and input scripts are given as Supporting Information.

RESULTS AND DISCUSSION

In our experiments, PGK was preincubated with PG so the starting material was the binary E·PG complex. As under our conditions the affinity of PGK for PG is high [$K_d = 1.5$ μM (*2*)], saturation was ensured at low PG concentrations, typically 20−50 μM. Under this condition, and because of the unfavorable overall equilibrium [K_{eq} = ([ADP][bPG])/([ATP][PG]) = 10^{-4} (*2*)], little free bPG is liberated: such experiments are virtually single turnovers even if under multiturnover conditions. Unless otherwise stated, the PG concentration was low (50 μM). The buffer was 30% in methanol (7.4 M), and the temperature was 4 °C. Methanol does not appear to affect the PGK reaction pathway (*2*).

We first checked on the presence of the abortive E·PG·ADP complex under our conditions. We then report our transient kinetic data which we interpret according to the type of experiment. First, we followed the formation of bPG from ATP and PG using the pathway proposed in Geerlof et al. (*2*) but without the release of products:

Scheme 1

$$\text{E·PG + ATP} \overset{K_1}{\rightleftharpoons} \text{E·PG·ATP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E*·PG·ATP} \overset{K_3}{\rightleftharpoons} \text{E*·bPG·ADP}$$

where the asterisk denotes a different conformation of PGK and $K_i = k_{-i}/k_{+i}$.

By this scheme, our P_i measurements take account of only one intermediate, E*·bPG·ADP.

Second, we perturbed PGK reaction mixtures with ADP. This requires an extension of Scheme 1 to include ADP release (Scheme 2, below).

The transient kinetic time courses were first interpreted intuitively, treating each type of experiment at a time. In particular, on the time scale of our experiments (≥4 ms), we assume that ligand binding occurs in two steps: a rapid equilibrium followed by a relatively slow protein isomerization process (*27*). Each k_{obs} (k_{MTO} in bPG formation under multiturnover conditions, k_{PER} in ADP perturbations) is a reflection of such a process, modulated by the rapid equilibria that precede and follow it. In all cases, the data fit well to single exponentials. By varying the ligand concentrations (ATP in bPG formation, ADP in the perturbations), we obtained estimates for certain parameters of the dependences (slopes and intercepts), which we then interpreted with reference to a reaction pathway, depending on the type of experiments. This provided us with estimates for rate and equilibrium constants which we then refined by a global fitting procedure. Finally, we attempt to link the transient kinetics to the structural properties of PGK.

Affinity of PGK for ADP in the Presence or Absence of PG. With the binding of PG to PGK to form the E·PG complex, or nucleotide to form the E·nucleotide complex, there is a decrease in the inherent fluorescence of the PGK that is characteristic of tryptophan perturbation. The affinities of the ligands can be measured from the dependencies of the amplitude of the fluorescence signal upon the ligand concentration (*2* and references therein).

With PG as the ligand, the decrease in fluorescence at saturation in PG was 11%. When ADP was added to E·PG, there was a further decrease (∼6% at saturation in ADP) and it was possible to obtain an estimate of the affinity of the E·PG complex for ADP (not illustrated).

The K_d values for the interactions of ADP with PGK and the E·PG complex are in Table 1 together with those for ATP and PG. Under our conditions, PG has the effect of decreasing significantly the affinity of PGK for ADP. At ∼300 μM, the K_d for binding of ADP to the E·PG complex (to form the abortive E·PG·ADP complex) should not interfere with our experiments.

The antagonistic effect of PG upon the binding of ADP to PGK was first observed by Scopes (*28*), but in an aqueous buffer, the K_d for ADP increased only ∼2-fold (from 40 to 90 μM) in the presence of PG. Merli et al. (*29*) and Kovári et al. (*30*) discuss the weakening effect of PG upon nucleotide binding to PGK.

Transient Kinetics of bPG Formation. Multiturnovers (MTO) at a high PG concentration (5 mM) are biphasic: a rapid burst transient phase of bPG followed by a short steady state phase before the final equilibrium. Even at a high PG concentration, the steady state phases were short in duration (typically 400 ms) because of the unfavorable overall K_{eq}. The steady state rates obtained were very close to those measured by the coupled assay method in which free bPG is removed continuously (*2*).

Here, at low PG concentrations, the time courses of bPG production are monophasic, because little bPG is released. A typical experiment at 50 μM PG and 200 μM [γ-^{32}P]ATP is shown in Figure 1a. The time course fitted well to a transient burst phase with kinetics k_{MTO} of 82 s^{-1} and an amplitude B_{MTO} of 0.50 mol of [1-^{32}P]bPG/mol of PGK. Clearly, at low PG concentrations, these parameters are easier to obtain because they are not confounded by a steady state phase.

The ATP dependencies of B_{MTO} and k_{MTO} are shown in panels b and c of Figure 1, respectively. Also included are data obtained at 5 mM PG (*2*).

The B_{MTO} dependence fitted well to a hyperbola with an amplitude B_{MTO}^{max} of 0.58 mol of bPG/mol of PGK and an

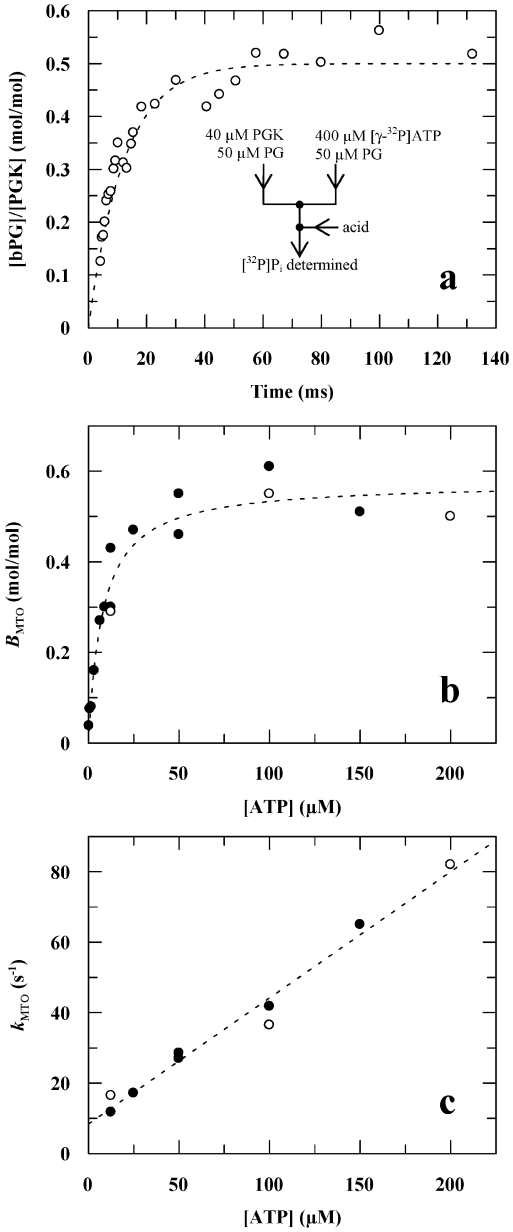


FIGURE 1: Time course for the formation of bPG under multiturnover conditions at low PG and high ATP concentrations in 30% methanol (a). The reactions (20 μM PGK, 200 μM [γ-^{32}P]ATP, and 50 μM PG) were quenched in acid at the indicated times, and the amount of [^{32}P]P_i was determined. (b and c) Effect of the PG concentration on the ATP dependences of the amplitude B_{MTO} (b) and kinetics k_{MTO} (c) of the transient burst phase in multiturnover experiments. The PG concentrations were 50 μM (○) and 5 mM (●; data from ref *2*). The constants obtained are in the text.

$ATP_{0.5}$ of 8 μM. That B_{MTO}^{max} is less than 1 can be explained by the several equilibria involving bound bPG complexes (Scheme 4) and by the presence of the inactive enzyme. The k_{MTO} dependence fitted well to a straight line up to 200 μM ATP with a slope of 0.36 μM^{-1} s^{-1}, which we interpret according to Scheme 1 as the second-order binding constant for ATP, k_2/K_1, and intercept on the k_{MTO} axis of 8 s^{-1} = $k_{-2}K_3/(1 + K_3)$. We estimate $K_1 \gg 200$ μM and $k_2 \gg 80$ s^{-1}. It is noteworthy that the concentration of PG seems to have little effect on these parameters.

Kinetics of ATP Formation: Perturbation of PGK Reaction Mixtures by ADP. So far, our experiments were confined to an extension of our previous work, i.e., an investigation of the kinetics of formation of bPG from ATP and PG at low PG concentrations. These experiments gave little information about the release of products. In particular, the model used to interpret the data obtained (Scheme 1) did not take account for ADP release. We now consider this step by perturbing PGK reaction mixtures (from [γ-^{32}P]ATP and PG) with ADP. Experimentally, this method is related to the tracer perturbation technique of Britton (*31*) which, however, is carried out on the minute time scale because it is concerned with the detection of relatively slow interconversions of unliganded enzyme (also see ref *32* as applied to triosephosphate isomerase). Here, we are interested in the kinetics of formation and decomposition of the intermediates on an enzyme reaction pathway which means experiments on the millisecond time scale.

Table 1: Dissociation Constants for the Binding of ADP, ATP, and PG to PGK

ligand			K_d (μM)	
variable	constant	complex formed	here[a]	ref *28*[b]
ADP	none	E·ADP	28 ± 3	40
ADP	PG	E·PG·ADP	300 ± 150	90
ATP	none	E·ATP	102 ± 5	105
PG	none	E·PG	1.5 ± 0.4	32

[a] Conditions: 20 mM triethanolamine, 0.1 M potassium acetate, 1 mM free Mg^{2+}, and 30% methanol at pH 7.5 and 4 °C. The interactions were assessed by fluorescence spectroscopy. The method used and the data for ATP and PG are in ref *2*. [b] In aqueous buffer.

The rationale for the perturbation experiments is as follows. We mix 10 μM PGK and 50 μM PG with 100 μM [γ-^{32}P]ATP (reaction mixture concentrations). Reaction occurs with the final equilibrium reached within 100 ms at 4 °C. At this equilibrium, the mixture contains 5.5 μM [1-^{32}P]-bPG which we assume to be essentially enzyme-bound because the PG concentration is low. Under these conditions, [1-^{32}P]bPG was stable for at least 2 h at 4 °C (A. Geerlof, unpublished results).

We carried out two experiments with this mixture, both in the rapid quench flow apparatus.

First, we diluted the equilibrium mixture with an equal volume of buffer. The diluted mixture was aged for different times, the reaction quenched in acid, and the amount of [1-^{32}P]bPG measured. As shown in Figure 2a, there was little change in the total amount of [1-^{32}P]bPG in the time range of 4−140 ms.

In the second experiment, the reaction mixture was perturbed by the addition of ADP and the decrease in the amount of [1-^{32}P]bPG followed with time. An experiment at 50 μM ADP is shown in Figure 2b: following the addition of ADP, the amount of [1-^{32}P]bPG decreased rapidly to a low plateau. We interpret this as the binding of ADP to a binary E·[1-^{32}P]bPG complex in the original reaction mixture to give the ternary E·[1-^{32}P]bPG·ADP complex which is then converted to the E·PG·[γ-^{32}P]ATP complex and finally free [γ-^{32}P]ATP, neither of which is measured. Thus, it appears that a binary E·[1-^{32}P]bPG complex is an important intermediate on the PGK reaction pathway.

The data in Figure 2b fitted well to a single exponential with kinetics k_{PER} of 92 s^{-1} and a final low plateau of 0.17 mol of [1-^{32}P]bPG/mol of PGK that represents the new equilibrium, B_{PER}.

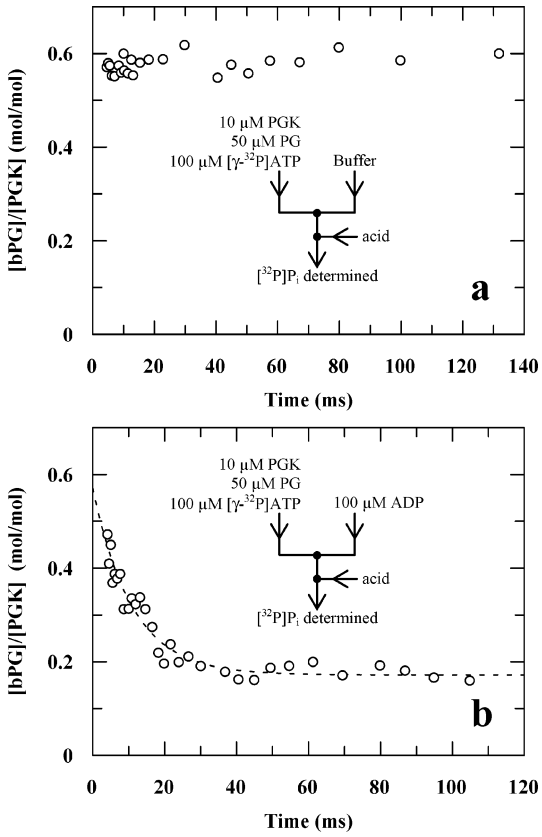


FIGURE 2: Perturbation of a PGK reaction mixture at equilibrium by dilution (a) or by 50 μM ADP (b) in 30% methanol at 4 °C. Reaction mixtures (initially 10 μM PGK, 50 μM PG, 100 μM [γ-^{32}P]ATP, and 25 μM Ap5A) were mixed with buffer (a) or 100 μM ADP (b) in the rapid quench flow apparatus and aged for the indicated times shown; the reactions were quenched in the acid, and the amount of [1-^{32}P]bPG was determined. Ap5A was included to inhibit any myokinase in the PGK preparation. The constants obtained are in the text.

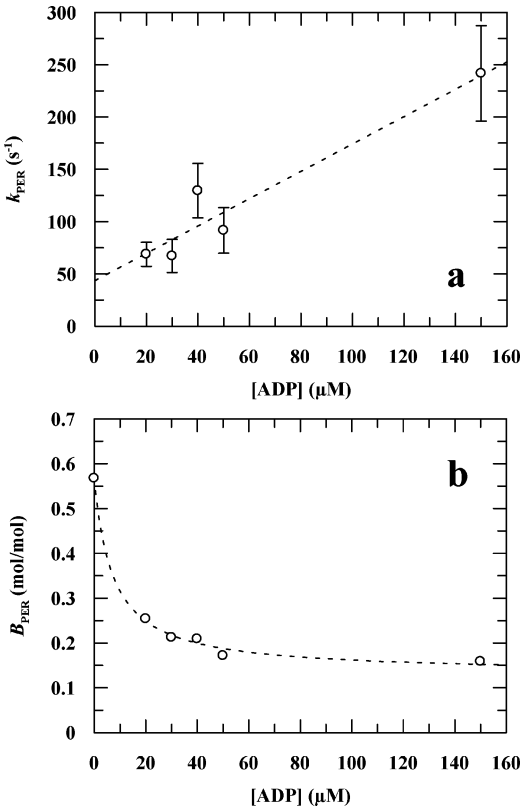


FIGURE 3: ADP perturbation experiments that reveal the dependences of k_{PER} (a) and B_{PER} (b) on the ADP concentration. PGK reaction mixtures (as in Figure 2) were perturbed by different concentrations of ADP and k_{PER} and B_{PER} obtained. For further details, see the legend of Figure 2.

The dependences of k_{PER} and B_{PER} on the ADP concentration are shown in Figure 3. In these experiments, we were limited to a narrow ADP concentration range: at low ADP concentrations (below 20 μM), the amplitude of the transient is low and a k_{PER} difficult to obtain, whereas at >50 μM ADP, the amplitude increases but k_{PER} becomes too fast for our equipment.

k_{PER} appears to increase linearly with the ADP concentration up to 50 μM; the linearity was confirmed by an experiment at 150 μM ADP. At 150 μM ADP, the kinetics were rapid ($k_{PER} \sim 240$ s^{-1}; first sampling at 4.5 ms at ~80% completion of the reaction; not illustrated). In the dependence (Figure 3a), the parameters of the ADP interaction were calculated with the data up to 50 μM ADP only. The slope is 1.3 μM^{-1} s^{-1} and the intercept on the k_{PER} axis 44 s^{-1}. The slope, related to the ADP "on" kinetics, seems to be too low to represent a diffusion-controlled process (*27*), which supports our assumption that ADP binds to the E·bPG complex in two steps, but as we did not attain saturation kinetics for k_{PER}, we could not confirm this.

The dependence of B_{PER} upon the ADP concentration was interpreted by a hyperbola with a limiting value at high ADP concentrations of 0.13 mol of bPG/mol of PGK and an $ADP_{0.5}$ of 7 μM (Figure 3b).

To conclude, these ADP perturbation experiments suggest strongly that a binary E·bPG complex is an important intermediate on the PGK reaction pathway. The experiments also show that it is possible to study the PGK reaction in the direction of ATP formation, i.e., in the physiologically important direction, without having to prepare and handle the labile bPG. Accordingly, we extend Scheme 1 to include the ADP release step:

Scheme 2

$$\mathrm{E{\cdot}PG + ATP} \overset{K_1}{\rightleftharpoons} \mathrm{E{\cdot}PG{\cdot}ATP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E^*{\cdot}PG{\cdot}ATP} \overset{K_3}{\rightleftharpoons} \mathrm{E^*{\cdot}bPG{\cdot}ADP} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{E{\cdot}bPG{\cdot}ADP} \overset{K_5}{\rightleftharpoons} \mathrm{E{\cdot}bPG + ADP}$$

By this scheme, our P_i measurements take account of three intermediates: E*·bPG·ADP, E·bPG·ADP, and E·bPG. We interpret the parameters obtained in the k_{PER} dependence as follows: slope $= k_{-4}/K_5 = 1.3$ μM^{-1} s^{-1} (where $K_5 = k_5/k_{-5}$) and the intercept on the k_{PER} axis $= k_4(1 + K_3)/K_3 = 44$ s^{-1}.

Estimates of the Individual Constants in Scheme 2 by Global Fitting: PGK Reaction Pathway at Low PG Concentrations. The experimental data obtained at different concentrations of ATP (in the direction of bPG formation, Figure 1) or of ADP (ATP formation, Figure 3) were fitted simultaneously assuming the model in Scheme 2 and using Scientist (version 2.0, MicroMath Research). The differential equations describing the time-dependent change in concentration for all species were entered, and curves were derived by numerical integration and nonlinear minimization of least-squares deviations (*33*). The following constraints were

Table 2: Estimates of the Kinetic Constants Describing Scheme 2[a]

constant[b]	estimate from experimental data: how obtained	estimate from experimental data: value	estimate from global fit
From Kinetics of bPG Formation (Figure 1)			
K_1 (mM)	linearity of k_{MTO} dependence	$\gg 0.2$	2.8 ± 0.5
k_2 (s^{-1})	linearity of k_{MTO} dependence	$\gg 80$	1000 ± 200
k_2/K_1 (μM^{-1} s^{-1})	slope of k_{MTO} dependence	0.36 ± 0.02	0.36[c]
k_{-2} (s^{-1})	intercept of k_{MTO} dependence	28 ± 7	28 ± 6
K_2	k_{-2}/k_2	$\ll 0.35$	0.028 ± 0.012
From Kinetics of ATP Formation (Figure 3)			
K_5 (mM)	linearity of k_{PER} dependence	$\gg 0.15$	7.5 ± 7.5
k_{-4} (s^{-1})	linearity of k_{PER} dependence	$\gg 250$	$(1 \pm 0.9) \times 10^4$
k_{-4}/K_5 (μM^{-1} s^{-1})	slope of k_{PER} dependence	1.3 ± 1.2	1.3[c]
k_4 (s^{-1})	intercept of k_{PER} dependence	13 ± 11	25 ± 5
K_4	k_4/k_{-4}	$\ll 5.2 \times 10^{-2}$	$(2.5 \pm 2.5) \times 10^{-3}$
Constant Not Obtained Experimentally			
K_3	–	–	0.4 ± 0.1[d]

[a] In 20 mM triethanolamine, 0.1 M potassium acetate, 1 mM free Mg^{2+}, and 30% methanol at pH 7.5 and 4 °C. [b] K_1, K_2, and K_3 are from the relation $K_i = k_{-i}/k_{+i}$; K_4 and K_5 are from the relation $K_i = k_{+i}/k_{-i}$. [c] Imposed. [d] In ref *2*, the value is 0.25.

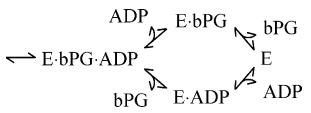
imposed: (1) on the time scale of the experiments, steps 1, 3, and 5 are rapid equilibria; (2) all the kinetic constants are positive; (3) active site titration, i.e., equivalents of active site per mole of PGK, ≤ 1 (the global fits gave 0.85 ± 0.15; also see ref *2*); (4) the second-order constants k_2/K_1 and k_{-4}/K_5 constrained to the slopes of the dependence of k_{MTO} on ATP concentration (Figure 1c) and k_{PER} on ADP concentration (Figure 3a), respectively.

In the estimates of the kinetic constants (Table 2), the errors were given by the computer analysis and they do not include experimentally introduced errors. The problem of errors is discussed by Lionne et al. (*34*).

It is noteworthy that the intuitive and global fit estimates agree well, especially k_{-2} and k_4. This is surprising because in the intuitive estimates, we did not consider steps far removed from those observed, whereas in the global fits, all of the steps were taken into account. The agreement could be due to the imposition of the two slopes of the dependences (k_2/K_1 and k_{-4}/K_5) in the global fits. It could also be due to our model (Scheme 2) in which steps involving relatively slow and therefore observable kinetics are separated by rapid equilibria. Further, we cannot exclude the possibility that our data can be fitted to other sets of constants in addition to that in Table 2. Also, even if the progress curves, whether describing bPG formation (e.g., Figure 1) or disappearance (Figure 2), fitted well to single exponentials, we cannot exclude multiple exponentials with similar rate constants for each process and, therefore, additional intermediates.

Order of Release of ADP and bPG. In Scheme 2, we assume that ADP leaves before bPG, and we must address the validity of this assumption. In Scheme 3, ADP is released before bPG in the upper pathway and bPG before ADP in the lower pathway:

Scheme 3



Scopes (*35*) wrote that "the rate of dissociation of bPG is the controlling factor in the enzyme's activity", but the order of release of ADP and bPG has not been established experimentally (*36*). Here, we did not study directly the product release steps, but our data support our assumption.

First, consider the experiments with reaction mixture at low PG concentrations, carried out under multiturnover conditions. A typical time course of bPG formation is shown in Figure 1a: there was a transient burst phase but no steady state phase of bPG production. Under the same condition but at a high PG concentration (5 mM), the transient phase was followed by a steady state phase of bPG production. This phase was short in duration but proceeded to more than 1 mol of bPG/mol of PGK so almost certainly represents free bPG (*2*). It is noteworthy that the amplitudes of the transients in the two experiments are identical and that in the experiment at low PG concentrations, the data fitted well to a single exponential. Taken together, these observations confirm that at low PG, little free bPG is liberated.

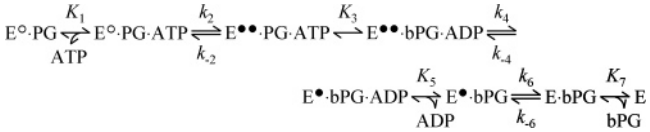
Second, PGK reaction mixtures at equilibrium are sensitive to ADP perturbation which is evidence that a binary E·bPG complex is an important intermediate (Figure 2b).

Finally, we note that PGK binds bPG much more tightly than ADP (*28*) which suggests that the k_{OFF} for bPG (directed by k_6 in Scheme 4) is less than the k_{OFF} for ADP (k_4).

In conclusion, our data support the assumption that ADP leaves before bPG and that E·bPG types of complexes are important on the PGK reaction pathway. Whether this order is obligatory, in that bound ADP impedes the exit of bPG, or whether it is merely a question of relative leaving kinetics remains to be determined.

Correlation of Kinetics with Structure: Structural Arguments for an Overall Reaction Pathway for PGK. Scheme 4 is an extension of Scheme 2 as it includes a two-step release of bPG (implicit in ref *28*). As our experiments were carried out at low PG concentrations, we did not study the bPG release kinetics here but the two steps are included (in gray) to aid the discussion.

Scheme 4



In the scheme, open conformations are shown by E° and E and closed conformations by E• and E••. For a recent discussion of these conformations of PGK, see ref *1*.

There are three types of step in Scheme 4: formation of collision complexes (rapid equilibria defined by K_1, K_5, and K_7), phospho-transfer [rapid equilibrium K_3 (*37*)], and protein conformational changes (k_2, k_{-2}, k_4, k_{-4}, k_6, and k_{-6}). We assume that on the time scale of our experiments, the kinetics obtained are reflections of the conformational changes: k_2 and k_{-2} in bPG bursts (k_{MTO} in Figure 1), k_4 and k_{-4} in ADP perturbations (k_{PER} in Figures 2 and 3), and k_6 in steady state experiments (also see ref *2*).

In the discussion below, the numbering of the amino acid residues is based on the yeast PGK primary structure (*38*).

PG binds to the N-terminal domain and ATP to the C-terminal domain of PGK. In the apoenzyme, the two sites are separated by a cleft and are 14 Å apart. ATP binds to the apoenzyme so that its α-phosphate interacts with Lys-218 and its β-phosphate (through a water molecule) with Asp-217 (*39*). However, because PG induces a conformational change (E to E° in Scheme 4; e.g., refs *40* and *41*), it leads to a different binding mode for ATP, but the two sites remain apart (in the E°•PG complex, the starting material in our experiments). Now, following the formation of the E°•PG•ATP collision complex, there appears to be a substantial structural change, or hinge bending motion, that leads to the approximation of the N- and C-domains, i.e., to the closing of the cleft to the closed structure in the E••PG•ATP complex (*42*, *43*; also see ref *2* and references therein). In this intermediate, the γ-phosphate is bound to Lys-214. Lys-214 is a key residue [it is highly conserved (*44*)] because the γ-phosphate is transferred directly from it to the carboxyl group of PG forming bPG (in E••bPG•ADP). Since the nascent bPG is also bound to the N-terminal domain, it bridges the two domains, thus keeping the enzyme in its closed conformation which appears to be kept locked by a salt bridge between Arg-66 and Asp-217 (*4*, *44*, *45*).

Here we propose that the E••bPG•ADP complex undergoes a small conformational change to the E••bPG•ADP complex in which the enzyme remains in the closed conformation; i.e., the bPG remains bound by its phosphate groups to the two domains. This is a key feature of Scheme 4, as it allows the ADP to escape (or to enter in the direction of ATP formation in the ADP perturbation experiments) from a closed conformation of PGK. It could be that the ADP escapes by a "backdoor", a mechanism proposed for certain other enzymes [e.g., ADP from another ATP handling enzyme, myosin ATPase (*46*, *47*); other backdoor enzymes reviewed in ref *48*].

After the release of the ADP, the enzyme remains in a closed conformation in the binary E••bPG complex. This implies that bPG on its own induces hinge bending (also suggested in refs *4*, *28*, *41*, and *44*) which would explain its tight binding.

Finally, we propose that the E••bPG complex isomerizes slowly to the E•bPG complex in which the PGK is in an open conformation. This may be the rate-limiting step on the overall reaction pathway. It is noteworthy that with pig PGK anion activation requires domain closure (*49*) which implies that with this enzyme also bPG release from a E• bPG complex is rate-limiting.

CONCLUSIONS AND IMPLICATIONS

From this and previous studies (*2*, *19*), we propose a seven-step reaction pathway for PGK that includes four bPG-containing intermediates (Scheme 4). Of these, we propose that the binary E••bPG complex is a key intermediate. In it, the PGK is in a closed conformation; it accumulates because bPG release (directed by k_6) appears to be the slowest step in the direction of bPG formation. In the direction of ATP formation, the rate-limiting step could be k_{-2}, which at 28 s^{-1} is relatively slow but faster than k_6 (~8 s^{-1}; ref *2*). This implies that in the forward direction (i.e., ATP formation), the maximum velocity of PGK is ~3 times greater than that in the reverse direction, in agreement with Scopes (*50*).

Further, the E••bPG complex is an important intermediate because according to Scheme 4 it interacts with ADP that may have entered the active site by a backdoor. This interaction perturbs the rapid equilibrium K_5 and leads to the formation of ATP. Therefore, the transient kinetics of disappearance of bPG is a reflection of ATP formation: by perturbation of PGK reaction mixtures at equilibrium with ADP, it is possible to study the kinetics of ATP formation without having to handle the unstable bPG, i.e., in the physiologically important forward direction. We are extending these perturbation studies to analogues of ADP, in particular those of medical interest such as L-nucleoside diphosphates. Further, we view the E••bPG complex as a target for the development of uncompetitive inhibitors of PGK.

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SUPPORTING INFORMATION AVAILABLE

Additional information about the global fittings. This material is available free of charge via the Internet at http://pubs.acs.org.

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