Transfer of 1,3-Diphosphoglycerate between Glyceraldehyde-3-phosphate Dehydrogenase and 3-Phosphoglycerate Kinase via an Enzyme–Substrate–Enzyme Complex†

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ABSTRACT: On the basis of the alternatives of direct inter-enzyme transfer vs. dissociation followed by random diffusion, two kinetic models for metabolite transfer between consecutive enzymes are developed. These two models are readily distinguishable experimentally for the transfer of 1,3-diphosphoglycerate (1,3-P_2G) between glyceraldehyde-3-phosphate dehydrogenase (GPDH) and 3-phosphoglycerate kinase (PGK). Since 1,3-P_2G is exceedingly tightly bound to PGK, the kinetics of its transfer to GPDH are predictably different for each of these two models. Our experiments unambiguously demonstrate that 1,3-P_2G is directly transferred between these two enzymes via an enzyme–substrate–enzyme complex. This direct transfer is described by a Michaelis–Menten scheme in which PGK·1,3-P_2G is the "substrate" for GPDH. At high concentrations of PGK·1,3-P_2G, the transfer reaction becomes nearly PGK·1,3-P_2G concentration independent. The rate of the transfer reaction is activated 3.5-fold by saturating quantities of ATP and 20-fold by saturating quantities of 3-PG. Evidence is presented that the PGK·1,3-P_2G complex is structurally distinct from either PGK itself or other PGK·ligand complexes.

The regulation of directed metabolic pathways such as glycolysis has been widely studied. Interest has centered on the interactions which govern the catalyzed flow of metabolites along the coupled reaction pathway. Much of this interest has emphasized the potential role of enzyme–enzyme or enzyme–effector mediated interactions between consecutive enzymes.

In glycolysis, the mechanism of metabolite transfer between enzymes is uncertain. Does a released intermediate metabolite disperse freely in the cytosol before proceeding along the pathway, or does the common metabolite transfer directly from one enzyme site to the next? There are several reports of interactions between glycolytic enzymes. These reports suggest that glyceraldehyde-3-phosphate dehydrogenase (GPDH)[1] interacts with other enzymes in the glycolytic pathway, both in muscle and in erythrocytes (Ovadi & Keleti, 1978; Bartha & Keleti, 1979; Ovadi et al., 1978; De & Kirtley, 1977; Yu & Steck, 1975; Strapazon & Steck, 1976). MacGregor et al. (1980) have presented strong evidence for a 1:1 complex between aldolase and fructose 1,6-bisphosphatase. The functional consequences of all of these interactions are, however, obscure.

In muscle sarcoplasm, the glycolytic enzymes are present at concentrations of 0.1 mM or greater, so that the molar concentrations of enzyme sites are in excess of the molar concentrations of the three-carbon intermediates of glycolysis. GPDH, as well as lactate dehydrogenase, is present in about 10-fold excess (in equivalents of sites) over other glycolytic enzymes. In resting muscle sarcoplasm, Bloch et al. (1971) showed that a substantial fraction of GPDH sites contains covalently bound 3-phosphoglyceroyl enzyme. Thus, this acyl-enzyme (thiol ester) is the major stoichiometic "3-carbon" intermediate in glycolysis.

We thought that the GPDH–PGK enzyme–enzyme system would be an interesting pair for the investigation of the mechanism of metabolite transfer for several reasons. A variety of spectroscopic signals for the study of the kinetics of the GPDH-catalyzed reaction are available. Discrete ^{31}P NMR signals for investigating all substrate interactions with the two enzymes have been identified (Nageswara Rao et al., 1978; Huskins, 1979; Huskins et al., 1982). The three-dimensional structures of both of the enzymes (from several sources) have been determined to atomic resolution by X-ray crystallographic techniques (Watson et al., 1972; Wendell et al., 1972; Buehner et al., 1973; Moras et al., 1975; Blake et al., 1972; Banks et al., 1979). The binding of the common metabolite 1,3-diphosphoglycerate (1,3-P_2G) to PGK is extremely tight, which facilitates monitoring its transfer to GPDH (see below).

A maximal estimate for the dissociation constant of 1,3-P_2G from both *yeast* and *halibut* PGK is about 1–10 nM (Nageswara Rao et al., 1978; Scopes, 1978; Huskins, 1979; Huskins et al., 1982). Such tight binding in the isolated PGK·1,3-P_2G complex demands a dissociation rate constant for the complex of at most $\sim 1\ s^{-1}$. This specific rate is far slower than that usually found for the turnover of substrate in enzyme catalysis.

We attempted to determine the rate of dissociation of 1,3-P_2G to solvent by using a coupled enzyme reaction. It soon became apparent that this determination depends on the assumed microscopic mechanism by which 1,3-P_2G is transferred between enzymes. Conversely, the dependence of the coupled reaction rate on enzyme and substrate concentration provided direct information concerning the microscopic mechanism. Although we still do not know the precise rate constant for the dissociation of the PGK·1,3-P_2G complex, we are certain that in the absence of effectors this rate constant must limit the rate of metabolite transfer in any coupled reaction which proceeds via 1,3-P_2G dissociation into aqueous solution.

PGK activity is conventionally assayed by coupling the kinase reaction to the NADH-dependent reduction of acyl-GPDH. The turnover number for the coupled reaction, when

† From the Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403. *Received January 8, 1982.* This work was supported by grants from the U.S. Public Health Service of the National Institutes of Health (GM 10451) and from the National Science Foundation (PCM 8016 249).

[1] Abbreviations: 3-PG, 3-phosphoglycerate; 1,3-P_2G, 1,3-diphosphoglycerate; PGK, 3-phosphoglycerate kinase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; G-3-P, glyceraldehyde 3-phosphate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-4189$01.25/0

PGK is rate limiting, is 2 orders of magnitude greater than the maximal estimate for 1,3-P_2G dissociation. Hence, there must be a special mechanism for enhancing the rate of acyl transfer between enzymes (eq 1) or for increasing the rate of

$$\text{PGK·1,3-P}_2\text{G} + \text{GPDH} \rightleftharpoons \text{3-phosphoglyceroyl-GPDH} + \text{P}_i + \text{PGK} \quad (1)$$

1,3-P_2G dissociation into the aqueous environment in the presence of other reaction components.

The preceding paper (Huskins et al., 1982) shows that high concentrations of all components of the PGK-catalyzed reaction do not substantially increase the PGK·1,3-P_2G dissociation rate in the absence of GPDH. This paper reports on the mechanistic details of this acyl transfer reaction. We present kinetic evidence for a direct transfer of the intermediate metabolite, 1,3-diphosphoglycerate, between 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase via an enzyme–substrate–enzyme complex.

Materials and Methods

Materials

Sodium salts of NADH (grade III), ADP (Sigma grade), ATP (grade I), DL-glyceraldehyde 3-phosphate diethyl acetal monobarium salt, and 3-phosphoglycerate (grade I) were obtained from Sigma Biochemicals. The sodium salt of NAD^+ (Chromatopure grade) was obtained from P-L Biochemicals. Reagent-grade chemicals and glass-distilled water were used to prepare all buffers and solutions. A pH 7.4 (pH adjusted with HCl) 0.05 M 2-methylimidazole–2×10^{-4} M Na_4EDTA buffer was used for all experiments.

1,3-Diphosphoglycerate. 1,3-Diphosphoglycerate (1,3-P_2G) is unstable at neutral pH and room temperature in aqueous solution with a reported half-life of about 30 min (Negelein, 1974). Thus, 1,3-P_2G, which was prepared and assayed by the method of Furfine & Velick (1965), had to be stored at −80 °C and thawed at 0 °C immediately before use. 1,3-P_2G in an excess of PGK was stable in stock buffer at room temperature for days and could be handled easily.

Enzymes. (A) 3-Phosphoglycerate kinase (PGK) from *halibut* muscle was used for all experiments. It was prepared according to the procedure of Huskins (Huskins, 1979; Huskins et al., 1982). Yields have been increased from 300 to 370 mg of PGK/kg of muscle by including 1×10^{-4} M phenylmethanesulfonyl fluoride in the extract buffer.

(B) Glyceraldehyde-3-phosphate dehydrogenase (GPDH) from *halibut* muscle was prepared from the 83–100%-saturated ammonium sulfate fraction left over from the PGK procedure described above. Purified enzyme was obtained from this fraction in a manner similar to that described for *sturgeon* GPDH (Seydoux et al., 1973). GPDH from rabbit muscle was obtained as an ammonium sulfate suspension from Boehringer Mannheim.

Methods

Since both PGK and GPDH were stored as ammonium sulfate suspensions, they were desalted on a Sephadex G-25 column equilibrated with 2-methylimidazole buffer before use. Only the peak fractions containing enzyme at greater than 100 μM were taken for use in experiments.

Assays. PGK was assayed by the method of Bücher (1955). GPDH was assayed by the method of Ferdinand (1964).

Kinetics experiments were carried out at 25 °C on a Cary Model 14 or Model 16 recording spectrophotometer. Kinetics were followed by monitoring the disappearance of the NADH absorption peak at 340 nm. An extinction coefficient of 6200 (Horecker & Kornberg, 1948) was assumed at this wavelength.

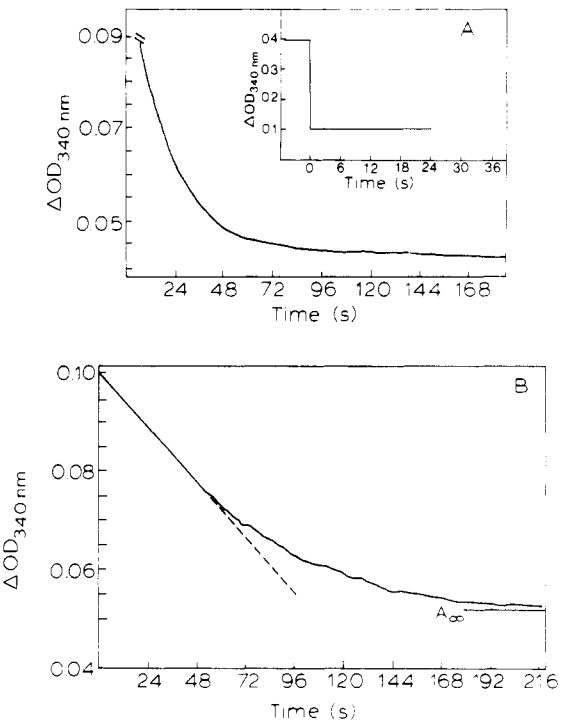

FIGURE 1: (A) Typical whole reaction trace of the transfer reaction. *Rabbit* muscle [GPDH] = 5×10^{-7} M, [PGK·1,3-P_2G] = $[PGK]_{free}$ = 8.4×10^{-6} M, [NADH] = 2×10^{-4} M, pH 7.4, 25 °C. Insert illustrates burst titration of free 1,3-P_2G using GPDH at this same concentration. (B) Typical whole reaction trace of the transfer reaction using *halibut* muscle [GPDH] = 4×10^{-7} M, [PGK·1,3-P_2G] = 8.4×10^{-6} M = $[PGK]_{free}$, [NADH] = 2×10^{-4} M, pH 7.4, 25 °C. The constant velocity deviation is emphasized with the dashed line.

Irreversible thermal denaturation of PGK was carried out by incubating enzyme (with or without additional ligands) at elevated temperature, usually 45 °C, in standard 2-methylimidazole buffer (pH 7.4 at 25 °C). Aliquots were removed at intervals and quenched into buffer at 0 °C. Quenched enzyme solutions were assayed for residual activity at 25 °C by using the standard assay of Bücher (1955). No changes in enzyme activity were detected over periods of 3 h in quenched solutions held at 0 °C.

Results

Kinetics of Coupled Reaction. For investigation of the coupled reaction mechanism, the reaction shown in eq 2 was used. In the following experiments, the concentration of PGK

$$\text{NADH} + \text{PGK·1,3-P}_2\text{G} \xrightarrow{\text{GPDH}} \text{G-3-P} + \text{NAD}^+ + \text{HPO}_4^{2-} + \text{PGK} \quad (2)$$

is greater than or equal to twice the 1,3-P_2G concentration and is very much greater than the GPDH concentration. Under these conditions, virtually all the 1,3-P_2G is tightly bound in the PGK complex. Thus, the steady-state conditions for 1,3-P_2G concentration and PGK·1,3-P_2G concentration are met. For the reaction of eq 2, a slow decay in the NADH concentration was observed (Figure 1A). In contrast, when PGK was left out of the reaction mixture and GPDH was allowed to react freely with 1,3-P_2G, the reaction was over instantly at these concentrations of GPDH (Figure 1A, insert). Since the slow decay rate in NADH absorbance extrapolates back to the absorbance at zero time, there is no fast reaction process which precedes this slow decay rate. This decay rate was measured as a function of the concentration of various components of the reaction process (eq 2). As is discussed in detail below, such concentration-dependent kinetic experiments

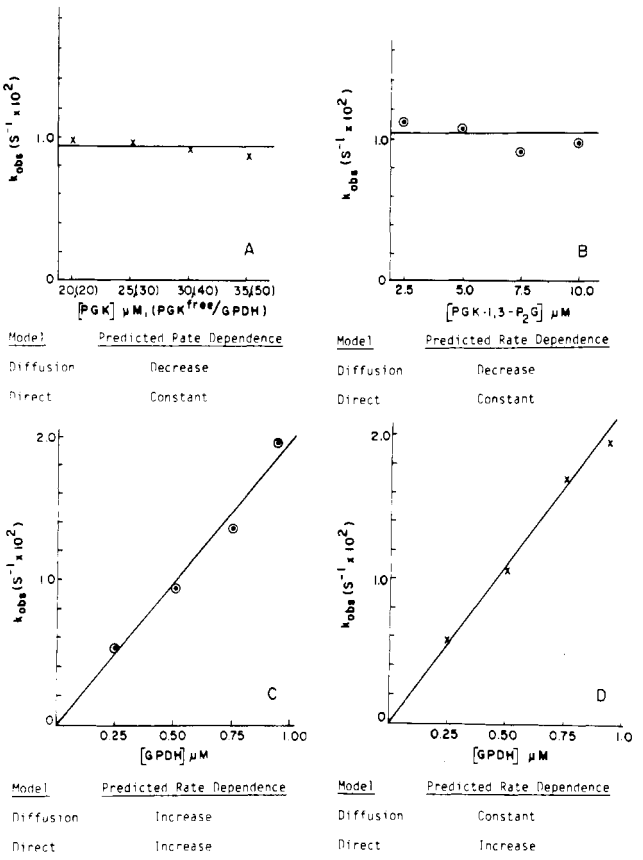

FIGURE 2: Transfer reaction rate dependence on [PGK] and [GPDH] (rabbit muscle); [NADH] = 2×10^{-4} M, pH 7.4, 25 °C. Below each plot, the predicted rate dependence on the concentration of the ordinate species is indicated for each model. (A) [GPDH] = 0.5 μM protein, [1,3-P_2G] = 10 μM; (B) [GPDH] = 0.5 μM protein, [PGK] = 2[1,3-P_2G]; (C) [PGK] = 20 μM, [1,3-P_2G] = 10 μM; (D) [PGK]:[1,3-P_2G]:[GPDH] = 2:1:0.1.

distinguish unambiguously between two basic models of metabolite transfer between the two enzymes.

Our early experiments utilized *halibut* muscle PGK and *rabbit* muscle GPDH. In all these experiments, a first-order decay in NADH is observed over at least 90% of the reaction. The pseudo-first-order rate constant is determined from linear plots of log OD vs. time. When the GPDH concentration is held constant and the PGK concentration or the PGK·1,3-P_2G concentration is varied, the pseudo-first-order rate constant remains invariant (Figure 2, parts A and B, respectively). That is, the pseudo-first-order rate constant is independent of PGK concentration and PGK·1,3-P_2G concentration. In contrast, when the PGK concentration is held constant and the GPDH concentration is varied, the pseudo-first-order rate constant varies linearly with the GPDH concentration (Figure 2C). In a dilution experiment (where the ratios of [PGK] to [GPDH] to [1,3-P_2G] are held constant), the pseudo-first-order rate constant varies linearly with the concentration of the mixture (Figure 2D). This demonstrates the dependence of the rate constant on a single reaction component, which from the data of Figure 2C must be GPDH.

Experiments with *halibut* GPDH lead to qualitatively different results at high concentrations of *halibut* PGK·1,3-P_2G. At these high concentrations, the NADH decay deviates from simple first-order kinetics (Figure 1B). The initial reaction velocity remains constant for a large part of the whole reaction, indicating that there is nearly no dependence of the velocity on substrate concentration. This allowed us to measure initial velocities at high concentrations of PGK·1,3-P_2G. The

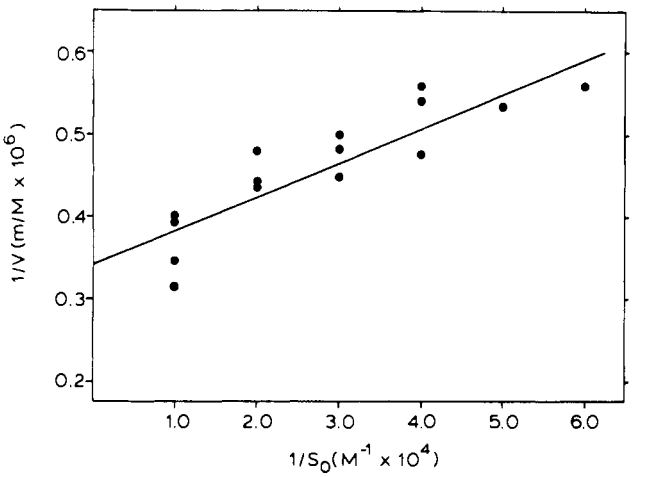

FIGURE 3: Double-reciprocal plot of initial velocity vs. [1,3-P_2G·PGK] (S_0). [NADH] = 2×10^{-4} M, $[GPDH]_{halibut}$ = 0.0042 mg/mL, $[PGK]_{free}$ = [1,3-P_2G·PGK], pH 7.4, 25 °C.

initial velocity exhibits a hyperbolic dependence on initial PGK·1,3-P_2G concentration. A double-reciprocal plot of $1/V_0$ vs. $1/S_0$ appears linear and intersects the abscissa at a finite value (Figure 3). If Michaelian behavior is assumed, a value of V_{max} = 6.9 ± 0.7 μmol min^{-1} (mg of GPDH)$^{-1}$ and a value of K_m = 12 ± 2 μM are calculated, where K_m refers to the GPDH·1,3-P_2G·PGK complex. In contrast, no deviation from single exponential decay of NADH was observed when *rabbit* muscle GPDH rather than *halibut* muscle GPDH was used, even at concentrations of *halibut* PGK·1,3-P_2G up to 90 μM.

Since the limiting V_{max} at saturating concentrations of PGK·1,3-P_2G is slow ($k_{cat} \sim 16 \pm 3$ s^{-1}; see discussion below), compared to the turnover number for PGK in the coupled assay system (Huskins et al., 1982), we undertook studies to examine the potential influence of other effectors on the rate of 1,3-P_2G transfer. Operating at near-saturating concentrations of PGK·1,3-P_2G transfer ([E·S] = 40 μM) and utilizing *halibut* GPDH, we tested the potential activation by Mg^{2+}·ATP, Mg^{2+}·ADP, and 3-phosphoglycerate (3-PG) at concentrations in great excess of their equilibrium dissociation constants (Huskins et al., 1982). At 4 mM, Mg^{2+}·ATP caused a 3.5-fold increase in velocity, a substantial increase, but vastly insufficient to account for the discrepancy in velocity. At 3 mM, Mg^{2+}·ADP virtually blocks the transfer reaction, presumably due to the PGK-catalyzed reaction to form aqueous 3-PG and ATP (see discussion below). At a concentration of 10 mM, 3-PG greatly accelerates the transfer reaction by 20-fold. Illustrated in Figure 4 is a titration of the activation of the transfer rate by 3-PG. Whether a pseudo-first-order reaction is observed at low concentrations of PGK·1,3-P_2G or a zero-order reaction is observed at high concentrations of PGK·1,3-P_2G, the transfer rate follows a hyperbolic dependence on 3-PG concentration. Half-maximal rate activation occurs at 0.58 mM 3-PG concentration.

Thermal Denaturations. PGK greatly protects bound 1,3-P_2G against hydrolysis. 1,3-P_2G is exceedingly tightly bound to PGK. Together these observations suggest the reciprocal stabilization of the structure of PGK by 1,3-P_2G. The potential stabilization of PGK by 1,3-P_2G was investigated by thermal denaturation of enzyme. Thermal denaturation of PGK was studied at 45 °C in the absence and in the presence of a stoichiometric excess of 1,3-P_2G. The residual PGK activity (assayed at 25 °C) decreased exponentially with time. A specific rate of 0.25 min^{-1} was calculated from the activity vs. time plot in the presence of 1,3-P_2G (Figure 5). When PGK was incubated in the absence of 1,3-P_2G at 45

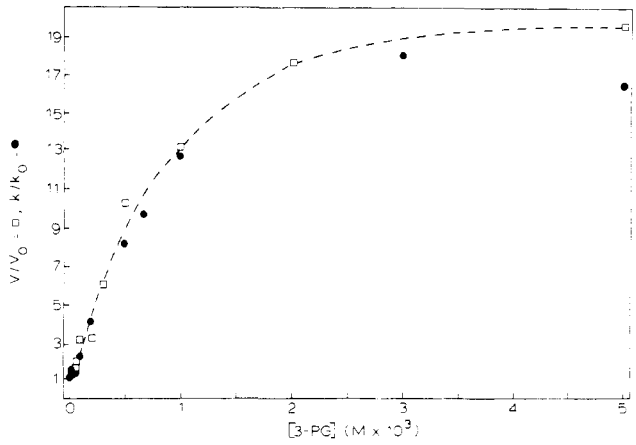

FIGURE 4: Transfer reaction rate dependence on 3-PG concentration ([NADH] = 2×10^{-4} M, pH 7.4, 25 °C) normalized to the rate in the absence of 3-PG (either V_0 or k_0). (●) Low substrate concentration pseudo-first-order conditions: [PGK·1,3-P_2G] = 5×10^{-6} M = $[PGK]_{free}$, $[GPDH]_{halibut}$ = 3.5×10^{-9} M, k_0 = 0.42 s^{-1}. (□) High substrate concentration zero-order conditions: [PGK·1,3-P_2G] = 3.5×10^{-5} M = $[PGK]_{free}$, $[GPDH]_{halibut}$ = 8×10^{-10} M, V_0 = 0.23 μM/min.

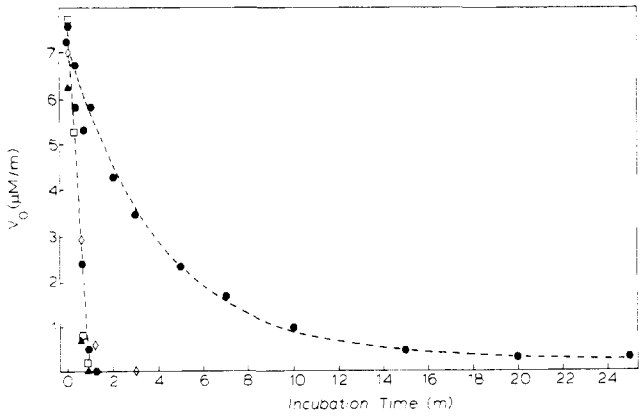

FIGURE 5: Thermal denaturation of PGK at 45 °C. Residual activity as a function of incubation time at 45 °C assayed at 25 °C. The dashed line drawn for PGK + 1,3-P_2G is the mean least-squares fit of the data to a single exponential. (◊) PGK alone; (●) PGK + 1,3-P_2G; (●) PGK + Mg·ADP; (□) PGK + Mg·ATP; (▲) PGK + Mg·ATP + 1,3-P_2G.

°C, there was no detectable PGK activity after ~80 s, which gives a denaturation rate of at least 2.6 min^{-1}. There was no detectable protection against denaturation at 45 °C over PGK alone when PGK was incubated in the presence of 2 mM Mg^{2+}·ADP or 5 mM Mg^{2+}·ATP or in the presence of 2 mM Mg^{2+}·ADP plus 50 μM 1,3-P_2G, concentrations which are sufficient to virtually saturate PGK with ternary complex (Huskins et al., 1982) (Figure 5). At 40 °C, the denaturation rate in the presence of 1,3-P_2G is ~0.07 min^{-1}.

Discussion

Halibut muscle PGK·1,3-P_2G complex has a dissociation constant of at most 1–10 nM (Huskins et al., 1982). Similar extremely high affinity has been found for *yeast* PGK·1,3-P_2G (Scopes, 1978; Nageswara Rao et al., 1978). The necessarily slow rate of dissociation of the PGK·1,3-P_2G complex permits us to distinguish between two basic models for metabolite transfer. Either the intermediate metabolite dissociates from the first enzyme into solution and diffuses freely through the solvent medium between the two enzymes or the metabolite is transferred directly between the two enzyme sites in an enzyme–substrate–enzyme complex. These two models are illustrated in Schemes I and II.

Scheme I: Random Diffusion Model

$$E_1 \cdot S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1 + S + E_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2 \cdot S \xrightarrow{k_3} \text{products}$$

Scheme II: Direct Transfer Model

$$E_1 \cdot S + E_2 \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} E_1 \cdot S \cdot E_2 \xrightarrow{k_b} \text{products}$$

In the random diffusion model of Scheme I, substrate dissociates from the first enzyme. Then, both enzymes compete for the same substrate. The fraction of catalytically competent substrate complexes (E_2S) is proportional to $E_2S/(E_1S + E_2S)$.

In the direct transfer mechanism of Scheme II, there is an obligatory formation of an enzyme–substrate–enzyme complex. This scheme is similar to a Michaelis–Menten scheme in which the enzyme–substrate complex (E_1·S) is the substrate for the second enzyme (E_2).

The reaction involving the transfer of 1,3-P_2G between PGK and GPDH (eq 2) is conveniently followed by monitoring the disappearance of NADH. We found that at lower concentrations of PGK·1,3-P_2G, there is a single exponential decay in NADH concentration. We can derive rate expressions for both of these model schemes which are consistent with this first-order decay in NADH provided that only small steady-state concentrations of S, in Scheme I, and E_1·S·E_2, in Scheme II, build up in solution. Due to the minute value for the dissociation constant of PGK·1,3-P_2G, this first proviso is always satisfied.

In the random diffusion model, we know that the specific rates of acylation and phosphorylation (k_2 and k_{-2}) (Seydoux et al., 1973) and the specific rate of acyl reduction (k_3) (J. P. Weber and S. A. Bernhard, unpublished results) are fast. Hence, under steady-state conditions, $[E_1 \cdot S] \gg [E_2]$, the resultant expression for the first-order rate constant is given by eq 3, where E_2 = GPDH and E_1 = PGK. Thus, the random

$$k = \frac{k_1 k_2 k_3 [E_2]}{k_2 k_3 [E_2] + (k_3 k_{-1} + k_{-2} k_{-1})[E_1]} \tag{3}$$

diffusion model predicts that the observed pseudo-first-order rate constant will be dependent on the concentration of both enzymes and should vary in proportion to the fraction of the total enzyme sites which are GPDH sites.

In the direct transfer mechanism, 1,3-P_2G need not dissociate from PGK. Hence, under our experimental conditions, we can derive the velocity expression (eq 4) according to the

$$V = \frac{k_b [E_2][E_1 \cdot S]}{(k_{-a} + k_b)/k_a + [E_1 \cdot S]} = \frac{k_b [E_2][E_1 \cdot S]}{K_m + [E_1 \cdot S]} \tag{4}$$

simple Michaelis–Menten model in which E_1·S rather than S is substrate and where E_2 is the total site concentration of GPDH. If the $[E_1 \cdot S] \ll K_m$, eq 4 reduces to a pseudo-first-order form where the macroscopic first-order rate constant is given by eq 5. Accordingly, we expect the pseudo-first-order

$$k = \frac{k_a k_b [E_2]}{k_{-a} + k_b} \tag{5}$$

rate constant to be dependent on the GPDH concentration and to be independent of the PGK concentration (within limits; since PGK is a "product" of the reaction, product inhibition might become substantial at sufficiently high unliganded PGK concentration). At high concentrations of PGK·1,3-P_2G complex, the "substrate" concentration may become substantial relative to K_m (eq 4). Under this condition, according to eq 4, an $[E_1 \cdot S]$-independent (zero-order) velocity should be observed.

All of our kinetic data (Figure 2) for the *halibut* PGK–*rabbit* GPDH catalyzed reaction are consistent with the direct transfer pathway. They are inconsistent with the random diffusion pathway. The concentration rate dependence predictions of each model are compared with the observed results in Figure 2.

At sufficiently high concentrations of *halibut* PGK·1,3-P_2G, a substrate concentration independent (zero-order) rate is observable over an extended portion of the reaction catalyzed by *halibut* GPDH (Figure 1B). This observation further confirms the direct transfer model. The affinity of *halibut* PGK·1,3-P_2G complex suggests that there may be species selectivity in the interaction. *Halibut* GPDH has at least a 10-fold higher affinity for *halibut* PGK·1,3-P_2G than does *rabbit* GPDH.

The structure of PGK is 1,3-P_2G dependent. Our thermal denaturation studies indicate that stoichiometric binding of 1,3-P_2G to PGK greatly stabilizes the enzyme against thermal denaturation. No indication of protection against thermal denaturation could be detected in the presence of bound Mg^{2+}·ADP or Mg^{2+}·ATP. Addition of Mg^{2+}·ADP to PGK·1,3-P_2G complex results in a sensitization of the PGK·1,3-P_2G complex toward denaturation, presumably due to the partial restoration of the relatively unstabilized PGK·ATP complex.

Using ion-exchange chromatography, Scopes & Algar (1979) found that the pH-dependent binding of PGK to the ion-exchange resin was substantially shifted when PGK was liganded with 1,3-P_2G. Binding 3-PG, ATP, or ADP to PGK caused only a small shift in the affinity of PGK for the ion-exchange resin. Whatever the actual structural changes are upon ligation, it is clear that 1,3-P_2G has a much more intense effect than other ligands. Structural studies with the PGK·1,3-P_2G complex have almost certainly been limited previously by the reported instability of the 1,3-P_2G in solution. This report and that of Huskins et al. (1982) show that this extreme caution toward the stability of enzyme-bound 1,3-P_2G is unwarranted.

Clearly, 1,3-P_2G causes a structural change in PGK. It is tempting to speculate that this structural transition is involved either in the recognition of PGK by GPDH or in the transfer of 1,3-P_2G between PGK and GPDH in the enzyme–enzyme complex.

The rate constant for (unimolecular) direct transfer of substrate (k_b) as calculated from Figure 3 is 16 ± 3 s^{-1}, assuming one site per GPDH tetramer. This rate is very much slower than the *halibut* PGK-limiting turnover rate, $k_{cat} = 570$ s^{-1}, in the GPDH-coupled assay in the direction of 1,3-P_2G production (Huskins et al., 1982). The limiting rate of 1,3-P_2G utilization in the oxidation of NADH catalyzed by GPDH is 250 s^{-1} (J. P. Weber and S. A. Bernhard, unpublished results) and cannot be limiting the transfer rate to such a small value. Therefore, the slow rate of transfer of 1,3-P_2G between PGK and GPDH must be activated by the presence of the other substrates or products during the overall coupled reaction. Our results demonstrate that ATP promotes a small but possibly important rate activation of about 3.5-fold for the intersite transfer of 1,3-P_2G. 3-PG, however, provides a much larger rate activation of about 20-fold. The rate activation by 3-PG can account for the steady-state V_{max} for the PGK-catalyzed turnover of ATP via the GPDH-coupled reaction ($k_{cat} = 570$ s^{-1}).

We can envisage two general mechanisms for the 3-PG assistance of 1,3-P_2G transfer. Either 3-PG causes a change in mechanism to the diffusion model of Scheme I or 3-PG increases the efficiency of 1,3-P_2G transfer in the enzyme–enzyme complex.

The following analysis argues against the former possibility. At these concentrations of 3-PG, one would expect a trivial dissociation of 1,3-P_2G from PGK to be caused by direct competitive displacement. For example, at 5.8×10^{-4} or 7×10^{-3} M 3-PG, when [PGK] = 10 μM and [1,3-P_2G] = 5 μM (conditions of the low substrate concentration experiments shown in Figure 4) and assuming dissociation constants of 150 μM for 3-PG and 10 nM (as an upper limit) for 1,3-P_2G (Huskins et al., 1982), the percentage displacement of 1,3-P_2G would be 0.76% and 8.4%, respectively. When [PGK] = 70 μM and [1,3-P_2G] = 35 μM (high substrate concentration conditions of Figure 4), the percentage displacement of 1,3-P_2G is now 0.1% and 1.3%, respectively. There cannot be any substantial 1,3-P_2G (>1% of the total 1,3-P_2G) which is free in solution. Since GPDH is added last to a solution in which PGK, 3-PG, and 1,3-P_2G are preincubated, any free 1,3-P_2G will react rapidly and cause a burst of NADH oxidation. In the absence of PGK, this burst is used to assay the 1,3-P_2G concentration (Figure 1A, insert). No such burst is detected even in the presence of 7×10^{-3} M 3-PG. The rate of the transfer reaction for the random diffusion model is given by eq 3. Under the high substrate concentration conditions of the experiment in Figure 4, to obtain the 20-fold rate activation observed, we calculate that the off rate, k_1, must be enhanced about 10^6-fold. Given that the affinity of 3-PG for the tightest site on PGK is 0.15 mM (Huskins et al., 1982), it is thermodynamically unreasonable that the energy available from 3-PG binding at these concentrations of 3-PG can drive a 10^6-fold rate activation. Therefore, we conclude that the activation mechanism demands the second alternative.

3-PG must therefore increase the rate of 1,3-P_2G transfer from PGK to GPDH in the enzyme–enzyme complex. The transfer rate of 1,3-P_2G in the unactivated enzyme–enzyme complex is comparable to or not much faster than the calculated off rate of 1,3-P_2G from the PGK·1,3-P_2G complex. This rate constant is much smaller than the rate of acyl-enzyme reduction by NADH and is very much smaller than the rate of acylation of GPDH by bound 1,3-P_2G (Seydoux et al., 1973). One advantage of forming the complex is that a high percentage of the 1,3-P_2G transfers are effective regardless of the high concentration of free PGK in solution. This high PGK concentration would compete with GPDH for ligand, decreasing the efficiency of transfer, if the transfer had to rely on diffusion to occur. This is probably more important physiologically in the direction of glycolysis, for the formation of complex guarantees that the right or most effective transfer of this unstable metabolite occurs.

It is not implausible that 3-PG plays a structural role in activating metabolite transfer. Anderson et al. (1979) have proposed that there is a cleft-closing structural change in kinases involving movement around the "hinge" region connecting the two folding domains of the single polypeptide. Evidence for this conformation change has been obtained X-ray crystallographically (Banks et al., 1979) where a substantial change in the difference map—too large for structure determination—was observed when 3-PG was bound to crystallized PGK. Low-angle X-ray scattering of PGK in solution (Pickover et al., 1979) showed a decrease in the radius of gyration of about 1 Å when 3-PG plus ATP were bound to PGK. This was interpreted in terms of a cleft closure about the hinge of 8–14 Å.

In this study, we have provided concrete evidence for a direct transfer of 1,3-P_2G between 3-phosphoglycerate kinase and

glyceraldehyde-3-phosphate dehydrogenase via an enzyme–substrate–enzyme complex. The rate of 1,3-P_2G transfer approaches a maximum value at high concentrations of PGK·1,3-P_2G complex. The binding of 3-phosphoglycerate dramatically activates the transfer rate. The concentration of enzymes required for substantial enzyme–enzyme complex formation and the concentration of 3-PG required for substantial rate activation all lie within the physiological range.

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