Kinetics of the Activation of Rat Liver Pyruvate Kinase by Fructose 1,6-Diphosphate and Methods for Characterizing Hysteretic Transitions

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1. Kinetics of fructose 1,6-diphosphate activation of liver pyruvate kinase type I inhibited with MgATP and L-alanine are described as a function of enzyme and fructose 1,6-diphosphate concentrations. These results can be explained by a single pseudo-first-order transition of the enzyme into an active form, independent of the enzyme concentration. This rate constant, $k_{app} = 0.24s^{-1}$ with 0.02mM-fructose 1,6-diphosphate ($t_{0.9} \approx 10s$ where $t_{0.9}$ is the time for 90% conversion), is an increasing function of fructose 1,6-diphosphate concentration far beyond that needed to maximally activate enzyme equilibrated with fructose 1,6-diphosphate (about 20 μM). 2. The model equations are best analysed with numerical techniques which are described. These techniques are useful in studying similar slow transients frequently observed in stopped-flow studies of enzymes. 3. Shorter transients ($t_{0.9} = 0.5$ –1.5s) were observed in the kinetic response of the enzyme to the addition of MgATP or phosphoenolpyruvate, but were not further characterized.

Hysteretic transitions in enzyme forms which occur during catalysis, but which are slow relative to the times of conversion of significant quantities of substrate into product, have been described by Frieden (1970), in general, and noted by other authors in particular cases (Shill & Neet, 1971; Hofer, 1971). With the wider use of stopped-flow spectrophotometers to measure properties of more concentrated enzymes, corresponding to cellular concentrations (Srere, 1967, 1970), hysteretic transitions as defined by Frieden (1970) have been observed more frequently. This is expected since the lifetimes of hysteretic transitions are generally independent of enzyme concentration whereas the lifetimes ($t_{0.9}$) of the initial-velocity period decrease with increasing enzyme concentration. (The extent of reaction corresponding to the initial-velocity period, $t_{0.9}$, will be defined in the present paper by the quantity of product produced (reactant converted) beyond which the catalytic reaction rate is less than 90% of the earliest steady-state velocity.)

Hysteretic transitions may have physiological significance (Frieden, 1970) and must be correctly interpreted to elucidate catalytic mechanisms. The present paper describes transitions of rat liver kinase and least-squares curve-fitting methods for analysing them. Analysis of hysteretic transitions of glutamate dehydrogenase by computer simulations with visual

criterion of fit has been described by Bates & Frieden (1973).

Experimental

Materials

Chemicals, enzymes and kinetic measurements were as described by Flory *et al.* (1974).

Calculations

Product concentrations [P], were calculated from the photographic records shown in Fig. 1 as:

$$[P] (\mu M) = 10^6[(\log V_t - \log V_0)/\epsilon l]$$

where V_t and V_0 are recorded voltages at reaction times t and zero respectively, $\epsilon = 6.22 \times 10^3 M^{-1} \cdot cm^{-1}$ is the molar absorptivity and $l = 0.234 cm$ is the cell path length. Voltages were calculated as $V (mV) = \text{bias} + \text{voltage sensitivity} \times \text{curve ordinate}$.

Simulations of eqns. (1) and (2) (see the Results and Discussion section), the model for a hysteretic transition from inactive to active enzyme, were made with the IBM System/360 Continuous System Modeling Program (CSMP) (1968). Least-squares curve fitting of eqn. (2) (see below) was accomplished by using the computer routine STEPIT (Chandler, 1965), which seeks a local minimum of a smooth function of several parameters by a direct-search method. Since an explicit expression for the dependent variable [P] in eqn. (2) is not possible in terms of standard functions, a subroutine (PEGAS) of Dowell

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& Jarratt (1972), was incorporated into the program, which determines simple roots of real functions of one variable. Standard deviations of the parameters K_a and k_{app} were determined by the method of support planes (Chandler *et al.*, 1972). When a Hill coefficient was added to eqn. (1) (see below) the direct-search routine was replaced with a Marquardt gradient method (Chandler *et al.*, 1972) of minimizing residuals in the least-squares analysis to improve the speed of convergence to the best fit. Eqn. (3) in the Appendix below, the velocity equation for a slow transition in K_m , was fitted to data by the program CRICF (Chandler *et al.*, 1972), which combines efficient numerical integration of differential equations with least-squares optimization.

Listings and further explanations of the programmes in this paper will be sent to interested readers on request and card or tape copies will be made available at cost.

Data ordinates (concentration of reaction products, P) were weighted by $(\sigma[P])^{-2}$, where the standard deviation values, σ , were calculated from the precision in reading the photograph records (about ± 0.05 cm). They are a measure of the random error only. The $\sigma[P]$ values calculated (~ 0.7 and $1.4 \mu\text{M}$ for 10 and 20 mV/division sensitivities respectively) were constant within 10% over the range of results, and were in good agreement with the standard-deviation values calculated from the best-fit residuals. Nevertheless, systematic errors of nearly the same magnitude (0.5–0.1 cm) result from the 2% errors in oscilloscope linearity alone. Thus probable errors of approx. $3 \times$ calculated s.d. values are likely.

The transient times for coupling pyruvate production to NADH oxidation with lactate dehydrogenase must be made considerably shorter than the transient times for activation (or inhibition) of pyruvate kinase, to permit accurate measurements of the latter. We used 20 units of lactate dehydrogenase/unit of pyruvate kinase, with the units of pyruvate kinase defined from its maximum activity in the standard assay (0.6 mM-phosphoenolpyruvate, 1.5 mM-MgADP and 1 mM-fructose 1,6-diphosphate). The times taken to reach a fraction, α , of the steady-state velocities in oxidation of NADH are then:

$$t_\alpha = (-K_B/V_2) \ln(1-\alpha)$$

(McClure, 1969) where K_B and V_2 are the Michaelis constant for pyruvate, and the velocity constant of lactate dehydrogenase (mm/min) respectively. Values of $t_{0.9}$ calculated from the above equation are 16, 0.17 and 0.035 s for 0.18, 17.6 and $51.2 \mu\text{g}$ of pyruvate kinase/ml respectively, when $K_B = 1.20$ mM (Pesce *et al.*, 1964) is used. The enzyme-coupling times are therefore adequate for analysing the pyruvate kinase transients at 17.6 and $51 \mu\text{g}/\text{ml}$, but probably not at $0.18 \mu\text{g}/\text{ml}$. We have therefore eliminated the data in the transient phase of the progress curve with

$0.18 \mu\text{g}$ of enzyme/ml from analysis (data below $4 \mu\text{M}$ product with $0.18 \mu\text{g}$ of enzyme/ml in Figs. 1 and 2).

Results and Discussion

Fructose 1,6-diphosphate activation of inhibited pyruvate kinase

Results and qualitative explanations. Physiological concentrations of MgATP (e.g., 1.75 mM) and L-alanine (0.5 mM) cause virtually complete inhibition (99.7%) of purified pyruvate kinase I at pH 7.5 in the absence of fructose 1,6-diphosphate (Table 1 in the present paper and Flory *et al.*, 1974). If the enzyme which was incubated with inhibitors was rapidly mixed with substrates and $20 \mu\text{M}$ -fructose 1,6-diphosphate as described in the legend to Fig. 1, 90% of the maximum velocity of the reaction progress curve was attained after a time designated $t'_{0.90}$, whose values are shown in Table 1. With $0.18 \mu\text{g}$ of enzyme/ml the maximum-velocity region of the progress curve corresponds in specific activity and extent of reaction ($60 \mu\text{M}$) to those of the fully activated enzyme achieved by incubation of enzyme and inhibitors with fructose 1,6-diphosphate before

Table 1. Response times for fructose 1,6-diphosphate activation of enzyme

Expt. A, dependence on enzyme concentration, $[E_t]$ where t denotes total enzyme. Expt. B, dependence on 1,6-diphosphate concentration. Lag times, $t'_{0.9}$ are times taken to reach 90% of the maximum velocity value of each curve, whereas $t_{0.9}$ are times taken to reach 90% of full activation of the enzyme ($190 \mu\text{mol}/\text{min}$ per mg of enzyme). Values of $t_{0.9}$ are related to the first-order rate constant, k_{app} , for enzyme activation by $t_{0.9} = 2.30/k_{app}$. s.d. values, σ , given for the $t_{0.9}$ values in Expt. B are based on an assumed precision of 0.75 mm in reading the photographic traces. A single $t_{0.9}$ value equal to that in Expt. B with 0.02 mM-fructose 1,6-diphosphate characterizes all the experiments in Expt. A. Experimental conditions were the same as those described in the legend of Fig. 1, except for enzyme and fructose 1,6-diphosphate concentrations as indicated. Lactate dehydrogenase was maintained as 20 units/unit of pyruvate kinase based on 388 units of pyruvate kinase/mg.

Expt. A with [fructose 1,6-diphosphate] = 0.02 mM

$[E_t]$ ($\mu\text{g}/\text{ml}$)	0.181	17.6	51.2
$t'_{0.9}$ (s)	9.4	2.0	1.6
Maximum velocities ($\mu\text{mol}/\text{min}$ per mg)	190	80	39

Expt. B with $[E_t] = 51.2 \mu\text{g}/\text{ml}$

[Fructose 1,6-diphosphate] (mM)	0	0.02	0.05	0.10	1.0
$t_{0.9}$ (s)	None	1.6	0.44	0.34	0.09
$t_{0.9}$ (s)	—	11	1.5	0.6	0.19
$\sigma(t_{0.9})$ (s)	—	1	0.2	0.1	0.05
Maximum velocities ($\mu\text{mol}/\text{min}$ per mg)	0.61	39	111	164	207

Fig. 1. Stopped-flow measurements illustrating rates of fructose 1,6-diphosphate activation of inhibited enzyme at pH 7.5 and 25°C

(a)–(c), Effects of various enzyme concentrations, $[E_t]$, at 0.02 mM-fructose 1,6-diphosphate; (c)–(f), effects of various fructose 1,6-diphosphate concentrations at $[E_t] = 51.2 \mu\text{g/ml}$. $[E_t]$ was 0.181, 17.6 and $51.2 \mu\text{g/ml}$ for (a), (b) and (c) respectively. Fructose 1,6-diphosphate concentrations were 0.05, 0.10 and 1.0 mM for (d), (e) and (f) respectively. Identical labelled curves above the lowest recorded ones represent continuation of reactions and time. See the Experimental section for method of calculating progress curves from the data given. However, qualitative features of the above transmittance records and calculated progress curves (product concentrations) are very similar, owing to the small non-linearity in the log transmittance function for the changes in recorded values of transmittance. Enzymes were stored in drive syringe 1; inhibitors (MgATP and L-alanine), MgSO_4 and buffer components were equally distributed between syringes, and substrates, fructose 1,6-diphosphate and NADH were stored in drive syringe 2. A minimum of 1.5 min preceded stopped-flow mixing, but reaction velocities were reproducible for longer than 1 h of incubation. Concentrations in reaction mixtures common to all experiments were 20 units of bovine heart lactate dehydrogenase/unit of pyruvate kinase, 1.5 mM-MgADP [except for 3.00 mM-MgADP in experiments of (d)–(f)], 0.15 mM-phosphoenolpyruvate, 1.75 mM-MgATP, 0.5 mM-L-alanine, 0.02 mM-fructose 1,6-diphosphate, and 8 mM-MgSO₄ in 10 mM-phosphate buffer, pH 7.5. Conditions unique to each experiment and not identified above were:

Photo	(a)		(b)		(c)		(d)	(e)		(f)	
Curve	A	B	A	B	B	C		A	B	A	B
V_c (mV/Div.)	10	20	20	20	10	20	20	20	20	20	20
Sweep (s/Div.)	5	5	0.2	0.2	0.2	0.5	0.2	0.1	0.1	0.1	0.1
Bias (mV)	355	355	500	510	260	290	260	260	270	260	270
[NADH] (μM)	244	244	131	131	500	500	500	500	500	500	500

where V_c , Sweep, Bias and Div. are voltage sensitivity of ordinate scale, sweep rate on abscissa scale, potential at zero cm deflexion and scale division respectively. Additional experiments in both Coleman and stopped-flow spectrophotometers showed that NADH concentrations, within the above range gave the same pyruvate kinase activities. NADH concentrations were varied in the above data to demonstrate this and permit adjustment of initial absorbance values to an optimum range (0.2–0.7) for the different path-length cells (stopped-flow and Coleman cells of 0.234 and 1.0 cm respectively). The sharp increase in slope of the uppermost trace in (b) resulted from a change in oscilloscope sweep rate at that point. Trace C in (c) starts late on the time-scale owing to an inadvertent start (trigger) of the oscilloscope sweep before mixing of the reactants.

starting the reaction with substrates. With the higher enzyme concentrations (17.6 and $51.2 \mu\text{g/ml}$, not incubated with fructose 1,6-diphosphate), the apparent constant-velocity regions are maintained for as long or longer in the extent of the reaction as with the lowest concentration of enzyme, but with smaller

specific activities (Table 1 and Fig. 2). Incubation of enzyme with $20 \mu\text{M}$ -fructose 1,6-diphosphate abolishes these transients and gives full enzyme activation to about $190 \mu\text{mol/min}$ per mg of enzyme for all enzyme concentrations (Table 2 and Flory *et al.*, 1974). Enzyme at the high concentration shown

Fig. 2. Enzyme activation by 0.02mM-fructose 1,6-diphosphate with progress curves at different enzyme concentrations $[E_t]$ and their relation to reaction velocities, v

Time axis is normalized for enzyme concentration such that progress curves would normally superimpose (see the text). Data are from Fig. 1, photos (a)–(c). Experimental (symbols) and calculated (—) product concentrations (μM) are with enzyme concentrations in $\mu\text{g/ml}$ of: $\circ$, 0.181; $\triangle$, 17.6; $\square$, 51.2. $\bullet$, $\blacktriangle$ and $\blacksquare$ denote data considered to be outside the model and these were omitted from least-squares analysis by eqn. (2). ---, illustrates a portion of the normalized reaction-velocity curve ($v/[E_t]$) for 17.6 μg of enzyme/ml. Velocity curves at other enzyme concentrations are related to their progress curves in a qualitatively similar manner. The smooth curves were calculated from eqns. (1) and (2) by using best-fit constants $K_a = 0.133 \text{ mM}$ and $k_{app} = 0.241 \text{ s}^{-1}$. Lifetimes for activation of the fraction α of the total enzyme are indicated by arrows on the progress curves and calculated as $t_a = -\ln(1-\alpha)/k_{app}$.

Table 2. Approximate lag times for 90% of maximum enzymic response to the addition of substrates and MgATP

Stopped-flow experiments, $[A] + [B]$, represent mixing solutions A against solution B. All solutions are in the buffer described in the legend of Fig. 1 with 8mM-MgSO₄. Although response times are characterized with concentrated enzyme, they represent full transients in contrast with transients in the fructose 1,6-diphosphate activation of concentrated enzyme (see the text).

Response tested to	Approx. $t_{0.90}$ (s)	Conditions of $[A] + [B]$
MgADP	<0.02	(34 μg of pyruvate kinase/ml, 1320 units of lactate dehydrogenase/ml, 0.6mM-phosphoenolpyruvate, 1mM-fructose 1,6-diphosphate)+(0.6mM-phosphoenolpyruvate, 3.0mM-MgADP, 0.83mM-NADH)
Phosphoenolpyruvate	0.4–0.5	(114 μg of pyruvate kinase/ml, 884 units of lactate dehydrogenase/ml)+(0.6–1.2mM-phosphoenolpyruvate, 3.0mM-MgADP, 0.4mM-NADH)
MgATP+phosphoenolpyruvate	1.5	(114 μg of pyruvate kinase/ml, 884 units of lactate dehydrogenase/ml)+(1.2mM-phosphoenolpyruvate, 3.0mM-MgADP, 7.0mM-MgATP)

in Fig. 2 therefore is in a transient or metastable state relative to the fully activated form.

Initially it is difficult to understand, how lower specific rates can be maintained for as long as they appear to be in the experiments shown in Figs. 1 and 2, without invoking long-lived metastable states, with specific activities which vary with the enzyme concentration. Yet the apparently constant but low velocities observed with a high concentration

of enzyme may be adequately explained, in general, by a single slow enzyme transition with a rate constant and fractional lifetimes that are independent of enzyme concentration. No metastable enzyme forms need be invoked. This follows from the hypothesis that with a sufficiently high concentration of enzyme only a fraction (α) of the total enzyme need be transformed to obtain a catalytic rate that is large relative to a further rate of change in α . Thereafter an

apparently constant catalytic rate may be observed until beyond the initial-velocity range (in product concentration, as defined in the introduction). The 'constant velocity' observed will be lower than that attained with low concentrations of enzyme, or enzyme incubated with fructose 1,6-diphosphate, by the factor $\bar{a}$, i.e. some average extent of enzyme transformation during the period of observation.

These ideas were more quantitatively tested by considering the following scheme of the model reaction:

where an enzyme form E' with essentially no activity is converted by the modifier M into an active form ME with catalytic activity k_c ; A and P represent a substrate and product respectively. In our experiments M is fructose 1,6-diphosphate, E' represents the enzyme inhibited by MgATP and L-alanine and A is phosphoenolpyruvate (the limiting substrate). The inhibitors are not explicitly represented in complex with enzyme since generality of the above scheme is desired and also since it is not known whether these inhibitors are present in the ME complex of pyruvate kinase. An unspecified number of intervening steps in enzyme activation are shown in the scheme, emphasizing our ignorance of the precise mechanism. Nevertheless, with $[\text{M}] \gg [\text{E}]$, (brackets denote molar concentrations and subscript t denotes total enzyme) enzyme activation may, and will be assumed to be first-order in time (t) with a pseudo-first-order rate constant $k_{\text{app.}}$. If transitions among active enzyme intermediates are considerably faster than the activation step, steady states in the former will be achieved giving a time-dependent reaction velocity:

$$v_t = \frac{d[\text{P}]}{dt} = V_m(1 - e^{-k_{\text{app.}}t}) \left(\frac{[\text{A}]}{K_a + [\text{A}]} \right) \quad (1)$$

where V_m and K_a are apparent the maximum velocity ($k_c[\text{E}_t]$) and the apparent Michaelis constant for substrate A respectively. The last term in eqn. (1), $[\text{A}]/(K_a + [\text{A}])$, might suffice for an appreciable fraction of the progress curve of a multisubstrate enzyme if the initial concentration of substrate A , $[\text{A}_0]$, is much less than concentrations of other substrates, as with our experiments. With the substitution $[\text{A}_0] - [\text{A}] = [\text{P}]$, eqn. (1) may be integrated giving the progress curve:

$$[\text{P}] = V_m t + \ln \left(\frac{[\text{A}_0] - [\text{P}]}{[\text{A}_0]} \right) - \frac{V_m}{k_{\text{app.}}} (1 - e^{-k_{\text{app.}}t}) \quad (2)$$

where $\ln$ is the natural logarithm. The first two terms on the right side of eqn. (2) are equivalent to the integral of the Michaelis-Menten equation and the last term accounts for the hysteretic transition. Curves were generated from eqn. (2) for total enzyme concentrations, $[\text{E}_t] = 0.181, 1.76, 17.6$ and $51.2 \mu\text{g/ml}$ with constants known or roughly estimated from our data with $0.181 \mu\text{g}$ of pyruvate kinase/ml and a specific activity, $V_m/[\text{E}_t] = 388 \mu\text{mol/min}$ per mg of enzyme. The calculated curves were very similar to those of our data. In the first simulations, $[\text{A}_0] \approx K_a = 0.15 \text{ mM}$, but essentially the same results are predicted when the enzymic reaction operates within the zeroth-order range of the Michaelis-Menten equation, as demonstrated by simulations with K_a decreased to negligible values relative to $[\text{A}_0]$, and V_m decreased to give approximately the same initial velocities.

Least-squares analysis

Effects of enzyme concentration at a fixed fructose 1,6-diphosphate (0.02 mM) concentration. Eqn. 2 was fitted to data from Fig. 1 [photos (a)–(c)] by using the STEPIT program with V_m fixed at the value obtained under standard-assay conditions with saturating concentrations of substrates and fructose 1,6-diphosphate. Data beyond $70 \mu\text{M}$ product with 0.181 and $17.6 \mu\text{g}$ of enzyme/ml and beyond $40 \mu\text{M}$ with $51.2 \mu\text{g}$ of enzyme/ml were omitted since inhibition becomes apparent beyond this extent of reaction (see below for justification of this). The earlier appearance of inhibition with $51.2 \mu\text{g}$ of enzyme is most likely due to the inadvertent use of 3.00 mM - rather than 1.5 mM - MgADP in this experiment. A good fit to combined data from all three enzyme concentrations was obtained (Fig. 2) where the time axis is normalized for enzyme concentration to permit comparisons as discussed below. Best-fit values were $K_a = 0.133 \text{ mM}$ and $k_{\text{app.}} = 0.241 \text{ s}^{-1}$. Virtually identical results were obtained when data from only 0.181 and $17.6 \mu\text{g}$ of enzyme/ml were analysed to obtain best-fit values and statistics, giving $K_a = 0.133 \pm 0.002 \text{ mM}$ and $k_{\text{app.}} = 0.241 \pm 0.003 \text{ s}^{-1}$. The quality of fit is good in view of the limiting systematic errors. A chi-square probability of 0.5 [confidence level, Hamilton (1964)] is obtained by assuming s.d. values of only $\pm 0.5 \text{ mm}$ of vertical deflexion in the photographic records. Because of systematic errors in the data, however, relative uncertainties in K_a and $k_{\text{app.}}$ values are more likely 10%, rather than the approximate value of 2% estimated from the regression analysis.

Data beyond $70 \mu\text{M}$ product are not accurately predicted by the model of eqns. (1) and (2), which contain only a single-substrate Michaelis-Menten term representing the activated enzyme. A more complex model, however, is neither justified nor needed for

our objective of characterizing the hysteretic transition since data confined to virtually initial substrate concentrations suffices. With K_a fixed at 0.133mM a $k_{app.}$ value of 0.252 ± 0.011 was in fact obtained with the data confined below 15 μ M product ($10^2 \times [A]/[A_0] < 10\%$). Further extents of reaction are shown in Fig. 2 to illustrate the concepts and demonstrate the surprisingly long reaction periods which appear to have constant velocities.

Lifetimes, $t_a = [\ln(1-\alpha)]/k_{app.}$, for activation of the fraction α of the total enzyme are shown on the curves in Fig. 2 with $k_{app.} = 0.24s^{-1}$. With low concentrations of enzyme (0.181 μ g/ml) enzyme activation is 90% complete (at $t_{0.9}$) before 5 μ M of product is produced. With 51 μ g of enzyme/ml, however, the bulk of the 'linear portion' of the catalytic reaction (between 40 and 100 μ M product on the theoretical curve) is achieved when only 30–50% of the enzyme is activated. Consequently the enzymic activity appears to be about 30% of the maximum specific activity of 180 units/mg with $[A_0] \simeq K_a$. Although deviations from the theoretical curve appear earlier with 51 μ g of enzyme/ml for reasons given above, linearity in the progress curve extends to 70 μ M product as with low concentrations of enzyme. The apparent linear portion of the reaction with both high concentrations of enzymes, therefore, is as long or longer in extent of reaction than with low concentrations of enzyme where the hysteretic transition is essentially complete by 5 μ M product. The dashed curve in Fig. 2 shows V_t from eqn. (1) for 17.6 μ g of enzyme/ml (the middle progress curve).

Steady-state kinetic theory requires that enzyme specific activities (slopes in Fig. 2) be independent of enzyme concentration $[E_t]$, thus all experimental curves in Fig. 2 should superimpose unless: (1) the condition, $[E_t] \ll$ concentrations of substrates and ligands, is not satisfied; or (2) slow (hysteretic) transitions of enzyme forms are occurring; or (3) enzyme concentration-dependent association of enzyme occurs producing oligomers with different steady-state kinetic properties. Since condition (1) was satisfied the above analysis shows that the transients and displacement of the curves in Fig. 2, observed when enzyme was not incubated with fructose 1,6-diphosphate, are adequately explained by a single hysteretic transition.

Proof of this superimposition follows from the fact that if $v/[E_t]$ is independent of $[E_t]$ then:

$$[P] = [E_t] \int_0^t \frac{v}{[E_t]} dt = \int_0^{[E_t] \cdot t} \frac{v}{[E_t]} d([E_t] \cdot t) = f([E_t] \cdot t)$$

where v is the steady-state reaction velocity. That is, since the definite integral of a continuous function is

a unique function of the limits of integration, the product concentration $[P]$ is a unique function of the normalized time-variable, $[E_t] \cdot t$.

More complex mechanisms

A more general mechanism for hysteretic transition would allow for appreciable activity of both enzyme forms. This was not necessary with the above data on pyruvate kinase in the presence of 1.75mM-MgATP and 0.5mM-L-alanine where enzymic activity was only 0.3% of that attained after the fructose 1,6-stimulated transition. In the absence of inhibitors and fructose 1,6-diphosphate, however, the enzyme has appreciable activity although fructose 1,6-diphosphate induces a transition to an enzyme form with a lower K_m value for phosphoenolpyruvate (Carminatti *et al.*, 1968).

Although eqns. (1) and (2) are easily adapted for a transition between enzyme forms differing in V_m values only (Frieden, 1971), a transition between enzyme forms differing in K_m values requires a catalytic reaction-velocity equation, derived in the Appendix (see below), whose integral is not easily obtained in terms of known functions unless all substrates remain at saturating concentrations. In this case we recommend analysis of progress curves by a program combining numerical integration with least-squares optimization (Chandler *et al.*, 1972), since the process of taking derivatives of data curves can greatly magnify errors (Magar, 1972). We emphasize that when the first enzyme form has negligible activity, eqns. (1) and (2) result whether the activation involves a transition in either K_m or V_m values.

Even though the relative activity of inhibited pyruvate kinase was only 0.3% of that achieved after the addition of fructose 1,6-diphosphate, the activity of the inhibited enzyme might contribute significantly during the early portions of the progress curves with high concentrations of enzymes when it is the predominant enzyme form. This possibility was excluded, however, by fitting eqn. (3) in the Appendix (see below) to our data, as well as by direct calculation of the product concentrations resulting from the inhibited enzyme form during the reactions.

Parameters expressing co-operativity in K_m values might be needed in some cases, though they do not appear necessary with our data. When a Hill coefficient n , was added to eqn. (1), its value was decreased from a starting estimate of 2.0 to a best-fit value of 1.03 and $k_{app.}$ was changed by less than 0.5%.

Dependence of activation times on fructose 1,6-diphosphate concentration

Data with 51.2 μ g of enzyme/ml [Fig. 1, photos

(d)–(f)] were analysed by eqn. (2) to obtain best-fit values of $k_{app.}$ at each fructose 1,6-diphosphate concentration. Results given in Table 1 (Expt. B), however, are too few and imprecise to deduce a rate law and further details of enzyme activation. However, it is clear that rates of enzyme activation depend strongly on fructose 1,6-diphosphate concentration far beyond $20\mu\text{M}$ where the equilibrium extent of activation is essentially complete.

Apparent transient times for enzymic response to rapid addition of substrates or MgATP

Less transient-state data are available to characterize enzymic response time to ligands other than fructose 1,6-diphosphate. Table 2 shows estimated lag times for the conditions tested. We have not attempted to measure inhibition transients with L-alanine. Transient times after the addition of MgADP to enzyme and phosphoenolpyruvate were too fast relative to the catalytic reaction to be observed in the progress curves of the latter at the enzyme concentrations used. Transient times after the rapid addition of phosphoenolpyruvate and MgATP to high concentrations of enzyme without inhibitors are probably close to the times needed to achieve full activation or inhibition, since the same maximum velocities were obtained with lower concentrations of enzyme.

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APPENDIX

Reaction-velocity equation for a hysteretic transition between enzyme forms with different K_m values, both forms having appreciable activity

A model allowing both enzyme forms, E' and complex ME to have catalytic activity may be obtained by the addition of catalytic steps to reactant enzyme E' in the scheme shown in the Results and Discussion section. The catalytic rate constants are k_{c1} and k_{c2} and the Michaelis constants are K_{a1} and K_{a2} for the enzyme complex ME and enzyme E' respectively with substrate A. By assuming (1) steady states in all enzyme transformations except the hysteretic transition, and (2) that the rate of the hysteretic transition is the same for all the enzyme forms, the steady-state reaction velocity, v , can be deduced from:—

$$v = k_{c1}[E'A] + k_{c2}[MEA]$$

$$= \frac{k_{c1}[E']_t[A]}{K_{a1} + [A]} + \frac{k_{c2}[ME]_t[A]}{K_{a2} + [A]}$$

giving:

$$v = \left(k_{c1}[E_T]e^{-k't} \cdot \frac{[A]}{K_{a1} + [A]} \right) + \left\{ k_{c2}[E_T](1 - e^{-k't}) \cdot \frac{[A]}{K_{a2} + [A]} \right\} \quad (3)$$

where $k' = k_{app.}$, t is reaction time, subscript t denotes the total concentrations of either E' or ME enzyme forms, and subscript T means the sum of both forms. If the velocities examined are confined to negligible changes in $[A]/[A_0]$, eqn. (3) is equivalent to that derived by Frieden (1971).