Transient and Equilibrium Kinetic Studies on Yeast 3-Phosphoglycerate Kinase. Evidence That an Intermediate Containing 1,3-Bisphosphoglycerate Accumulates in the Steady State†

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ABSTRACT: The structure of the key glycolytic enzyme 3-phosphoglycerate kinase (PGK) is known in detail, but there is little information on its reaction pathway. We have studied its equilibrium and transient kinetics in the direction of 1,3-bisphosphoglycerate (1,3-bis-P-glycerate) production: ATP + 3-P-glycerate ↔ ADP + 1,3-bis-P-glycerate. We devised a sensitive method for following this production. PGK is mixed with 3-P-glycerate and [γ-^{32}P]ATP in a rapid flow quench apparatus. The reaction mixtures are aged for 4 ms or more and then quenched in acid in which any [1-^{32}P]-1,3-P-glycerate decomposes to 3-P-glycerate and $^{32}P_i$, which is determined specifically. The P_i reflects accurately the 1,3-bis-P-glycerate in the original reaction mixture, and the k_{cat} obtained is identical to that obtained by the conventional linked assay method with glyceraldehyde-3-phosphate dehydrogenase. This does not support the postulate of a rapid direct transfer of the 1,3-bis-P-glycerate between the kinase and the dehydrogenase [Srivastava, D. K., & Bernhard, S. A. (1986) *Science 234*, 1081−1086]. We fitted our data to a simple scheme with the formation of binary complexes, the interconversion of substrates to products via ternary complexes, and the release of products. Because of the high turnover of PGK, the work was carried out under cryoenzymic conditions with 40% ethylene glycol in the buffer. The glycol decreased k_{cat} from 80 to 8.5 s^{-1} (pH 7.5, 4 °C), but the K_m for 3-P-glycerate and ATP and the equilibrium constants in the scheme were little affected. We carried out two types of experiment. In *equilibrium studies* we obtained estimates for the equilibrium constants and evidence for the accumulation of the intermediate PGK•1,3-bis-P-glycerate. This supports the proposal that the kinetics of the release of 1,3-bis-P-glycerate are slow [Scopes, R.K. (1978) *Eur. J. Biochem. 85*, 503−516]. In *transient kinetics* experiments, we confirmed the accumulation of the binary complex by showing that there was a transient burst phase of 1,3-bis-P-glycerate. The kinetics of the phase were determined, but because of the rapidity of the processes they were difficult to exploit fully, even at −15 °C. The equilibrium constant for enzyme-bound substrates and products is E•ADP•1,3-bis-P-glycerate/E•ATP•3-P-glycerate = 0.1, compared with the 0.8 value obtained by NMR [Nageswara-Rao, B. D., Cohn, M., & Scopes, R. K. (1978) *J. Biol. Chem. 253*, 8056−8060], a difference that we explain by the NMR measurement including the binary complex E•1,3-bis-P-glycerate.

An important problem in enzymology is to correlate structure and function. This requires a detailed knowledge of the structure of an enzyme and its reaction pathway. Ideally, one must obtain the structures of all the reaction intermediates and their rates of interconversion. The problem is how to obtain this information. Douzou and Petsko (1984) proposed that by the use of high-resolution techniques such as X-ray crystallography and NMR at subzero temperatures one can obtain "stop-action" pictures of enzyme reaction pathways. One can exploit genetic engineering to design enzymes that accumulate selected intermediates in their reaction pathways [e.g., Avis and Fersht (1993)].

Experimenting with enzymes at subzero temperatures (cryoenzymology; Douzou, 1977) is a powerful means for obtaining kinetic information. It is a technique that we have used with several systems handling ATP [reviewed in Barman and Travers (1985)], and here we applied it to another ATP-handling enzyme, 3-phosphoglycerate kinase (PGK).[1] This enzyme catalyzes the first ATP generating reaction in anaerobic glycolysis, i.e. the reversible transfer of phosphate between ADP and 1,3-bis-P-glycerate (bPG):

$$ADP + bPG \leftrightarrow ATP + PG$$

This enzyme has received much attention from protein chemists, but there is little information on its reaction pathway and, in particular, on the rates of interconversion of the intermediates. Detailed steady-state kinetics have been carried out with PGK [reviewed in Scopes (1973); see also Scopes (1978a,b)]. These studies were mainly on the back reaction, i.e., the reaction with ATP and 3-phosphoglycerate (PG) as substrates. Work on the forward reaction is hampered by the instability of bPG. Furthermore, in the back reaction, one can link PGK to glyceraldehyde-3-phosphate dehydrogenase, which allows one to follow conveniently bPG production by the oxidation of NADH at 340 nm.

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; Ap5A, P1,P5-di-(adenosine-5′) pentaphosphate; ATP, adenosine 5′-triphosphate; bPG, 1,3-bisphosphoglycerate; PG, 3-phosphoglycerate; PGK, 3-phosphoglycerate kinase; P_i, inorganic orthophosphate.

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The steady-state kinetics of PGK are complex because the nucleotide substrates (as well as other anions such as sulfate) have two binding sites: a catalytic site and a site termed "anionic" situated in a "basic patch" region on the enzyme surface (Scopes, 1978a; Wrobel & Stinson, 1978; Ray & Nageswara Rao, 1988; Joao & Williams, 1993). It is thought that the anionic site plays a regulatory role in that its occupancy aids the release of the tightly bound product, bPG, which has the effect of increasing k_{cat} (Scopes, 1978a). However, at high concentrations anions inhibit by competing with the substrates at the catalytic site.

At reasonable substrate concentrations, it appears that the binding of the substrates (ATP and PG) is random and that the binding of one substrate does not affect the binding of the other. At very high concentrations of one substrate the binding of the other is affected but only to a small extent (Scopes, 1978a). This secondary effect is not taken into account here. The results of NMR studies suggest that, as is common with a number of kinases, the equilibrium constant for the enzyme-bound reactants and products is about 1 (Nageswara Rao et al., 1978; Burbaum & Knowles, 1989). There is little evidence for a phosphoryl enzyme intermediate, and it is thought that transfer occurs directly between the substrate molecules within a ternary complex [e.g., Scopes (1973), Johnson et al. (1976), and Knowles (1980)].

Extensive structural studies have been carried out on the yeast enzyme both by X-ray (e.g. Watson et al., 1982) and by NMR [reviewed in Joao and Williams (1993)]. PGK is a single polypeptide chain of about 45 kDa, and the probing of the roles of several amino acid residues in its catalytic function has been carried out by site-directed mutagenesis [e.g., Ballery et al. (1990), Sherman et al. (1992), and Walker et al. (1992); reviewed in Joao and Williams (1993)]. From these structural studies the binding of either substrate induces a conformational change, that with PG being the larger. Both substrates must bind for the complete conformational change needed for the in-line transfer of phosphate (Dryden et al., 1992; Jao & Williams, 1993; Pineda et al., 1993). Because of its very tight binding (K_d about 0.05 μM), Scopes (1978b) has predicted that the kinetics of the release of bPG is the rate-limiting step on the PGK reaction pathway, but a transient burst phase (or for that matter, a lag) has not been reported. This is presumably in part due to the rapidity of the overall k_{cat} which makes transient phase measurements difficult. Despite these several works, therefore, there is little information on the PGK reaction pathway. For a full description of this, the different intermediates must be identified and their kinetics of formation and decomposition obtained. To our knowledge, a transient kinetic study on PGK has not been carried out.

In this work we applied to the PGK system the techniques that we used with another system that handles ATP: myosin ATPase [reviewed in Barman and Travers (1985)]. In that work, we used [γ-^{32}P]ATP and the rapid flow quench method (chemical sampling in the millisecond to several seconds time range). Here we developed a chemical sampling method for studying the transient and equilibrium kinetics of PGK in the direction of bPG formation by quenching reaction mixtures in acid and determining the $^{32}P_i$ produced by the decomposition of any [1-^{32}P]bPG.

The kinetics of PGK are difficult to study for several reasons, and we addressed these problems as follows. First, we slowed down its high turnover rate by applying cryoenzymology (solvent and temperature perturbation; Douzou, 1977).

Second, by choosing to study the back reaction, we choose the thermodynamically unfavorable one (Scopes, 1973). This presents little problem with the linked assay method, as the tightly bound product bPG is continuously removed. However, with the chemical sampling method this artifice cannot be used; the chemical equilibrium is reached rapidly, and kinetic measurements are difficult. We overcome this problem by sampling reaction mixtures in the millisecond range, i.e., well before the attainment of the final equilibrium.

Third, as discussed above, kinetic studies on PGK are confounded by interference from secondary anionic sites. To reduce this interference, we kept the ATP concentrations low (<0.5 mM). We did not study the role of these sites here (for instance, the effect of sulfate).

Fourth, the reaction involves two substrates, ATP and PG, which complicates kinetic analysis, and we used as the starting material the binary complex PGK•PG. Finally, to prevent problems with Mg^{2+}, we kept its free concentration constant at 1 mM.

In this study we present evidence that intermediates containing bPG accumulate on the PGK reaction pathway in the steady state, and in agreement with Scopes (1978a) we give evidence that the kinetics of the release of bPG are relatively slow. Further, by our chemical method, it appears that the equilibrium constant for the interconversion of the ternary complexes (E•ATP•PG/E•ADP•bPG) is about 0.1 which is lower than the 0.8 obtained by Nageswara Rao et al. (1978) by NMR.

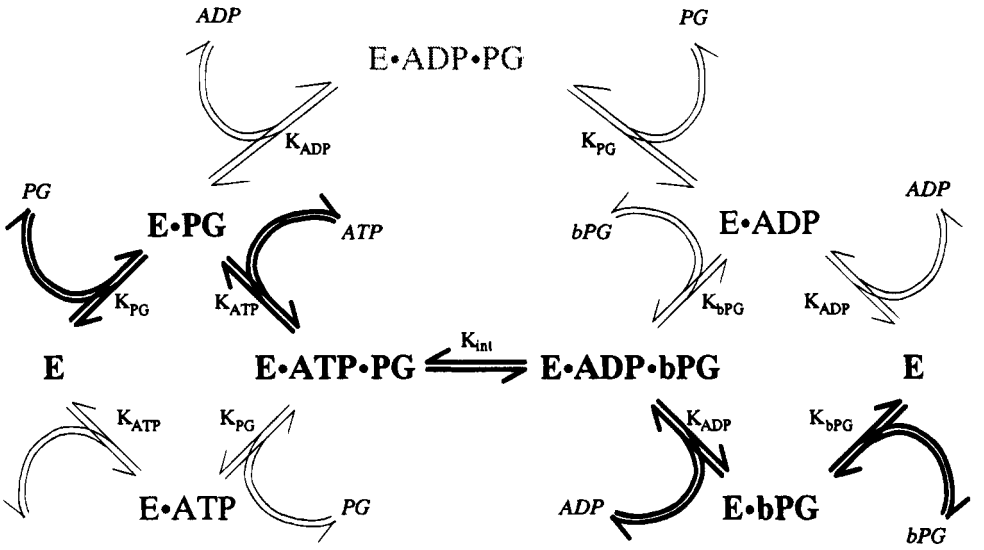
MATERIALS AND METHODS

Proteins and Reagents. PGK was prepared as in Scopes (1971), and myosin, as in Weeds and Taylor (1975). PG, NADH, and yeast glyceraldehyde-3-phosphate dehydrogenase were from Boehringer Mannheim, and [γ-^{32}P]ATP was from Amersham International. P1,P5-di(adenosine-5′) pentaphosphate (Ap5A) was from Sigma Chemical Co. PGK concentrations were estimated at 280 nm with $E^{1\%,\,1\,cm} = 4.9$ (Graham et al., 1991).

Experimental Conditions. Our experiments were carried out in the absence of sulfate, and to reduce complications arising from the anionic site in PGK, we considered carefully the experimental conditions. To reduce interference from free ATP (which binds strongly to both the catalytic and anionic sites, thus competing with the catalytically active Mg-ATP), the free Mg^{2+} concentration (as Mg-acetate) was kept at 1 mM, as advocated by Scopes (1978a). To reduce further anionic site complications, the ionic strength was moderate and was made up of the noninterfering 0.1 M potassium acetate (K-acetate) (Scopes, 1978a). The concentrations of ATP were kept below 2 mM, and that of PG, at 5mM (Scopes, 1978a; Sherman et al., 1990). The concentrations of ATP and PG in the figure captions refer to the final reaction mixture concentrations. The solutions were buffered by 20 mM triethanolamine, adjusted to pH 7.5 with acetic acid. In certain experiments the buffer contained 40% ethylene glycol.

PGK Activity by the Coupled Enzyme System. The equilibrium constant for the PGK reaction is strongly in favor of the formation of ATP (Nageswara Rao et al., 1978). However, when coupled to glyceraldehyde-3-phosphate dehydrogenase, the reaction can be followed conveniently

Scheme 1: Phosphoglycerate Kinase Reaction Pathway



in the reverse direction, i.e., by the formation of bPG. The method used is a modification of that of Scopes (1978a). The assay mixture (500 μL) contained 0.15 mM NADH and 300 nM glyceraldehyde-3-phosphate dehydrogenase in the buffer under Experimental Conditions, above. The concentrations of ATP and PG are specified in the different experiments.

PGK Activity by Chemical Sampling. We also studied the back reaction by chemical sampling using a method that is based upon the extreme instabilities in acid of acylphosphates (Jencks, 1969) and here of bPG (Johnson et al., 1976). PGK plus PG and [γ-^{32}P]ATP reaction mixtures were incubated for specified times, quenched in acid (by the addition of 22% trichloroacetic acid plus 1 mM K_2HPO_4), and kept on ice for less than 1 h before analysis. Under these conditions, ATP and PG are stable but any [1-^{32}P]bPG decomposes to PG and [^{32}P]P_i, which was measured by the method of Reimann and Umfleet (1978). *Thus, as a first approximation, we assume that the $^{32}P_i$ determined is equal to the [1-^{32}P]bPG produced by PGK.*

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 (cpm) in the [γ-^{32}P]ATP solutions were obtained by completely hydrolyzing portions of the solution with myosin. Zero-time points and total radioactivities were done in triplicate.

Transient Kinetic Experiments. These were carried out by rapid chemical sampling. PGK preincubated with PG (5 mM) was rapidly mixed with [γ-^{32}P]ATP in a flow quench apparatus. The reaction mixtures were aged for specified times and then quenched in acid and the [^{32}P]Pi determined, as above. Two apparatuses were used: a rapid flow quench apparatus for reaction times of 4−300 ms and a time delay flow quench apparatus for times of 0.3 s to several tens of seconds. Both apparatuses were fully thermostatically controlled and had been constructed in this laboratory (Barman & Travers, 1985).

The concentrations of PGK and [γ-^{32}P]ATP used in the different experiments are given in the captions to the figures or in the text.

Treatment of the Data. We interpreted our data by Scheme 1, in which K_{ATP}, etc. are dissociation constants. Following Scopes (1978a), we assume first that PGK (E in Scheme 1) binds randomly the substrates PG and ATP and the products bPG and ADP and that the binding of one substrate (or product) does not affect the binding of the other substrate (or product). Second, we assume a direct in-line transfer of the phosphoryl group via the ternary complexes E•ATP•PG and E•ADP•bPG. The abortive ternary complex E•ADP•PG is also included (see Results, K_{int}, below); as for the formation of the productive ternary complexes we assume that the presence of one ligand does not affect the binding of the other.

In Scheme 1 we do not take into account any two-step binding of ATP and PG (for which there is certain structural but little kinetic evidence).

We interpret our data by the pathway indicated in bold because first, in our experiments, the enzyme was incubated with saturating PG [K_{PG} is about 50μM (Scopes, 1978b); the concentration used here is 5 mM) and second, it is thought that ADP is released before bPG (Scopes, 1978a).

RESULTS

Steady-State Parameters of PGK by the Linked Assay Method

The steady-state parameters in the backward reaction were obtained by the linked assay method at two temperatures and in two solvents (Table 1). The somewhat higher k_{cat} with ATP as the variable substrate is presumably due to a small activation by the high concentration of PG used (5 mM).

At 25 °C and in water, the k_{cat} and K_m values are in good agreement with previous work obtained under similar conditions [e.g., Scopes (1978a) and Mas et al. (1987)]. At 4 °C, the high k_{cat} of PGK was reduced to about 50% of its value at 25 °C, and most of our experiments were carried out at this temperature. We discuss the effect of 40% ethylene glycol below.

Also included in Table 1 are values for the apparent second-order constant k_{cat}/K_m. The value of this constant informs us of the value in searching for intermediates on a reaction pathway. Thus, if it is high ($>10^3$ μM^{-1} s^{-1}), the reaction is diffusion controlled [e.g., Blacklow et al. (1988)]; i.e., the substrate binding kinetics are rate limiting, and clearly no intermediate accumulates. The low value for k_{cat}/K_m with PGK suggests that with this enzyme the kinetics of

Table 1: Effect of Temperature and Solvent on Steady-State Parameters of PGK[a]

variable substrate	temp (°C)	kinetic parameters k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
in water				
PG[b]	25	160 ± 6	170 ± 25	0.94 ± 0.16
	4	79 ± 3	240 ± 40	0.33 ± 0.07
ATP[c]	25	195 ± 5	125 ± 15	1.6 ± 0.2
	4	83 ± 3	110 ± 20	0.75 ± 0.15
in 40% ethylene glycol				
PG[b]	25	27.5 ± 1.0	215 ± 30	0.13 ± 0.02
	4	8.1 ± 0.4	225 ± 40	0.04 ± 0.01
ATP[c]	25	27 ± 4	115 ± 30	0.23 ± 0.10
	4	9.4 ± 0.7	110 ± 20	0.09 ± 0.02

[a] The buffer was 20 mM triethanolamine–acetate and 0.1 M potassium acetate, pH 7.5. The free Mg^{2+} concentration was kept constant at 1 mM. [b] [ATP] was kept constant at 5 mM. [c] [PG] was kept constant at 5 mM.

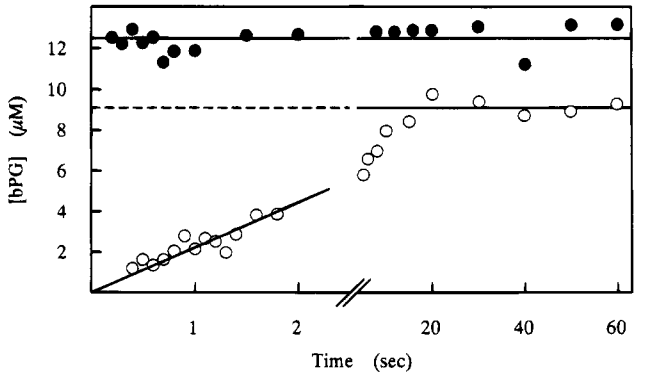


FIGURE 1: Time courses for the formation of bPG by PGK at 4 °C in the seconds time range. The reaction mixtures (0.05 μM PGK, ○; 10 μM PGK, ●) were quenched in acid, and the $^{32}P_i$ was determined. The other reagent concentrations were 100 μM [γ-^{32}P]-ATP and 5 mM PG. In the experiment at 0.05 μM PGK the line covering 0–2 s represents the initial velocity obtained by the linked assay method under the same conditions. The buffer was 20 mM triethanolamine, 0.1 M K-acetate, and 1 mM free Mg^{2+}, pH 7.5.

steps other than those of substrate binding are rate limiting and, therefore, that certain intermediates may accumulate in the steady state.

Since the turnover of PGK is high, the linked assay method cannot be used to investigate the kinetics of its reaction intermediates. Further, by this method the product bPG is continuously removed, so equilibrium constants cannot be studied. We approached the problem by exploiting a chemical sampling method.

Kinetics of PGK by Chemical Sampling: Steady-State and Equilibrium Studies

As with the linked assay, these experiments were carried out in the direction of the formation of bPG. Typical experiments at 4 °C and at two PGK concentrations are shown in Figure 1.

The first was at a catalytic concentration of PGK (0.05μM) and the progress curve consists of a rise to the final equilibrium plateau of bPG. The points on the rise agreed very well with the initial velocity obtained by the linked assay method which gave a state rate of 35 (±5) s^{-1} (continuous line). The implication of this identity in connection with the proposal of direct transfer of bPG in a PGK-glyceraldehyde-3-P-phosphate dehydrogenase complex is discussed below (Discussion).

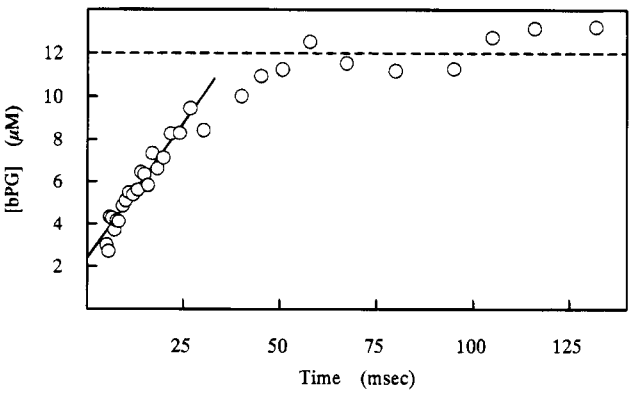


FIGURE 2: Time course for the formation of bPG by PGK at 4 °C in the milliseconds time range. The reaction mixtures (10 μM PGK, 100 μM [γ-^{32}P]ATP, and 5 mM PG) were quenched in acid at the times indicated, and the $^{32}P_i$ was determined. For buffer, see Figure 1. The data were fitted to an initial burst of 2.5(±0.5) μM, a linear phase of 25(±5) s^{-1}, and a plateau of 12(±1) μM bPG.

As the concentration of enzyme was catalytic, the plateau (8.8 μM bPG) gives the overall equilibrium constant (K_{eq}) at 4 °C for the reaction with the relationship for free substrates and products:

$$K_{eq} = ([ADP][bPG])/([ATP][PG]) = 1.5 \times 10^{-4}$$

The second experiment was at a reagent concentration of PGK (10μM). Here the plateau of bPG was reached before the first time point (0.35 s). This rapidity at a high enzyme concentration is, of course, as expected but the high plateau at 12.7 μM bPG is noteworthy. It is 3.9μM higher than the plateau at catalytic PGK and is evidence of intermediates containing bPG. Thus, at reagent concentrations of PGK the bPG plateau appears to contain both free and PGK bound bPG. To confirm this protein bound bPG, we must obtain its kinetic of formation which means transient kinetics.

Transient Kinetics of bPG Formation

In these experiments the choice of reagent concentrations is critical. For good accuracy the [γ-^{32}P]ATP to PGK molar ratio must be low but when it is too low the linear steady state is difficult to discern as the final equilibrium is reached so rapidly. Here we used a 10:1 ratio.

A typical experiment at 4 °C is shown in Figure 2. It was carried out by preincubating the PGK with PG and then mixing this with [γ-^{32}P]ATP also containing PG. The time course could be fitted to three phases:

First, there was a rapid rise of amplitude about 0.25 mol bPG/mol PGK. This is further evidence that intermediates containing bPG accumulate in the steady state. However, the kinetics of the burst were too fast to be measured with the apparatus used, which confirms the conclusion of Nageswara Rao et al. (1978) that the formation of bPG on PGK is very rapid. Second, there was a linear phase which was over at about 25msec giving a rate constant of 25 (±5) s^{-1}. Since it was squeezed between a rapid transient and the kinetics of the approach to equilibrium, it was difficult to extract a rate constant for this phase but its value is in good agreement with the 35 (±5) s^{-1} obtained by the linked assay under the same conditions. Third, the final plateau of 12μM bPG accords well with the overall K_{eq}.

Clearly the kinetics of the formation of the putative bPG enzyme complexes are very rapid, even at 4 °C, and to try to measure these we resorted to cryoenzymology.

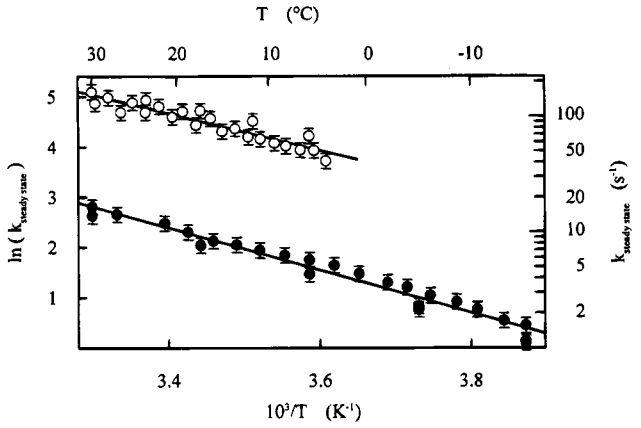


FIGURE 3: Arrhenius plots of the steady-state rates of PGK in water (○) and 40% ethylene glycol (●). The reaction mixtures contained 1 mM ATP and 2 mM PG. The data were obtained from steady-state experiments using the linked assay system, as described in the text. The dependences are linear with $\Delta H^{\ddagger} = 30.5 \pm 2.4$ kJ/mol in water and 35.0 ± 1.3 kJ/mol in 40% ethylene glycol.

Experiments in 40% Ethylene Glycol

A powerful method for increasing the time resolution is to experiment under cryoenzymic conditions which means the addition of an antifreeze, usually an organic solvent (Douzou, 1977). The artifice of merely adding an antifreeze to the system may be enough to increase the time resolution (e.g. Barman and Travers, 1985). Here we used 40% ethylene glycol to slow down the transient kinetics of PGK. But first let us consider the effect of the glycol on the overall PGK reaction.

Steady-State and Equilibrium Experiments. The effect of 40% ethylene glycol on the *steady state parameters* of PGK at 4 °C are summarized in Table 1. This solvent did not affect significantly the K_m values for ATP or PG but k_{cat} was reduced by a factor of about 8. Interestingly, similar decreases of k_{cat} by 40% ethylene glycol were found with three other ATP handling systems − arginine kinase (Barman et al., 1978), creatine kinase (Barman et al., 1980) and actomyosine ATPase (Biosca et al., 1984).

The temperature dependence of the k_{cat} for PGK in 40% ethylene glycol is compared with that in water in Figure 3. In these experiments, we did not obtain the kcat values at each temperature. Instead, we determined the steady state rate at only one set of concentrations (1mM ATP and 2mM PG). These concentrations were choosen to be well above the respective K_m values and yet low enough not to activate. We note that the K_m values are little affected by the temperature (Table 1).

The energies of activation (30.5 kJ mol^{-1} in water and 35 kJ mol^{-1} in 40% ethylene glycol) are similar which suggests that 40% ethylene glycol does not affect significantly the overall mechanism of PGK. It is noteworthy that the plots are linear in both solvents. Dryden et al. (1992) determined the temperature dependence of yeast PGK in water and they found a "break", depending on the pH. Thus, at pH 5.6 the dependence was linear ($\Delta H^{\ddagger} = 37$kJ mol^{-1}) but at pH 6.8 there was a break at 15 °C with $\Delta H^{\ddagger} = 22$ kJ mol^{-1} above and 46 kJ mol^{-1} below the break.

The *equilibrium experiments* were carried out by quenching manually as described in Materials and Methods. Here we only considered the final equilibrium plateau which is made up of free (amount given by K_{eq}) plus bPG bound to PGK. We determined the dependencies of this plateau upon the PGK and ATP concentrations and the results are illustrated in Figure 4. There are three features of these experiments.

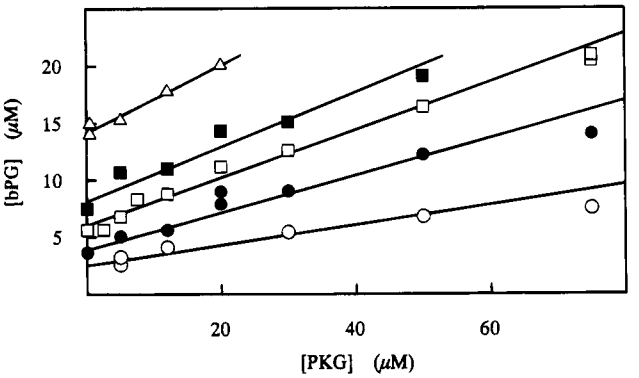


FIGURE 4: The dependence of the bPG plateau upon the PGK and ATP concentrations in 40% ethylene glycol. The plateaux were obtained as in Figure 1. The ATP concentrations were 10 μM (○), 50 μM (●), 75 μM (□), 250 μM (■), and 500 μM (△). For full details, see the text.

Table 2: Values for Certain Equilibrium Constants on the PGK Reaction Pathway[a]

	solvent[b]	
constant	water	40% ethylene glycol
K_{ADP} (μM)	40 ± 5[c]	40 ± 5[d]
K_{PG} (μM)	33 ± 3[c]	33 ± 3[d]
K_{ATP} (μM)	15 ± 3	14 ± 3
K_{bPG} (nM)	16 ± 4	13 ± 4
K_{int}	0.11 ± 0.02	0.075 ± 0.01
K_{eq}	(1.5 ± 0.1) × 10^{-4}	(0.8 ± 0.2) × 10^{-4}

[a] For the definition of each constant, see Scheme 1. K_{eq} is the overall equilibrium constant for the chemical reaction (see text). K_{ATP}, K_{bPG}, and K_{int} are from Figure 7 (see text). [b] The buffer was 20 mM triethanolamine, pH 7.5, 0.1 M potassium acetate, 1 mM free Mg^{2+}, 5 mM PG, and 5 or 10 mM ADP. [c] Values from Scopes (1978b). [d] Assumed (see text).

First, the intercepts on the abscissa (i.e. the bPG at catalytic PGK) represents the free product from which K_{eq} can be calculated in each case. Taking the intercepts at [ATP] ≥ 50μM, the mean $K_{eq} = 8.3\ (\pm 2) \times 10^{-5}$. Thus, the effect of ethylene glycol is to reduce this constant by only about 40% (Table 2).

Second, as the [PGK] was increased so the bPG increased linearly. This is further evidence of enzyme bPG intermediates on the PGK reaction pathway. Finally, consider the slopes of the dependences. These give the fraction of PGK as bPG intermediates and they increase with the ATP concentration. This dependence is illustrated in Figure 5 and if we assume an hyperbolic relationship, the fraction is 0.37 at saturating ATP. The $[ATP]_{0.5}$ is 50 (±10) μM which is similar to the K_m for ATP (110 ± 20 μM).

The dependencies of the bPG plateau on the PGK and ATP concentrations were also determined in water and the results were very similar to those in 40% ethylene glycol (results included in Figure 5 at only certain concentrations). These experiments were with 5mM PG; identical results were obtained at 1mM PG (in 40% ethylene glycol, data not shown).

Taken together, these results showed that ethylene glycol does not perturb significantly the equilibrium parameters of the PGK reaction. We then investigated the effect of 40% ethylene glycol on the kinetics of the formation of enzyme-bound bPG.

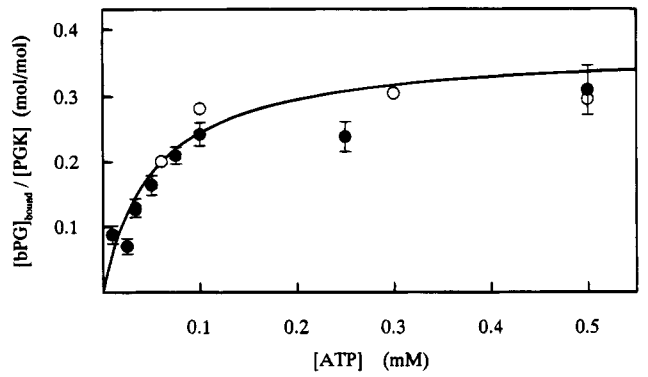


FIGURE 5: Dependence of the amount of bPG bound to PGK on the ATP concentration at 4 °C. The buffer was with (●) or without (○) 40% ethylene glycol.

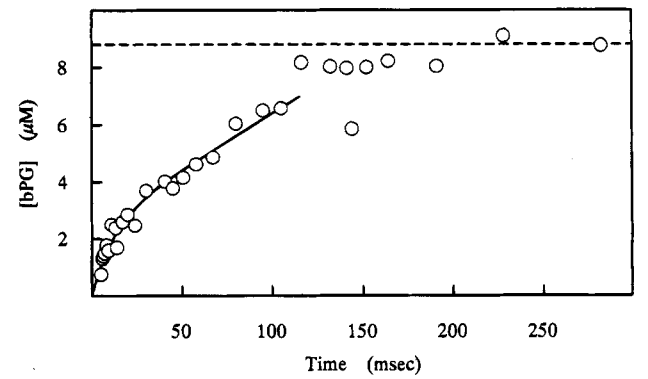


FIGURE 6: Time course for the formation of bPG at 4 °C in 40% ethylene glycol in the milliseconds time range. The reaction mixtures (10 μM PGK, 100 μM [γ-^{32}P]ATP, and 5 mM PG) were quenched in acid and the $^{32}P_i$ was determined. The data were fitted to an initial burst of amplitude 2.5(±0.2) μM bPG, kinetics of 120 (±10) s^{-1}, a linear phase of 4.5 (±0.5) s^{-1}, and a plateau of 8.8-(±0.2) μM bPG.

Kinetics of Formation of PGK-Bound bPG. A typical time course for the formation of bPG by PGK is shown in Figure 6. As in the experiment in water (Figure 2), the PGK had been preincubated with PG. By fitting this curve to an exponential phase followed by a linear one, we obtained a transient burst of bPG of amplitude 0.25 mol of bPG/mol of PGK and kinetics about 120 s^{-1}, followed by a steady-state rate of 4.5 s^{-1} and, finally, the approach to equilibrium at times above 150 ms.

We note, first, that the value for the steady-state rate is very close to that obtained by the linked assay method under the same conditions (4.3 s^{-1}). Second, the amplitude of the burst phase agrees well with that obtained from the equilibrium experiment, also at 100 μM ATP (Figure 4), and it was very similar to that in water (Figure 2). This agreement shows that the acid quench does not introduce a shift in the chemical equilibria of PGK. (In Figure 6, the quenching was performed by the rapid flow quench apparatus; in Figure 4, by hand.)

Finally, since the *kinetics* of the initial burst of bPG were obtained, we confirmed that bPG intermediates accumulate on the PGK pathway in the steady state.

Except for the inclusion of ethylene glycol, this experiment was carried out under the same conditions as those in Figure 2. The glycol had the important effect of reducing the rate of formation of the bPG intermediates so that an estimate for their kinetics could be obtained.

Measurements of K_{int}

Burbaum and Knowles (1989) developed a method for measuring the equilibrium constant for the interconversion of the ternary complexes on phosphokinase reaction pathways (K_{int} for PGK; Scheme 1). The procedure is to choose for measurement a substrate−product pair and then to determine the dependence of their ratio upon the enzyme concentration. The successful outcome of the procedure depends upon having the enzyme concentration much higher than that of the chosen substrate (and, of course, the associated product).

Here we choose the pair ATP·bPG. In our experiments, we added [γ-^{32}P]ATP to the system ($[E]_o \gg [ATP]_o$) and we measured the product [1-^{32}P]bPG (as $^{32}P_i$). The concentration of [γ-^{32}P]ATP remaining (free or bound) was obtained by difference.

The purpose is to obtain K_{int} ([E·ADP·bPG]/[E·ATP·PG]). This ratio is obtained when all of the added [γ-^{32}P]ATP is partitioned between E·[γ-^{32}P]ATP·PG and E·ADP·[1-^{32}P]bPG. However, to ensure that all of the ATP is indeed confined to the two ternary complexes is not easy, but this situation can be approached by taking certain precautions. In particular, the concentrations of the binary complexes E·ATP and E·bPG must be reduced to a minimum. This can be achieved by adding PG and ADP at concentrations well above their dissociation constants and the highest PGK concentration used. However, this device leads to new problems: high PG activates PGK; its concentration was therefore a compromise at 5 mM. Further, high ADP has the danger of trapping the enzyme as the abortive complex E·ADP·PG. This complex is not measured by our method, but if important it would reduce the amount of enzyme available for the formation of the central ternary complexes.

Following the procedure of Burbaum and Knowles, one obtains experimentally

$$r = ([1\text{-}^{32}P]bPG)_t/([\gamma\text{-}^{32}P]ATP)_t$$

where t (total) means that all the species are included, whether protein bound or not. The dependences of r upon the PGK concentration (in water) at two ADP concentrations are illustrated in Figure 7a. The problem is to use these results to obtain an estimate for K_{int}.

Burbaum and Knowles (1989) studied K_{int} for creatine kinase, and in their data treatment they obtained r in terms of equilibrium and substrate concentrations. By adapting their expression to PGK we obtain

$$r = \frac{[bPG]_t}{[ATP]_t} = \frac{[bPG] + [E{\cdot}bPG] + [E{\cdot}ADP{\cdot}bPG]}{[ATP] + [E{\cdot}ATP] + [E{\cdot}ATP{\cdot}PG]} = \frac{(K_{bPG} + [E])\dfrac{K_{ADP}}{[ADP]} + [E]}{(K_{ATP} + [E])\dfrac{K_{PG}}{[PG]} + [E]} \quad (1)$$

As already mentioned above, we could not use higher PG concentrations in our experiments, and as the PGK concentration had to be less than that of PG, we could not attain plateaux in the r versus [PGK] dependence. Nevertheless, by making certain assumptions and by using literature values for certain of the equilibrium constants, we were able to obtain an estimate for K_{int}.

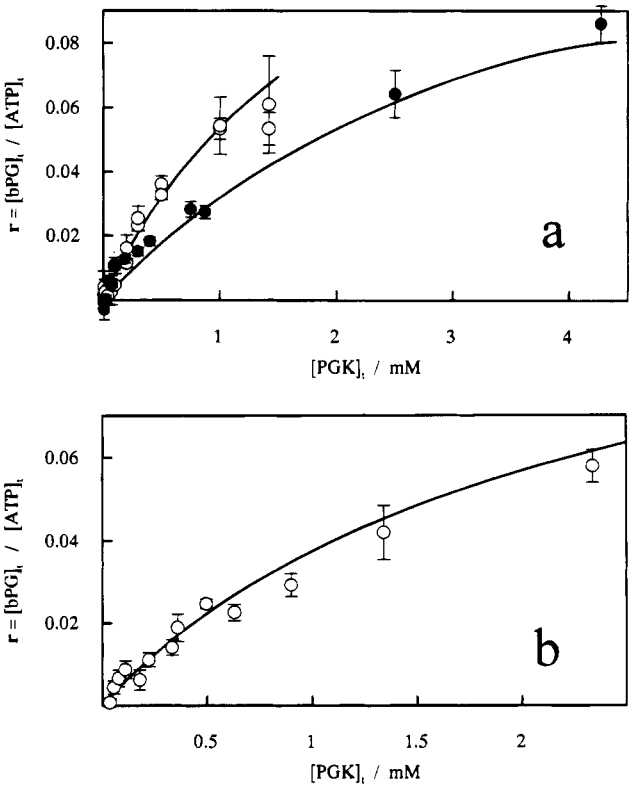
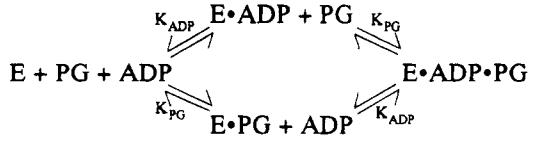


FIGURE 7: Determination of K_{int} from the variation of r ($[bPG]_t/[ATP]_t$) with the PGK concentration in water (**a**) and in 40% ethylene glycol (b). In (a), the ADP concentrations were 5 (○) or 10 mM (●); in (b), 5 mM. In all cases the PG was 5 mM and the [γ-^{32}P]ATP was 0.5 μM. The data were obtained and fitted as described in the text, and the estimated values of K_{int} obtained are in Table 2.

First, we assumed random binding for the substrates and products (Scopes, 1978a), which means that five constants had to be adjusted: K_{ATP}, K_{ADP}, K_{PG}, K_{bPG}, and K_{int} (Scheme 1). Then, to decrease the number of constant to be adjusted, we used for K_{ADP} and K_{PG} the values of Scopes (1978b), 40 and 33 μM, respectively (we note that in the final fitting procedure, below, the fits were not very sensitive to these values). Furthermore, we used the relationship $K_{eq} = (K_{ADP}{\cdot}K_{bPG}{\cdot}K_{int})/(K_{ATP}{\cdot}K_{PG})$. Thus, the number of constants to be adjusted were reduced to two : we choose K_{ATP} and K_{int}.

But we also had to have estimates for the [ADP], [PG], and [E] in eq 1. Since $[ATP]_o \ll [E]_o$, Scheme 1 can be reduced to



[ADP], [PG], and [E] were obtained from the relevant quadratic equations with the initial values for the reagent concentrations ($[ADP]_o$, $[PG]_o$ and [E]o) and the values for K_{ADP} and K_{PG} given by Scopes (1978b).

In the final analysis, the dependence of r upon the PGK concentration was fitted with the above assumptions and eq 1 using the program GraFit (Erithacus Software Ltd). The estimates are summarized in Table 2. $K_{int} = 0.11(\pm 0.02)$, and the optimized values for K_{ATP} (14 μM) and K_{bPG} (0.016 μM) are in the same range as those obtained by Scopes (1978b). As illustrated in Figure 7a, experiments were carried out at two concentrations of ADP, and the fitting procedure was carried out in each case, but the values obtained did not differ significantly.

The effect of 40% ethylene glycol upon the r versus [PGK] dependence is illustrated in Figure 7b. The data were treated as above, assuming the same values for K_{ADP} and K_{PG} (the effect of ethylene glycol on the K_m values appears to be very small; Table 1). The constants are summarized in Table 2, from which it is seen that the glycol has little effect on the equilibrium constants considered.

DISCUSSION

Our objective was to obtain kinetic information on a plausible reaction pathway of PGK (Scheme 1). In particular, we wished to identify and kinetically characterize any intermediates that accumulate in the steady state. Our procedure was to obtain evidence for such intermediates from steady-state and equilibrium studies and then to confirm this evidence by transient kinetics. PGK has a high turnover rate, and to make the transient kinetics measurable, we exploited temperature and solvent perturbation.

Before we discuss our results, we consider the validity of our method for determining the product bPG.

Validity of the bPG Assay Method. Our reaction mixtures, which contained [γ-^{32}P]ATP, were quenched in acid, and the $^{32}P_i$ content was determined by a specific method. We argued that any [1-^{32}P]bPG formed is hydrolyzed quantitatively to $^{32}P_i$ and PG. Does the P_i determined represent accurately the bPG present in the original reaction mixture? We believe that it does.

First, the P_i is probably not derived from an acid-labile phosphoenzyme (e.g., one involving acylphosphate or phosphohistidine), as there is little evidence for such an intermediate on the PGK reaction pathway (Johnson et al., 1976). The P_i is not derived from the hydrolysis of ATP: under our quenching conditions (11% TCA, ice, less than 1 h) less than 4% is hydrolyzed, and in any event this was taken into account in the blanks.

Second, the steady-state rates obtained by the acid quenching method agreed well with those obtained from the linked enzyme system, which is highly specific for bPG.

Third, consider experiments under equilibrium conditions at catalytic PGK concentrations. As shown in Figure 1, the equilibrium of the overall reaction is attained rapidly with only about 9% of the ATP as bPG, and from this, the calculated equilibrium constant ($K_{eq} = 1.5 \times 10^{-4}$) agreed well with that obtained by NMR, i.e. about 3×10^{-4} (Nageswara Rao et al., 1978). Furthermore, when the same experiment was carried out in the presence of the linked enzyme system, all the [γ-^{32}P]ATP was hydrolyzed to $^{32}P_i$ and ADP (result not shown). Similarly, when the reaction mixture contained 0.7 M hydroxylamine, we obtained the same result, which is explained by hydroxylamine decomposing acylphosphates such as bPG (Johnson et al., 1976). Unfortunately, the effect of hydroxylamine was too slow to be exploited in transient kinetic experiments.

Which Are the Intermediates That Predominate in the Steady State? Our results suggest strongly that intermediate(s) containing the product bPG accumulate in the steady state. This is in accord with the suggestion of Scopes (1978a) that the kinetics of release of bPG is the rate-limiting step on the PGK reaction pathway.

First, from equilibrium studies the amount of bPG measured increased with the PGK concentration (Figures 1 and 4). In these experiments, the end plateaux (e.g., Figure 1) are the sum of free and protein-bound bPG.

Second, in transient kinetic experiments there was a transient burst phase of bPG that was followed by a short steady state and then the final equilibrium of the reaction (defined by K_{eq}, Figure 2). We assume that the burst represents protein bound rather than a rapid release of bPG because its amplitude agrees well with that predicted for an intermediate from the equilibrium measurements (Figure 4). Further, the bPG is bound much more tightly than ADP (Scopes, 1978b) and is presumably released slowly. Finally, by exploiting cryoenzymology, we obtained the kinetics of the formation of bPG.

By considering our data with reference to Scheme 1 and by making certain assumptions, we can calculate the concentrations of the intermediates that predominate in the steady state. We assume that the enzyme is 100% active. Under the conditions used, the PG concentration was much greater than its dissociation constant, and so the concentration of free enzyme was low. Further, as argued above, the concentration of E·ADP is negligible. Thus, we need to consider four intermediates: E·PG, E·ATP·PG, E·ADP·bPG, and E·bPG.

In the equilibrium experiments at reagent PGK concentrations, we measure the concentration of enzyme-bound as well as free bPG (Figure 1). The latter is defined by K_{eq}, giving [E·ADP·bPG] plus [E·bPG] by subtraction. This PGK-bound bPG increased hyperbolically with the ATP concentration (Figure 5), from which the two intermediates at saturation represent 0.37 mol/mol of PGK. Here the concentration of E·PG is negligible, so our complexes can be reduced to three, and from a K_{int} of 0.11 we can calculate their relative concentrations in the steady state: [E·ATP·PG] = 0.62; [E·ADP·bPG] = 0.07; [E·bPG] = 0.31. These estimates are based on the assumption that the three complexes are in rapid equilibrium and that the kinetics of the release of bPG is relatively slow (see above).

In 40% ethylene glycol we obtained an estimate for the kinetics of formation of the product intermediates E·ADP·bPG and E·bPG. Because of the smallness of the burst size and the rapidity of the kinetics, it was difficult to assign these kinetics to any particular constant(s) in Scheme 1.

Determination of the Equilibrium Constants for the Overall Reaction (K_{eq}) and for Enzyme Reactants and Products (K_{int}). The K_{eq} obtained by our chemical method (1.5×10^{-4}) is similar to that obtained by Nageswara Rao et al. (1978) using ^{31}P NMR (3×10^{-4}) under similar experimental conditions. Huskins et al. (1982) obtained $K_{eq} \leq 8 \times 10^{-4}$ at 7.5 °C. K_{eq} was not significantly reduced by 40% ethylene glycol.

K_{int} is the equilibrium constant for the central ternary complexes in Scheme 1, i.e., [E·ADP·bPG]/[E·ATP·PG]. We determined K_{int} by a chemical method and by following the analytical procedure of Burbaum and Knowles (1989) with certain modifications. Our value of 0.11 (± 0.02) is lower than the 0.8 (±0.3) reported by Nageswara Rao et al. (1978). This difference can be explained by Nageswara Rao et al. not having ADP in their reaction mixtures, so the binary complex E·bPG was presumably included in their measurements. If this is the explanation for the difference, their results are, in fact, in good agreement with ours. In their experiments, Nageswara Rao et al. incubated 3.3 mM PGK with 2.7 mM ATP and PG under conditions similar to ours. At these reagent concentrations the free substrate and product concentrations are low. They measured the $[ADP]_t/[ATP]_t$ ratio (by ^{31}P NMR), which is equivalent to the $[bPG]/[ATP]_t$ ratio that we measured. Now, this ratio includes both binary and ternary complexes (([E·ADP·bPG] + [E·bPG])/([E·ATP·PG] + [E·ATP])), and it can be expressed as $[bPG]_t/([ATP]_o - [bPG]_t)$. Nageswara Rao et al. obtained 0.8 for the ratio, and with $[ATP]_o = 2.7$ mM, $[bPG]_t/[PGK] = 0.36$. This is very close to the 0.37 value that we found for the amount of bPG bound to PGK at saturating substrate concentrations (Figure 5).

Thermodynamically speaking, the values for K_{int} are in broad agreement, and they show that, as with a number of enzymes, K_{int} is near 1 and very different from K_{eq}. The significance of this difference has been discussed [e.g., Gutfreund and Trentham (1976), Wilkinson and Rose (1979), and Burbaum and Knowles (1989)].

Experiments in 40% Ethylene Glycol. Cryoenzymology is a way of perturbing a system and of obtaining kinetic information (Douzou, 1977). Such experiments require the addition of an antifreeze (usually an organic solvent) whose use on its own may be sufficient to yield information. This was the case with the kinetics of the binding of ATP to myosin. Thus, in water buffers it was difficult to show saturation kinetics (i.e., that the ATP binds in two steps). Ethylene glycol (40%) had the effect of reducing both the equilibrium constant for the formation of the initial collision complex and the rate constant describing the following isomerization, which allowed for the determination of these constants (Biosca et al., 1984).

With PGK, the glycol reduces rate constants such as the overall k_{cat} and the kinetics of the formation of enzyme-bound bPG, but none of the equilibrium constants studied was significantly affected. In particular, the burst size (of bPG) remained low, and therefore its kinetics were difficult to exploit fully. It did not help matters to reduce the temperature: experiments at −7.5 and −15 °C gave progress curves with shapes identical to that at 4 °C (of course, on longer time ranges; experiments not illustrated), and the kinetics of the transient burst phase remained difficult to measure with precision.

With PGK, then, the artifice of including ethylene glycol in the solvent allowed for an estimate of the burst phase and therefore a confirmation of the accumulation of product(s) containing intermediates, but it did not allow for a full exploitation of these kinetics.

Is bPG Directly Transferred from PGK to Glyceraldehyde-3-phosphate Dehydrogenase? bPG is bound very tightly to PGK with a K_d of about 50 nM (Scopes, 1978b). Now, the k_{on} for a small ligand to an enzyme is typically 10^2-10^3 μM^{-1} s^{-1} [e.g., Fersht (1977)], from which we can calculate a k_{off} for the bPG of $5-50 s^{-1}$. This dissociation rate constant is much lower than the overall k_{cat} for PGK (Table 1). Scopes (1978b) explained the difference by taking bPG to be a double-headed compound that binds by both its PG and its 1-phosphoryl portion to adjacent sites on PGK. The substrates (ATP, PG) are envisaged to compete with the bPG by binding to either site, thus assisting in its complete displacement, i.e., by increasing k_{off}.

Another explanation for the difference is afforded by Weber and Bernhard (1982), who proposed that in the coupled assay the bPG is transferred directly and rapidly to the dehydrogenase rather than being released slowly into solution. For this to occur, the authors suggest that the two

enzymes form a complex [metabolite transfer via enzyme complexes; reviewed by Srivastava and Bernhard (1986)].

Here we determined the kinetics of the release of bPG (k_{off}) by a chemical method that measures directly the bPG (as P_i). These measurements in the absence of glyceraldehyde-3-phosphate dehydrogenase gave k_{cat} values identical to those obtained by the coupled assay, i.e., in the presence of the dehydrogenase (e.g., Figure 1). We conclude that whereas there may be a complex between PGK and glyceraldehyde-3-phosphate dehydrogenase, there is little evidence for this interaction from the kinetics of the release of bPG (by the chemical method) and the k_{cat} of PGK by the coupled assay. Of course, it cannot be excluded that the k_{on} for the bPG is $>10^3\ \mu M^{-1}\ s^{-1}$ i.e., that k_{off} is $>50\ s^{-1}$ and equal to the k_{cat} obtained in the glyceraldehyde-3-phosphate dehydrogenase system.

CONCLUSIONS

This is an attempt at obtaining information on the intermediates that populate the reaction pathway of PGK, an enzyme whose structure is known in considerable detail. The aim is to understand the structure−function relationship of PGK. Using a plausible reaction pathway (Scheme 1), we propose that, under our experimental conditions, two intermediates accumulate in the steady state: the ternary E·ATP·3PG and the binary E·bPG. The accumulation of E·bPG is in agreement with Scopes (1978a,b), who proposed that the kinetics of the release of bPG is a relatively slow step. However, because of the rapidities of the kinetics of the processes, it was difficult to study the kinetics of their interconversions. We are approaching the problem by screening genetically engineered PGKs for an enzyme that provides large and, therefore, exploitable phases of intermediates containing the product bPG.

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