Cryoenzymic Studies on Yeast 3-Phosphoglycerate Kinase. Attempt To Obtain the Kinetics of the Hinge-Bending Motion†

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ABSTRACT: This is a continuation of a study on the 3-phosphoglycerate kinase (PGK) reaction in the direction of 1,3-bisphosphoglycerate (bPG) formation: ATP + 3-phosphoglycerate (PG) ⇌ ADP + bPG [Schmidt, P. P., Travers, F., & Barman, T. (1995) *Biochemistry 34*, 824−832]. We showed that species containing bPG accumulate in the steady state, but their low concentrations and rapid kinetics of formation precluded a full study, even under cryoenzymic conditions in 40% ethylene glycol. Here we studied the PGK reaction in 30% methanol. The transient kinetics of bPG formation were obtained by chemical sampling: PGK was mixed with PG and [γ-^{32}P]ATP in a rapid flow quench apparatus, the mixture aged 4 ms up and quenched in acid, and the [1-^{32}P]bPG was determined. The time course consisted of a rapid rise of bPG (kinetics k_{obs}) and a steady state phase. In methanol, the amplitude of the rise was large (>50% of the PGK in the steady state), and k_{obs} was measurable. Fluorescence stopped flow was used to study the formation of the binary E·PG and E·ATP. The affinities of PGK for ATP and PG were high in methanol (K_d = 102 and 1.5 μM, respectively), but the kinetics of the formation of E·PG and E·ATP were too rapid to be measured. From these and the chemical sampling experiments, we propose a reaction scheme for PGK: a rapid formation of the collision complex E·PG·ATP (K_1), a slow isomerisation to E*·PG·ATP (k_2,k_{-2}), a rapid phosphorylation transfer step to E·bPG·ADP (K_3), and a slow release of the products (k_4). In our scheme, k_{obs} is the reflection mainly of k_2 and k_{-2} and the steady state of k_4. Using a computer simulation procedure, k_2/K_1 = 0.37 μM^{-1} s^{-1}, k_{-2} = 33 s^{-1}, K_3 = 4, and k_4 = 7.1 s^{-1}. We propose that k_{obs} measures the kinetics of the putative hinge-bending motion of PGK, i.e., the conformational change that is necessary for the substrates to line up for phosphoryl transfer.

3-Phophoglycerate kinase (PGK)[1] catalyzes the reversible transfer of phosphate between ATP and 3-phosphoglycerate (PG): ATP + PG ⇌ ADP + 1,3-bisphosphoglycerate (bPG). Extensive studies have been carried out on yeast PGK, but few works have been directed toward understanding its reaction pathway and, in particular, the rates of interconversion of the reaction intermediates (Schmidt et al., 1995, and references cited therein). There are several reasons for this paucity of kinetic information. PGK has a high turnover, its pathway is complex (two substrates, two products), and the reaction that it catalyzes is reversible. Further, in common with most kinases (Barman, 1969, 1974), its affinity for ATP is poor. This makes certain types of transient kinetic experiments difficult. Finally, kinetic studies on PGK are confounded by interference from secondary substrate binding sites (Joao & Williams, 1993, and references cited therein).

In our previous study on PGK we devised a chemical-sampling method to study the kinetics of the production of bPG, i.e., with ATP and PG as substrates. In this method, reaction mixtures containing PGK, PG, and [γ-^{32}P]ATP are aged in a rapid flow quench apparatus and quenched in acid, and the [^{32}P]P_i was determined. We showed that this P_i is equal to the [1-^{32}P]bPG produced by the enzyme. We reduced the turnover rate of PGK by including an antifreeze (ethylene glycol) in the buffer, and by this means we obtained the kinetics of formation of enzyme-bound bPG. However, because of the relatively low concentrations of the bPG-containing species, we could not exploit fully these kinetics. Further, the affinity of PGK for ATP (and PG) remained poor in the glycol.

Here we searched for a better cryosolvent, and after testing several (DMSO, methanol, ammonium acetate) we chose 30% methanol. This cryosolvent has the important effects of increasing significantly the affinities of PGK for its substrates and of decreasing the rapidity of the kinetics of formation of bPG. But as important, in 30% methanol, the concentration of bPG-containing species in the steady state increased significantly to at least 60% of the enzyme. These favorable conditions made it possible to carry out transient kinetic experiments and to obtain the kinetics of formation of bPG-containing species. In particular, we propose that these kinetics measure those of the putative hinge-bending motion of PGK (Banks et al., 1979), i.e., the conformational change that is necessary for the substrates to line up for phosphoryl transfer.

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 dehydroge-

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

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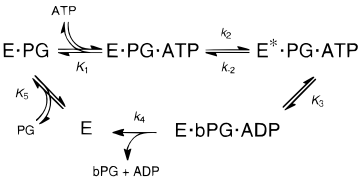
Scheme 1



nase were from Boehringer Mannheim, and [γ-^{32}P]ATP was from Amersham International. Ap_5A was from Sigma Chemical Co. PGK concentrations were estimated at 280 nm with $E^{1\%,1cm} = 4.9$ (Minard et al., 1990).

Experimental Conditions. We have already discussed the problem of substrate activation (Schmidt et al., 1995). Our experiments were carried out in a buffer containing 20 mM triethanolamine, pH 7.5, 0.1 M potassium acetate and 1 mM free Mg^{2+}. In most experiments, the buffer contained 30% (v/v) methanol. The concentrations of PGK, ATP, and PG in the figure legends or in the text refer to the final reaction mixture concentrations. Unless otherwise stated, the temperature was 4 °C.

PGK Activity by the Coupled Enzyme System. The PGK reaction was coupled to glyceraldehyde-3-phosphate dehydrogenase, as described previously (Schmidt et al., 1995).

PGK Activity by Chemical Sampling. PGK reaction mixtures containing [γ-^{32}P]ATP were quenched in acid (22% trichloroacetic acid plus 1 mM KH_2PO_4). Under these conditions, ATP and PG are stable, but any [1-^{32}P]bPG decomposes quantitatively to PG and [^{32}P]P_i. The [^{32}P]P_i was determined by the filter paper method of Reimann and Umfleet (1978).

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 done in triplicate.

Determination of K_{eq}. This is the overall equilibrium constant for the PGK reaction ([ADP][bPG]/[ATP][PG]). It was determined using catalytic amounts of PGK, as in Schmidt et al. (1995). Attempts were made to obtain the equilibrium constant for the interconversion of the ternary complexes (K_3 in Scheme 1), by the method of Burbaum and Knowles (1989), but these were unsuccessful. This is because the concentration of PGK required by this method (several micromoles per milliliter) cannot be attained in 30% methanol (limited to about 0.2 mM, see below).

Transient Kinetic Experiments. These were carried out in a home-built, thermostatically controlled rapid flow quench apparatus (Barman & Travers, 1985). The procedure was to mix in the apparatus PGK (unless otherwise stated preincubated with PG) with [γ-^{32}P]ATP plus PG, allowing the mixtures to age (4.5 ms and up, see figures) and quench them in 22% trichloroacetic acid plus 1 mM KH_2PO4, and the [^{32}P]P_i was determined as above. This [^{32}P]P_i is equal to the total [1-^{32}P]bPG produced by PGK, whether free or enzyme bound (Schmidt et al., 1995). The concentrations of PGK, PG, and [γ-^{32}P]ATP used in the different experiments are given in the legends to the figures or in the text.

Fluorescence Spectroscopy Studies. Experiments under *equilibrium conditions* were carried out at 4 °C in a SLM-Amicon Bowman Series 2 luminescence spectrophotometer. All measurements were carried out in the ratio mode with a 4 nm bandwidth for both the excitation and emission monochromators. The excitation and emission wavelengths were 295 and 335 nm, respectively. Background fluorescence from the buffer and the substrates was also recorded and subtracted from the protein signal. Experiments under *transient kinetic conditions* were carried out at 4 °C in a Hi Tech Scientific SF-61 DX2 Double Mixing Stopped Flow System using an excitation wavelength of 290 nm and a 320 nm cutoff filter.

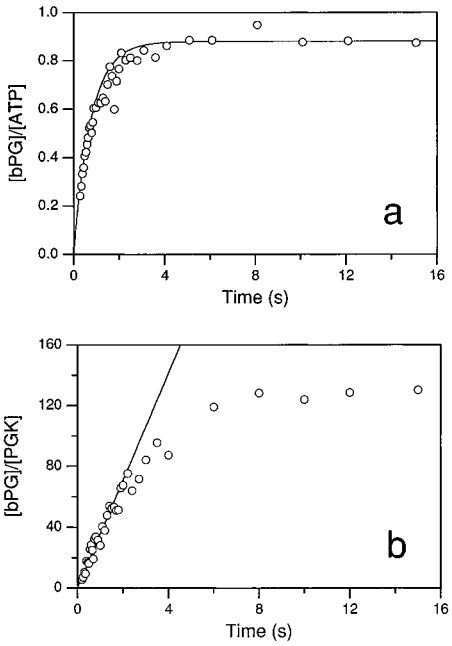


FIGURE 1: Progress curves for ATP binding (a) and bPG release (b) with PGK at 4 °C and in water. *ATP binding* was measured under single-turnover conditions (reaction mixture = 0.8 μM PGK, 0.08 μM [γ-^{32}P]ATP, and 5 mM PG) and *bPG release* under multiturnover conditions (0.1 μM PGK, 100 μM [γ-^{32}P]ATP, and 5 mM PG). The mixtures were quenched in acid at the times indicated, and the [^{32}P]P_i determined. The buffer was 20 mM triethanolamine, 0.1 M potassium acetate, and 1 mM free Mg^{2+}, pH 7.5. The constants obtained are in Table 1.

RESULTS

Choosing a Better Cryosolvent for PGK. A way of increasing the amount of species containing bPG is to increase the ratio of their rate of formation to their rate of decomposition (i.e., release of bPG). The kinetics of the *formation* of bPG were measured under single turnover conditions ([PGK] > [ATP]) at low concentrations of both PGK and ATP. Under these conditions, the kinetics of the formation of bPG are limited by those of the ATP binding. The kinetics of the *release* of bPG were measured under multiturnover conditions and at high ATP concentrations (Schmidt, 1995).

Typical single- and multiturnover experiments, carried out in water at 4 °C, are shown in Figure 1. In the single-turnover experiment, the data could be fitted to a single exponential, $k_{obs} = 1.3\ s^{-1}$, which provides a measure of the kinetics of total bPG formation. In the multiturnover experiment, the steady state rate, $k_{ss} = 35\ s^{-1}$, provides a measure of the kinetics of the release of bPG. We now carried out single and multiturnover experiments under the same conditions as in Figure 1 but in different solvents, and the results obtained are summarized in Table 1.

Clearly DMSO and ammonium acetate are unsatisfactory cryosolvents for PGK. 40% ethylene glycol had little effect

Table 1: Effect of the Different Solvents on the Kinetics of ATP Binding and of bPG Release at 4 °C[a]

solvent	concentration % (v/v)	concentration (M)	ATP binding k_{obs} (s^{-1})	bPG release k_{ss} (s^{-1})	relative ratio (k_{obs}/k_{ss})
water			1.3	35	1.0
ethylene glycol	40	7.2	0.14	3.5	1.1
methanol	20	4.9	0.43	4.7	2.5
	30	7.4	0.21	2.5	2.3
	40	9.9	0.079	1.1	2.0
DMSO	40	5.6	*b*	*b*	
ammonium acetate		7.2	*c*	*c*	

[a] ATP binding measurements were under single-turnover conditions and bPG release under multiturnover conditions. For full details, see the legend to Figure 2. [b] Kinetics affected and the experiments were unreproducible. [c] PGK inactivated.

on the ratio; this is as expected as, in it, the transient amplitudes of bPG formation are the same as they are in water (Schmidt et al., 1995). However, in methanol the ratio increased significantly and we chose 30% methanol for further studies.

Experiments in 30% Methanol

Overall Effects on PGK: Stability and Reaction Mechanism. The stability of PGK in 30% methanol was tested at 0, 22, and 30 °C by the linked assay method. The experiments at 0 and 22 °C were carried out at 0.4 μM PGK and those at 30 °C at 0.25 μM. At 0 and 22 °C, there was little loss of activity after 5 h incubation (>96% recovery). At 30 °C the recovery was about 65%. Therefore, PGK is reasonably stable in 30% methanol.

As the concentration of methanol used in our experiments was high (7.4 M), we checked that it did not interfere with the PGK phosphoryl transfer. Thus, a PGK reaction mixture (50 nM PGK, 5 mM PG, and 91 μM ATP) was incubated with 300 nM glyceraldehyde-3-phosphate dehydrogenase and 150 μM NADH at 22 °C (for the buffer, see Materials and Methods). From the final decrease in absorbance at 340 nm, the concentration of NAD at equilibrium was 90 μM. This is almost identical to the ATP originally in the reaction mixture, which confirms the stoichiometry of the PGK reaction in methanol. We note that in 30% methanol the catalytic activity of the dehydrogenase was about 35% of that in pure water but there was little further decrease following incubation for 45 min at 22 °C.

Methanol had little effect on the $\Delta H^{\ddagger}$ of k_{cat} (Figure 2) or on the overall K_{eq} for the PGK reaction: in water, $K_{eq} = (1.5 \pm 0.4) \times 10^{-4}$; in 40% ethylene glycol, $(0.8 \pm 0.2) \times 10^{-4}$; and in 30% methanol, $(1.2 \pm 0.1) \times 10^{-4}$. Taken together, these results suggest strongly that 30% methanol does not modify the PGK reaction. However, PGK was less soluble in methanol than in water or 40% ethylene glycol, and in it experiments were limited to a concentration of 0.2 mM.

Steady State Parameters. To obtain k_{cat} and K_m for ATP and PG (i.e., in the direction of bPG formation) is difficult. First, because of the low K_{eq}, the final equilibrium concentration of free bPG is low so the duration of the steady state is short, as illustrated by the transient kinetics experiments (see below). This difficulty is overcome by the use of the coupled enzyme system by which bPG is removed continuously. Second, when determining the saturation curve for one substrate (the other being constant), the dependence is anomalous because of substrate activation at high concentrations (Scopes, 1978). For a further discussion of these problems, see Schmidt et al. (1995).

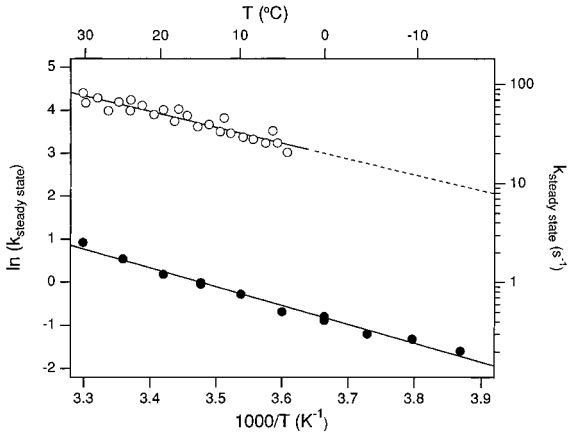


FIGURE 2: Temperature dependences of k_{cat} of PGK in 30% methanol (●) and water (○). The ATP and PG concentrations were, respectively, 200 and 100 μM (30% methanol) and 1 and 2 mM (water). The measurements in methanol were carried out by the rapid flow quench method and those in water by the linked assay method.

The effect of 30% methanol and 40% ethylene glycol on the steady state parameters of PGK are compared in Table 2. It is noteworthy that the linked assay (specific for bPG) and the acid quench method ([1-^{32}P]bPG determined as [^{32}P]P_i) gave similar values, as in water and ethylene glycol (Schmidt et al., 1995). This confirms the conclusions of Kvassman and Pettersson (1989a, b) and Wu et al. (1991) that there is no direct transfer of bPG between PGK and glyceraldehyde-3-phosphate dehydrogenase (substrate channelling; Huskin et al., 1982).

Methanol has interesting effects on the PGK steady state parameters. First, the activating effect of high substrate concentrations was greater in methanol than in water or ethylene glycol. At low concentrations (PG $\leq$ 150 μM, ATP $\leq$ 250 μM), there was little activation. Second, methanol lowers significantly the K_m values, especially for PG at low [ATP]. Finally, k_{cat} was reduced by a factor of about 15. These reductions in K_m and k_{cat} facilitated the transient kinetic experiments (see below).

The temperature dependence of k_{cat} for PGK in 30% methanol is compared to that in water in Figure 2. To prevent substrate activation, the experiments in methanol were carried out at low concentrations of the substrates, but these were well above the respective K_m values. As in water and ethylene glycol (Schmidt et al., 1995), the K_m for ATP is insensitive to the temperature: at 5 mM PG, it was 43 ($\pm$5) μM at 22 °C and 62 ($\pm$6) μM at 4 °C. The Arrhenius plot in 30% methanol was linear, and the $\Delta H^{\ddagger}$ was similar to that in water, 36.3 and 30.5 kJ mol^{-1}, respectively. In 40% ethylene glycol, $\Delta H^{\ddagger} = 35.0$ kJ mol^{-1} (Schmidt et al., 1995).

Measurements of the Interactions of ATP and PG with PGK by Fluorescence Spectroscopy. Yeast PGK has two tryptophan residues, one of which is near the ATP binding site (Trp 333, Banks et al., 1979; Szpikowska et al., 1994; Ritco-Vonsovici et al., 1995). Upon the binding of ATP or PG, to form the binary complexes E·ATP or E·PG, there is a decrease in the inherent fluorescence of PGK, which is characteristic of tryptophan perturbation.

The dependences of the amplitudes of the decrease in the fluorescence on the ATP and PG concentrations are shown

Table 2: Effect of Cryosolvents on the Steady State Parameters of PGK at 4 °C[a]

substrate		k_{cat} (s^{-1})			K_m (μM)			k_{cat}/K_m (μM^{-1} s^{-1})		
variable	constant	water	ethylene glycol[b]	methanol	water	ethylene glycol[b]	methanol	water	ethylene glycol[b]	methanol
ATP	PG (5 mM)	72 ± 2	9.4 ± 0.7	7.6 ± 0.2	109 ± 11	110 ± 20	62 ± 6	0.66 ± 0.07	0.09 ± 0.02	0.12 ± 0.01
	PG (5 mM)			5.3 ± 0.5[c]			44 ± 9[c]			0.12 ± 0.03
	PG (0.25 mM)	16 ± 1		1.05 ± 0.06	109 ± 18		19 ± 4	0.15 ± 0.03		0.06 ± 0.01
PG	ATP (5 mM)	79 ± 3[b]	8.1 ± 0.4		240 ± 40[b]	225 ± 40		0.33 ± 0.06	0.036 ± 0.007	
	ATP (2 mM)	31 ± 3		2.7 ± 0.1	118 ± 32		16 ± 2	0.26 ± 0.08		0.17 ± 0.02
	ATP (0.25 mM)	17 ± 1		0.71 ± 0.03	79 ± 10		5 ± 1	0.22 ± 0.03		0.14 ± 0.03

[a] Unless otherwise stated by the coupled enzyme method. The concentrations of ethylene glycol and methanol were 40 and 30%, respectively. [b] From Schmidt et al. (1995). [c] By chemical sampling.

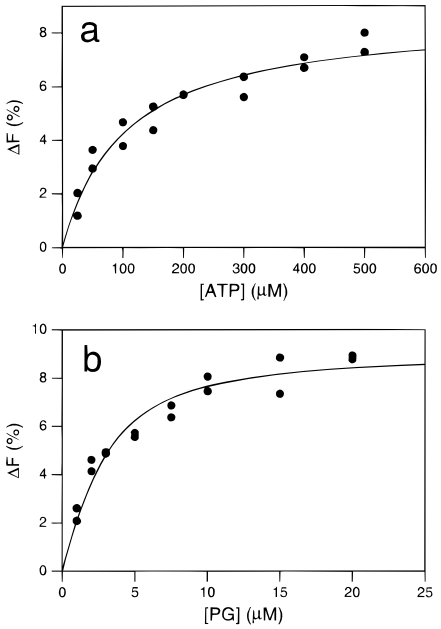


FIGURE 3: Dependences of the quenching of the tryptophan fluorescence of PGK on the ATP and PG concentrations at 4 °C in 30% methanol. PGK (2.5 μM) was mixed with different concentrations of (a) ATP or (b) PG and the decrease in fluorescence measured at 335 nm (the excitation wavelength was 295 nm). The data were fitted to hyperbolas: in panel a, $\Delta F_{max} = 8.6 \pm 0.3\%$ and $K_d = 102 \pm 5$ μM; in panel b, $\Delta F_{max} = 9.2 \pm 0.5\%$ and $K_d = 1.5 \pm 0.4$ μM.

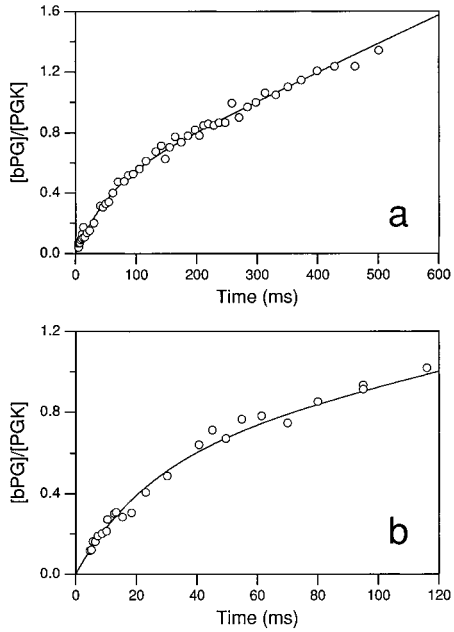


FIGURE 4: Time courses for the formation of bPG under multiturnover conditions at 4 °C in 30% methanol. The reaction mixtures (panel a, 2.5 μM PGK, 25 μM [γ-^{32}P]ATP, and 5 mM PG; panel b, 10 μM PGK, 100 μM [γ-^{32}P]ATP, and 5 mM PG) were quenched in acid at the times indicated, and the [^{32}P]P_i was determined. In each experiment, the data were fitted to a transient burst phase of kinetics k_{obs} and amplitude B followed by a steady state rate, k_{ss}. In panel a, $k_{obs} = 17$ s^{-1}, $B = 0.44$ mol of bPG/mol of PGK, and $k_{ss} = 1.9$ s^{-1}; in panel b, $k_{obs} = 42$ s^{-1}, $B = 0.56$ mol of bPG/mol of PGK, and $k_{ss} = 3.7$ s^{-1} (note the different time scales). For fitting procedures and other details, see the text.

in Figure 3. Under the conditions used (30% methanol, 4 °C) the K_d values were 102 ± 5 μM for ATP and 1.5 ± 0.4 μM for PG. The low K_d for PG is striking: in water it is 34 ± 3 μM (result not shown). It is in line with its low K_m (Table 2).

Transient Kinetics of bPG Formation. In these we were confronted with a dilemma: we were working with PGK in the thermodynamically unfavorable direction so, to induce it to turn over enough times to give a steady state rate, high substrate concentrations were needed. But, this activates the enzyme; furthermore, for precise measurements, the [γ-^{32}P]ATP concentration had to be kept at no more than 10 times that of the PGK, i.e., it was low. The only way out was to work at a high concentration of PG (5 mM) which, of course, is frankly activating.

Our aim was to make the kinetics as simple as possible, in particular, by eliminating the kinetics of E·PG formation. To ensure this, the PGK was incubated with an excess of PG before the rapid mixing with [γ-^{32}P]ATP in the flow quench apparatus. The starting materials were, therefore, E·PG and [γ-^{32}P]ATP.

Two typical time courses for bPG formation (at 25 and 100 μM ATP) are shown in Figure 4. As expected from the exploratory experiments (k_{obs}/k_{ss} ratio, Table 1), the transient phase amplitudes were considerably larger than in water or ethylene glycol. This made it possible to obtain with reasonable precision the kinetics of the transient which was difficult in ethylene glycol. However, the ATP concentration range was limited to 12.5−150 μM: at [ATP] < 12.5 μM, the transient amplitude became too low to be exploitable. At [ATP] > 150 μM, the steady state phase was fast, so it became short and difficult to measure as it was squeezed between the transient and the approach to the final equilibrium.

As a first approximation we fitted the data to two phases: a rapid transient followed by a linear steady state rate of bPG production. To facilitate the fitting procedure, the steady state phase, k_{ss}, was imposed for ATP with $K_m = 44$ μM and $k_{cat} = 5.3$ s^{-1} at [PG] = 5 mM (Table 2). Therefore, we considered two parameters: the kinetics of the rapid phase (k_{obs}) and its amplitude (B). The dependences of k_{obs} and B on the ATP concentration are shown in Figure 5. In the k_{obs} dependence, the slope gave an apparent second-order constant = 0.37 ± 0.02 μM^{-1} s^{-1} and an intercept on the

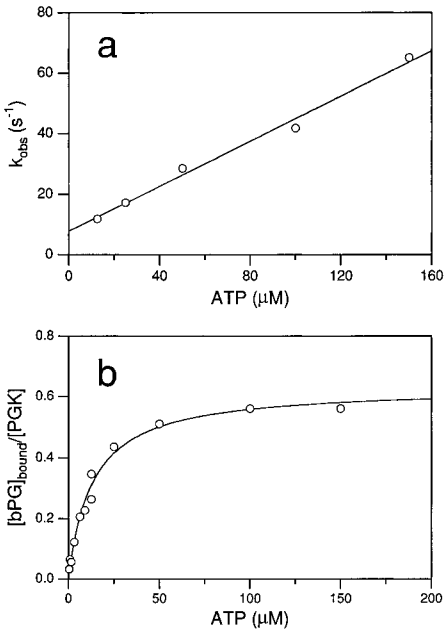


FIGURE 5: ATP dependences of the kinetics, k_{obs} (a), and amplitude, B (b), of the transient burst phase in multiturnover experiments. The parameters were obtained as in Figure 4 where the experimental conditions are also given. In panel a, the dependence fitted to a straight line, and in panel b, to a hyperbola. See text for the constants obtained.

k_{obs} axis, k_{-}, of 8 ± 2 s^{-1}. In the B dependence, the amplitude at saturation in ATP was 0.56 mol of bPG/mol of PGK with an $[ATP]_{0.5}$ of 13 μM. This high amplitude shows that, at ATP concentrations above 30 μM, species involving bPG predominate in the steady state.

Attempts to Measure the Kinetics of the Formation of the Binary Complexes, E·PG and E·ATP. In the experiments described above, the PGK had been preincubated with PG. This precaution turned out to be unnecessary as the kinetics of formation of the binary complexes E·PG and E·ATP appear to be very rapid.

Consider the kinetics of the formation of E·PG. First, we determined whether or not our PGK contained already bound PG (Johnson et al., 1976). Thus, 10 μM PGK was incubated with 100 μM [γ-^{32}P]ATP at 4 °C. Samples were removed at intervals from 1 to 60 mn and quenched in acid, and the $^{32}P_i$ was determined. This experiment revealed that PGK hydrolyses ATP very slowly (k_{ss} about 1×10^{-4} s^{-1}), but there was no significant initial burst of P_i (<0.01 mol of P_i/mol of PGK). When 1 μM PG was added to the reaction mixture, there was a rapid burst of 0.86 mol of P_i/mol of PG, which was followed by a slow phase of ATP hydrolysis. We conclude that our PGK contained less than 0.01 mol of PG/mol of protein, not unexpected as PG had not been used in its preparation.

In Figure 6 are shown two transient kinetic experiments at a low PG concentration (20 μM): one in which the PGK had been incubated with the PG and the other in which the PG had been added together with the [γ-^{32}P]ATP to the substrate-free enzyme. It is noteworthy that the time courses are almost identical (we are unable to explain the deviation in the 50 ms time range). In particular, a transient lag phase could not be discerned in either experiment, which suggests strongly that the PG binding kinetics are very rapid.

The rapidity of the PG binding is illustrated further in Figure 7. This shows two time courses of bPG formation, one at 20 μM and the other at 5 mM PG. It is striking that

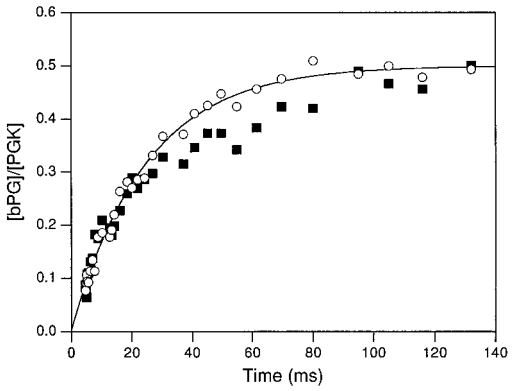


FIGURE 6: Time courses for bPG production with PG free PGK or PG·PGK as the starting material. The reaction were started by adding [γ-^{32}P]ATP plus PG to substrate free PGK (○) or [γ-^{32}P]ATP to PGK preincubated with PG (■). In both experiments, the final reaction mixtures (10 μM PGK, 100 μM [γ-^{32}P]ATP, and 20 μM PG) were quenched in acid at the times indicated and the $[^{32}P]P_i$ determined.

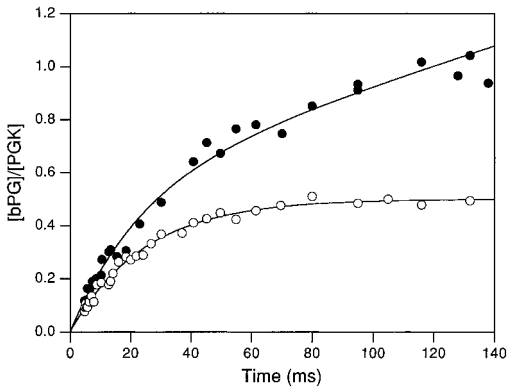


FIGURE 7: Time course for the formation of bPG under multiturnover conditions at different concentration of PG at 4 °C in 30% methanol. The reaction mixtures contained 10 μM PGK, 100 μM [γ-^{32}P]ATP, and 20 μM (○) or 5 mM PG (●). The mixtures were quenched in acid at the times indicated and the $[^{32}P]P_i$ determined. In (●), the data were fitted to a transient burst phase (k_{obs} = 42 s^{-1}, B = 0.56 mol of bPG/mol of PGK) followed by a steady state rate (k_{ss} = 3.7 s^{-1}); in (○), to a burst phase only (k_{obs} = 40 s^{-1}, B = 0.50 mol of bPG/mol of PGK). For fitting procedures and other details, see the text.

the kinetics of the transients are identical. This confirms at once the rapidity of the PG binding process and the tightness of this binding (Figure 3). Since 5 mM PG strongly activates k_{cat} (Table 2), our finding also supports the idea that high PG activates by aiding the release of bPG without affecting the substrate binding steps (Scopes, 1978).

We made attempts to study the kinetics of formation of E·PG and also E·ATP by the fluorescence stopped-flow technique. These were unsuccessful, even at low concentrations (20 μM PG, 10 μM ATP) and in 30% methanol at 4 °C: on mixing PGK (2.5 μM) with either substrate, we observed a decrease in fluorescence, but it occurred completely within the dead time of the apparatus (2 ms; results not illustrated).

Taken together, these results show that the kinetics of the formation of the binary complexes E·PG and E·ATP are very rapid.

Pertubation of the PGK Equilibrium Mixture by ATP. So far our study has been limited to the kinetics of formation of bPG. To gain further information, we now carried out a cold ATP chase on a PGK equilibrium mixture. The rationale of the experiment (illustrated in Figure 8) is as follows.

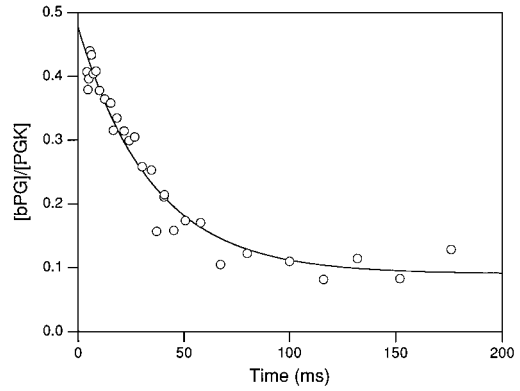


FIGURE 8: Cold ATP chase experiment with PGK at 4 °C in 30% methanol. Reaction mixtures (10 μM PGK, 100 μM [γ-^{32}P]ATP, and 20 μM PG plus 25 μM Ap_5A) were mixed with 11 mM cold ATP in a rapid flow quench apparatus, quenched in acid at the times indicated and the [^{32}P]P_i determined. The data were fitted to a single exponential of rate constant 29 ± 3 s^{-1}.

We mix 10 μM PGK with 100 μM [γ-^{32}P]ATP and 20 μM PG (reaction mixture concentrations). Reaction occurs, and the equilibrium is reached within 100 ms (Figure 6). At this equilibrium, the mixture contains about 5 μM [1-^{32}P]-bPG. Since the PG concentration is low, essentially all of the bPG present is bound, as E·bPG·ADP (Scheme 1). The mixture is now perturbed by the injection of a large molar excess of cold ATP (11 mM). The free [γ-^{32}P]ATP and that in the collision complex E·PG·ATP are diluted out immediately and on the time scale of the experiment no longer take part in the reaction. This instantaneous removal of these species perturbs the equilibria of the following steps. The [γ-^{32}P]ATP in the E*·PG·ATP complex and the [1-^{32}P]bPG in E·bPG·ADP are released (via K_3, k_{-2}, and K_1) as free [γ-^{32}P]ATP, which is diluted out in the bulk cold ATP. Thus, as the perturbed equilibrium mixture ages, the [1-^{32}P]bPG disappears. As shown in Figure 8, the kinetics of this disappearance can be fitted to a single exponential of $k_o = 29 \pm 3$ s^{-1}.

DISCUSSION

A first approach toward solving an enzyme reaction pathway is to identify some intermediate and then to obtain its kinetics of formation and decomposition. With PGK, the time course for bPG formation consists of an initial burst of bPG followed by the steady state phase (Schmidt et al., 1995). This shows that some intermediate(s) involving bPG accumulates on the PGK pathway. Unfortunately, the rapidity of the kinetics and low amplitude of the burst, even at 4 °C, precluded a study. Matters were improved somewhat by including ethylene glycol (40%) in the buffer: the rapidity of the kinetics were reduced but the amplitude remained too low for a full exploitation. So, how is the PGK system induced to accumulate its bPG intermediate(s) in sizeable quantities?

Here we perturbed the PGK system by testing other cryosolvents. Of these, 30% methanol was the most satisfactory as it at once reduced the rapidity of the kinetics and significantly increased the size of the burst of bPG. This allowed for a study of the kinetics of formation and decomposition of species containing bPG. But first we consider the overall effects of methanol on the PGK system.

Overall Effects of 30% Methanol on the PGK System. PGK lost little activity after several hours of incubation in methanol at 22 °C, but it was much less soluble in this solvent than in water or 40% ethylene glycol (solubility below 0.2 mM in methanol, above 5 mM in either water or ethylene glycol). The relatively low solubility of PGK in 30% methanol is an illustration of the use of primary alcohols in fractioning proteins [e.g., Scopes (1994)].

Whereas ethylene glycol had little effect on the affinities of PGK for its substrates, methanol reduced them significantly (Table 2). An explanation for this is not immediately obvious; on a molar basis, the concentrations of the two solvents are almost identical. Ethylene glycol (40%) and methanol (30%) have about the same low dielectric constant (about 75 compared with 88 for water at 0 °C), and we would expect that in them the strengths of electrostatic and hydrogen-bond interactions are increased by similar extents. Thus, both solvents should increase the affinities of PGK for its substrates (both highly charged). However, whereas methanol did indeed increase these affinities (especially for PG), ethylene glycol did not. This could be a consequence of their different sizes. Both ATP and PG are bound to PGK by a network of hydrogen-bonds and electrostatic interactions (Watson et al., 1982; Harlos et al., 1992). It could be that the smaller methanol, but not the larger ethylene glycol, has access to the substrate binding sites. This would reduce the microscopic dielectric constant around the sites and thereby increase the tightness of the substrate binding. Interestingly, 30% methanol reduced the K_m for the charged L-glutamate to glutamate dehydrogenase from 30 to 6 mM (Cohen et al., 1982) whereas 50% methanol did not affect the affinity of aspartate aminotransferase for cysteine sulfinate (Gehring, 1986) nor that of leucine amino peptidase for a specific substrate (Lin et al., 1988).

Reaction Pathway for PGK. The pathway in Schmidt et al. (1995) is at once too simple and too complex to be of use in interpreting our transient kinetic data (e.g., Figure 4). Instead we used Scheme 1.

In this pathway there are four types of steps: formation of collision complexes (rapid equilibria defined by K_1 and K_5), a protein conformational change (k_2, k_{-2}), phosphoryl transfer (a rapid equilibrium, K_3; Nageswara Rao et al., 1978), and release of bPG and ADP in one step (k_4). This pathway is obviously an oversimplification but it fits well our data. It is based on the following arguments.

First, the binary E·PG and E·ATP are formed very rapidly, but as a precaution in our experiments, PGK was preincubated with PG before the initiation of the reaction by the addition of the [γ-^{32}P]ATP. *Therefore, at $t = 0$ all of the enzyme is in the form E·PG.*

Second, consider the progress curves in Figure 4. During the experiment, the bPG formation was followed well before the final equilibrium was reached. We assume, therefore that the back reaction is insignificant, i.e., that the steady state phase is truly initial. This assumption is justified because the steady state rates obtained by the acid quench method (free bPG not removed) and coupled assay system (bPG removed) agree well [also see Schmidt et al. (1995)]. *Therefore, we assume that bPG and ADP leave in one step,* defined by k_4. It follows that the large transient burst phases are due to the accumulation of the ternary complex, E·bPG·ADP.

Third, there were two kinetics in the progress curves, the kinetics of the transient phase (k_{obs}) and the steady state rate (k_{ss}). To which steps in the Scheme 1 can we assign these kinetics?

Table 3: Rate and Equilibrium Constants of Scheme 1

constant	value
$[E]_o/[PGK]$ (mol of active site/ mol of enzyme)	0.7 ± 0.5 [a]
k_2/K_1 (μM^{-1} s^{-1})	0.37 ± 0.03 [b]
k_{-2} (s^{-1})	33 ± 4 [a]
K_3	4 ± 0.5 [a]
k_4 (s^{-1})	7.1 ± 0.6 [a]
K_5 (μM)	1.5 ± 0.4 [c]

[a] Estimated from computer simulation (see text). [b] The slope of the dependence of k_{obs} upon [ATP] (Figure 5a). [c] K_d for the formation of the binary E·bPG (Figure 3b).

In all our experiments, the kinetics of the transients fit well to single exponentials (k_{obs}). This suggests that k_{obs} is the reflection of a single step. Since k_{obs} is sensitive to the ATP concentration (saturation kinetics were not obtained) and the measured E·bPG·ADP concentration is high, the step is early on the reaction pathway, most probably that preceding a ternary substrates complex. Now, the ATP dependence of k_{obs} gave an apparent second-order constant of 0.37 μM^{-1} s^{-1} (Figure 5a). This is much too low to represent a diffusion-controlled process [typically > 100 μM^{-1} s^{-1}; e.g., Gutfreund (1995)]. Therefore, ATP almost certainly binds to E·PG in two steps, formation of a collision complex (K_1 in Scheme 1) followed by a protein isomerization (k_2, k_{-2}). k_{obs} is then a function mainly of k_2 and k_{-2}. The slope of the dependence of k_{obs} upon the ATP concentration is k_2/K_1 (Figure 5a).

We assign k_{ss} to the kinetics of the release of the products, i.e., k_4. This is justified by the accumulation of the products intermediate (E·bPG·ADP) in the steady state.

Determination of the Rate and Equilibrium Constants in Scheme 1. With Scheme 1 as a base, we now interpret the dependences of the transient kinetic parameters illustrated in Figure 5.

By the integration of the differential equation describing the Scheme 1 [e.g., Gutfreund (1995)] we obtain

$$([E\cdot bPG\cdot ADP] + [bPG])/[PGK] = k_{ss}t + B[1 - \exp(-k_{obs}t)]$$

(i) Kinetics. $k_{obs} = k_- + k_2[ATP]/(K_1 + [ATP])$.

Since the variation of $k_{obs} = f([ATP])$ is linear (Figure 5a), $K_1 \gg [ATP]$ and the slope is k_2/K_1. The intercept $k_- = (k_{-2} + k_4K_3)/(1 + K_3)$.

(ii) Amplitude. $B = \alpha(K_3/1 + K_3)(x^2 + ax)/(x + k_{obs})^2$, where $x = (k_2/K_1)[ATP]$, $a = k_{-2} + (k_4/1 + K_3)$, and α is the active site titration, i.e., moles of active site per mole of PGK. At saturation in ATP, $B = B_{max} = \alpha K_3/(1 + K_3)$.

(iii) Steady State. $k_{ss} = \alpha k_{cat}[ATP]/(K_m + [ATP])$ with $k_{cat}/K_m = (k_2/K_1)[k_4K_3/(k_{-2} + k_4K_3)]$.

From our experiments, we *obtained* k_{cat}/K_m, k_2/K_1, k_-, B_{max}, and $B = f([ATP])$. *To estimate* the unknown parameters k_{-2}, K_3, k_4, and α, we simulated the variation of $([E\cdot bPG\cdot ADP] + [bPG])/[PGK] = f(t)$ using the KINSIM program (Barshop et al., 1983) with Scheme 1 as the input model. Imposing the values obtained, the unknown parameters were adjusted to fit the simulation to the experimental results and the best values obtained are given in Table 3.

We have not devised a method for titrating PGK; an all or none assay such as equilibrium dialysis will not do as we are only interested in *fully active sites*. Since the maximum burst size was 0.56 mol of bPG/mol of PGK, the active site titration α is obviously greater than 0.56 mol of site/mol of PGK. During the adjustment of the fitting we varied α between 0.6 and 1, and the best appeared to be 0.7. This is a realistic value: an enzyme preparation is rarely fully active. We note that the data we used to estimate the constants were obtained from a single PGK preparation.

We confirmed the estimates for k_{-2} and K_3 by a cold-chase experiment. In this, as described in the Results, we perturbed a PGK equilibrium mixture by the injection of a large molar excess of cold ATP and then followed the disappearance of the [1-^{32}P]bPG. As the concentration of PG was low (20 μM), the concentration of free [1-^{32}P]bPG was low, so we follow essentially the disappearance of bPG from only one species, the ternary E·bPG·ADP. So, from the Scheme 1 we have

$$E\cdot bPG^+\cdot ADP \overset{K_3}{\longleftrightarrow} E^*\cdot PG\cdot ATP^+ \xrightarrow{k_{-2}} E\cdot PG\cdot ATP^+ \xrightarrow{K_1} E + PG + ATP^+$$

where the $^+$ indicates a radioactive species.

The key to the process is that the ATP^+ in $E\cdot PG\cdot ATP^+$ is diluted out rapidly by the cold ATP via the rapid equilibrium, K_1. The disappearance kinetics are then given by

$$k_o = k_{-2}[K_3/(1 + K_3)]$$

From Figure 8, $k_o = 29$ (± 3) s^{-1}. This agrees well with the k_o calculated from k_{-2} and K_3 in Table 3, i.e., 26.4 s^{-1}.

Equilibrium Constant for Enzyme-Bound Substrates and Products (K_3). Because of solubility problems, we were unable to measure K_3. The value estimated from computer simulation with Scheme 1 as the model (Table 3) is about 40 times higher than those obtained experimentally in water and 40% ethylene glycol (Schmidt et al., 1995). Why this difference?

It could be related to the effect of methanol in increasing the affinity of PGK for its substrates (see above). Another explanation is that by the method used by Schmidt et al. (1995) one does not measure specifically K_3, or in the terminology of Burbaum and Knowles (1989), K_{int}. The key assumption in this method is that all the [1-^{32}P]bPG is in the ternary E·bPG·ADP and the [γ-^{32}P]ATP in a single E·PG·ATP. If there are two ternary substrates complexes (as in Scheme 1), K_3 will be underestimated. This is an illustration of how difficult it is to measure the equilibrium constant of the phosphoryl transfer step on a kinase reaction pathway.

CONCLUSIONS

Does k_{obs} Measure the Kinetics of the Hinge Binding Motion of PGK? In most kinase reactions, water is not an acceptor substrate: very few kinases are also ATPases. To explain this lack of water reactivity, Koshland (1958) proposed that in the absence of the phosphoryl acceptor substrate, the catalytic site is incomplete. Upon substrate binding, there is a conformational change that leads to the proper geometry of the active site and the expulsion of water. This is the basis of the induced fit theory; for a further discussion see Janin and Wodak (1983).

Extensive studies have been carried out to determine whether or not there is a substrate-induced fit with PGK, and if so how extensive it is. Crystallographic studies agree that PG and ATP bind to sites that are far apart [e.g., Harlos

et al. (1992)]. To bring the sites into proximity and allow for an in-line phosphoryl transfer (Webb & Trentham, 1980), Banks et al. (1979) proposed that upon the binding of both the substrates there is a large hinge-bending motion which involves a relative movement of the two domains of PGK [also Joao and William (1993)]. Although each substrate on its own induces conformational changes [e.g., Joao and Williams (1993) and McPhillips et al. (1996)], these may not be sufficient for the final line-up of the substrates [but see May et al. (1996)]. Large substrate-induced domain movements may also occur in hexokinase (Bennett & Steitz, 1980) and adenylate kinase (Bilderback et al., 1996, and references cited therein).

Despite several structural studies on PGK [X-ray crystallography, NMR etc.; e.g., Joao and Williams (1993) and McPhillips et al. (1996)], there is little experimental evidence for the large conformational change implied by the proposed hinge-binding motion. The theoretical study of Guilbert et al. (1995) is in agreement with the motion. However, very recently, McPhillips et al. (1996) claimed that the substrates are nearer each other than was thought previously. A substrate-induced conformational change may still be necessary, but it could be less extensive than a hinge-bending motion. In any event, at appears that *both* substrates are needed for the change [e.g., Pickover et al. (1979) and Joao and William (1993)].

The kinetics of the putative hinge-bending motion are given by step 2 in Scheme 1. This step represents an induced fit process and allows the phosphoryl transfer to take place (step 3). Step 2 is, therefore, a key step on the PGK reaction pathway, and a knowledge of its kinetics would be of great interest. Because k_{obs} is a function mainly of k_{-2} and k_2, we may be near to this, at least under our experimental conditions.

We are investigating further k_{obs}, in particular the effect of intensive parameters such as temperature and viscosity. Thus, if the motion is extensive, as proposed by Banks et al. (1979), it should be sensitive to these parameters [e.g., Gutfreund (1995)].

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NOTE ADDED DURING MANUSCRIPT REVISION

Since this paper was submitted, a paper has been published which supports a large substrate-induced hinge-binding motion in PGK (Bernstein et al., 1997). Bernstein et al. (1997) present the 2.8 Å crystal structure of PGK from *Trypanosoma brucei*. They show that in the ternary complex [PGK·ADP·3PG] there is a dramatic closing of the large cleft that separates the two domains in the substrate free enzyme. If our kinetic interpretations are correct, then k_{obs} is the manifestation of this extensive conformational change by which the ATP and PG are engulfed and the catalytic site of PGK formed.

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