BBA 66086

MECHANISM OF FRUCTOSE DIPHOSPHATE ACTIVATION OF A MUTANT PYRUVATE KINASE FROM HUMAN RED CELLS

G. F. MUNRO AND D. R. MILLER

Department of Pediatrics, University of Rochester, School of Medicine and Dentistry, Rochester, N.Y. 14620 (U.S.A.)

(Received December 22nd, 1969)

SUMMARY

1. Normal human erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) and mutant pyruvate kinase from a propositus with congenital, nonspherocytic, hemolytic anemia were isolated by DEAE-cellulose extraction and $(NH_4)_2SO_4$ precipitation.

2. Fru-1,6-P_2 lowered the apparent Michaelis constant for phosphoenolpyruvate of the normal enzyme but did not substantially change the maximal velocity. This activator greatly elevated both the apparent Michaelis constant for phosphoenolpyruvate and the maximal velocity of the mutant enzyme.

3. Fru-1,6-P_2 did not affect the Michaelis constant for ADP or the optimal Mg^{2+} concentration in either the normal or the mutant enzyme.

4. Fru-1,6-P_2 altered the extremely sensitive interaction of the binding sites for the two substrates on the mutant enzyme.

5. The sensitivity of the normal enzyme to Fru-1,6-P_2 could be increased by either dilution or heating. With heating, dilute solutions of both normal and mutant enzymes decayed in two steps, indicating the presence of multiple conformations in both enzymes.

6. A molecular model is proposed for Fru-1,6-P_2 activation; Fru-1,6-P_2 acts directly on the phosphoenolpyruvate binding site; interaction of the two substrate sites then alters the conformation of the binding site for ADP.

INTRODUCTION

Erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) deficiencies are a well known cause of hereditary hemolytic anemia. They comprise a heterogeneous group of disorders characterized by both quantitative and qualitative enzyme abnormalities. The mutants described include enzymes with (1) decreased activity and normal apparent K_m (phosphoenolpyruvate (PEP))[1,2], (2) decreased activity and decreased apparent K_m (PEP)[3–6], and (3) slightly decreased or normal

Abbreviation: PEP, phosphoenolpyruvic acid.

activity and increased apparent K_m (PEP)[7,8]. In crude hemolysates PAGLIA *et al.*[7] identified a kinetically aberrant pyruvate kinase with an apparent K_m (PEP) 10 fold greater than normal, normal kinetics for ADP, pH optimum 1 unit lower than the normal enzyme, and markedly decreased stability on storage. Family studies revealed paternal heterozygosity for quantitative pyruvate kinase deficiency of the usual type (approx. 50% activity with normal apparent K_m (PEP)). The clinically unaffected mother had normal pyruvate kinase activity but an intermediate apparent K_m (PEP). Disease in the propositi appeared to require the simultaneous inheritance of a maternal gene for a kinetically aberrant pyruvate kinase and a paternal gene resulting in quantitative enzyme deficiency. This paper describes kinetic and stability studies of partially purified pyruvate kinase from normal erythrocytes, from a previously described patient (Propositus I)[7] with a kinetically aberrant enzyme, and from his parents.

MATERIALS AND METHODS

Chemicals

ADP (from equine muscle), PEP (tricylclohexylamine salt), AMP (sodium salt), Fru-1,6-P_2 (tetrasodium salt), ATP (disodium salt from equine muscle), 2,3-P_2-glycerate (pentacyclohexylammonium salt), dihydroxyacetone-*P* (dimethylketal dimonocyclohexylamine salt), NADH (disodium salt from yeast), and lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27; Type II from rabbit muscle) were obtained from the Sigma Chemical Co. Lactate dehydrogenase (A Grade from rabbit muscle), and dithiothreitol (A Grade) came from Calbiochem. Bio-Rad Laboratories supplied the DEAE-cellulose (Cellex-D; exchange capacity, 0.76 mequiv/g).

Purification procedure for human red cell pyruvate kinase

(1) Approx. 50 ml of blood were collected in heparinized tubes and processed within 8 h of collection. All subsequent operations were done at 4°. After centrifugation at 2000 × *g* for 10 min (International PR-2 centrifuge: head no. 253, 2800 rev./min), the plasma and buffy coat were drawn off. The cells were then 3 times suspended in approx. 50 ml of 0.85% NaCl and spun at 2000 × *g* for 10 min. After the third wash white cells always represented less than 0.02% of the total cells present. The cell pellet was then suspended in 1.0–1.5 vol. of cold, deionized, distilled water and freeze-thawed 2 times in a solution of dry ice and acetone. Aliquots of this hemolysate were stored at −20°.

(2) Most of the red cell enzymes, including pyruvate kinase, were adsorbed onto DEAE-cellulose by batch extractions[9], and the hemoglobin was removed by washing the DEAE-cellulose 6 times in 3 mM potassium phosphate buffer (pH 7.00). Pyruvate kinase was then removed from the DEAE-cellulose by three extractions in 0.125 M KCl[10]. Subsequent extractions in 0.400 M KCl did not result in any additional recovery of either normal or mutant enzyme.

(3) Pyruvate kinase was next precipitated from the pooled KCl extracts by adding enough cold saturated $(NH_4)_2SO_4$ solution to bring the extracts to 25% saturation[11]. The majority of the protein precipitated at less than 10% saturation. The solutions were kept at 4° for 2 h and spun at 10 000 × *g* for 30 min (International PR-2 centrifuge with multispeed attachment: head No. 296, 2520 rev./min). The supernatant, containing pyruvate kinase was brought to 40% saturation with satu-

rated $(NH_4)_2SO_4$ solution, allowed to precipitate in the cold for 2 h, and again spun at 10 000 $\times g$ for 30 min. The supernatant contained less than 5% of the amount of pyruvate kinase in the precipitate, which was dissolved in less than 10 ml of 0.125 M KCl–0.01 M potassium phosphate–7 mM $MgSO_4$– and 8 mM triethanolamine buffer (pH 6.98) (KCl–potassium phosphate–$MgSO_4$–triethanolamine buffer[10]). This final solution was stored at −20° and used within 5 days. In this solution purified pyruvate kinase has a half-life of more than 1 week.

Assay of pyruvate kinase activity

Pyruvate kinase was assayed at 37° by a modification of the method of BÜCHER AND PFLEIDERER[12], detailed in other reports[13,14]. The standard assay solution (3.0 ml) contained 8.3 mM triethanolamine · HCl (pH 7.50), 75 mM KCl, 8.0 mM $MgSO_4$, 0.4 mM ADP, 1000 Bucher units of lactate dehydrogenase, 0.2 mM NADH, pyruvate kinase, and 2.0 mM PEP. Alterations in the concentration of $MgSO_4$, ADP, and PEP for particular experiments are described in the legends to the tables and figures. Reactions were followed in a Beckman DU spectrophotometer equipped with a Gilford model 2000 multiple sample absorbance recorder and a Haake constant temperature circulator. Units of pyruvate kinase activity are expressed as μmoles NADH converted/min, using the extinction coefficient at 340 mμ, ε (NADH)$-\varepsilon$ (NAD^+) $= 6.3 \cdot 10^{-3}$ M^{-1} (ref. 15). All experiments were controlled for activity in the absence of added pyruvate kinase with inhibitors or activators present where appropriate; assays were also run with twice the normal activity of lactate dehydrogenase to assure that this enzyme was not rate-limiting. Protein was estimated as: protein concentration (mg/ml) = $(1.55 \times A_{280\ m\mu}) - (0.76 \times A_{260\ m\mu})$[16].

RESULTS

Isolation of pyruvate kinase from normal, propositus, and parental red cells

Table I shows specific activities and relative purifications for pyruvate kinase isolated from red cells of a normal person, the propositus, and his mother and father. Without Fru-1,6-P_2 present most crude hemolysates had a specific activity of 2.5–4.5 units/g of protein. DEAE-cellulose adsorption and $(NH_4)_2SO_4$ precipitation generally resulted in an approx. 500-fold purification of normal pyruvate kinase. DEAE-cellulose extraction produced purification of enzyme from the propositus and his parents either in the absence or presence of Fru-1,6-P_2. In the absence of Fru-1,6-P_2, $(NH_4)_2SO_4$ precipitation does not appear to increase the specific activity of the enzyme from the propositus; the enzyme appears to be purified only 35-fold. Pyruvate kinase from the parents was purified to an intermediate extent. Overall yields from normal red cells and from the parents' red cells ranged from 8.8 to 12.1%. Overall yield from the propositus was only 2.4% in the absence of Fru-1,6-P_2, and 8.9% in the presence of Fru-1,6-P_2.

Table I also demonstrates the effect of Fru-1,6-P_2 on the enzyme activities at different stages of purification. DEAE-cellulose adsorption and subsequent extraction with KCl did not sensitize the propositus enzyme to Fru-1,6-P_2; in contrast, after $(NH_4)_2SO_4$ precipitation, the enzyme was extremely sensitive to Fru-1,6-P_2. With Fru-1,6-P_2 present in the assay solution, $(NH_4)_2SO_4$ did effect a purification of the propositus enzyme and elevated its activity to the normal range. The mother's purified

TABLE I

SPECIFIC ACTIVITIES OF NORMAL, MUTANT, AND PARENTAL PYRUVATE KINASE

Assayed in the presence or absence of 1 mM Fru-1,6-P_2. All assays were run at 1.5 mM PEP. Other reactants were in the concentrations of the standard assay.

Source of enzyme	*Stage of purification*	*Specific activity (units/g protein)*		*Activation by Fru-1,6-P_2*
		Assayed without Fru-1,6-P_2	*Assayed with Fru-1,6-P_2*	
Normal	Crude hemolysate	4.31	4.69	1.09
	KCl extract	956.	1150.	1.20
	$(NH_4)_2SO_4$ ppt.	1960.	2310.	1.18
Propositus	Crude hemolysate	3.97	8.51	2.14
	KCl extract	125.	245.	1.96
	$(NH_4)_2SO_4$ ppt.	132.	1060.	8.03*
Mother	Crude hemolysate	2.48	6.06	2.44
	KCl extract	825.	1740.	2.11
	$(NH_4)_2SO_4$ ppt.	727.	2230.	3.07
Father	Crude hemolysate	6.08	7.25	1.19
	KCl extract	1350.	1590.	1.18
	$(NH_4)_2SO_4$ ppt.	1400.	2180.	1.56

* The extent of activation varied from 8- to 15-fold on three preparations. The sensitivity to Fru-1,6-P_2 was unstable and had a half life of about a week. This instability did not change the shape of the curves in the experiments shown later.

pyruvate kinase (carrier of the mutant gene) showed intermediate levels of Fru-1,6-P_2 activation. The father's enzyme (classical pyruvate kinase deficiency) and the normal enzyme were not sensitized to Fru-1,6-P_2.

Kinetics of Fru-1,6-P_2 activation of normal and mutant pyruvate kinase

Fig. 1a shows that, in the absence of Fru-1,6-P_2, the purified normal enzyme had a sigmoid saturation curve with increasing concentrations of PEP. The presence of Fru-1,6-P_2 converted this sigmoid curve to an hyperbolic curve, slightly raised v_{max}, and lowered the apparent K_m of the enzyme for PEP by almost 5-fold (*cf.* Table II). The mutant enzyme (Fig. 1b) also had a sigmoid curve in the absence of Fru-1,6-P_2; Fru-1,6-P_2 produced an hyperbolic curve, but v_{max} in this case was greatly elevated and the apparent K_m (PEP) was raised almost 4-fold. The fact that Fru-1,6-P_2 seems to raise the specific activity of the purified mutant enzyme to normal values (Table I) might suggest that Fru-1,6-P_2 causes the mutant to revert to the normal conformation. This is not true because the saturation curve for PEP is still distinctly abnormal.

Fig. 1c indicates that the mother's enzyme was kinetically normal in the absence of Fru-1,6-P_2. The curve with Fru-1,6-P_2 present had a normal configuration at low PEP concentrations but rose markedly at the highest concentration of PEP. Thus, it appears to be the sum of the curves for normal and mutant enzyme. The father's pyruvate kinase (Fig. 1d) exhibited normal kinetics. This indicates that patients with classical pyruvate kinase deficiency have low quantities of normal enzyme.

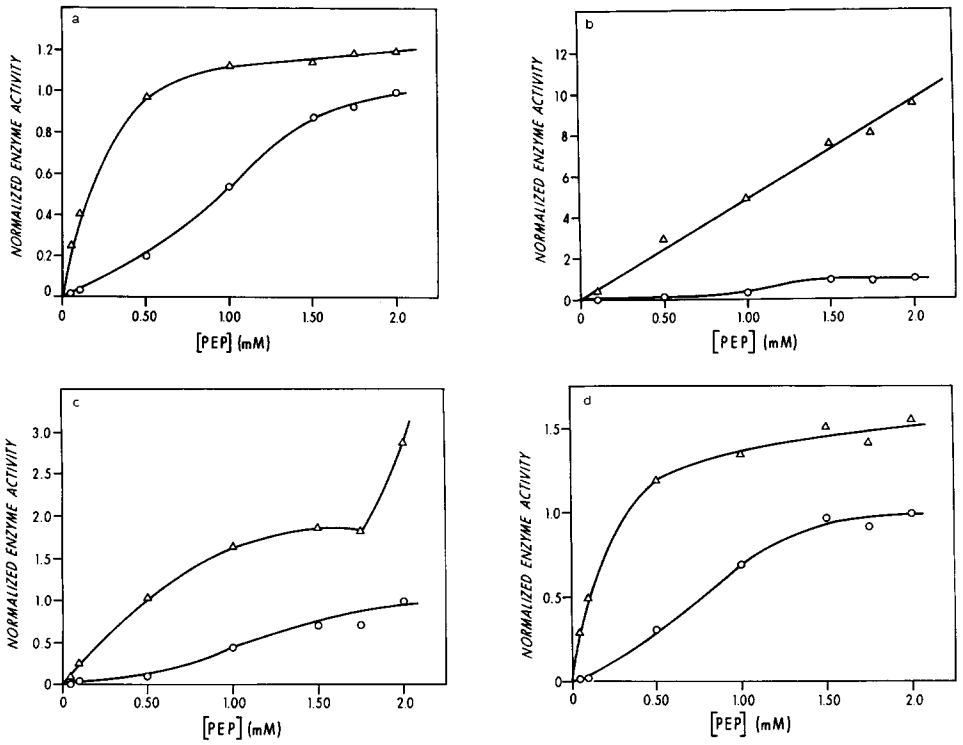


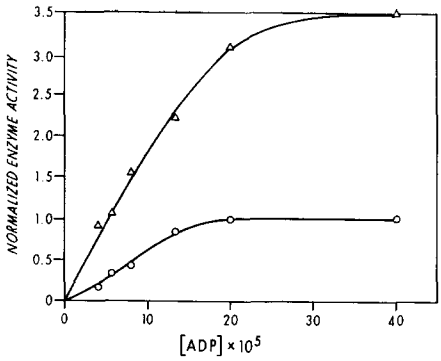
Fig. 1. Plot of the effect of increasing concentrations of PEP on the activity of purified pyruvate kinase from normal (a), propositus (b), maternal (c), and paternal (d) red cells, assayed with (△—△) and without (○—○) 1 mM Fru-1,6-P_2. In each graph all values are normalized to the activity at 2.0 mM PEP in the absence of Fru-1,6-P_2. All other reagents were present at the concentrations of the standard assay. At 2.0 mM PEP, half maximal activation of normal enzyme occurred at 0.8–0.9 mM Fru-1,6-P_2; in the mutant, half-maximal activation occurred at 0.68 μM Fru-1,6-P_2.

TABLE II

APPARENT MICHAELIS CONSTANTS FOR PEP CURVES SHOWN IN FIG. 1

Source of enzyme	*Apparent K_m (PEP) (mM)*	
	In the absence of Fru-1,6-P_2	*In the presence of 1 mM Fru-1,6-P_2*
Normal	0.96	0.21
Propositus	1.13	4.17*
Mother	1.08	0.81**
Father	0.76	0.20

* Calculated from a linear, double reciprocal plot.
** Calculated using the value at 2.0 mM PEP as v_{max}.



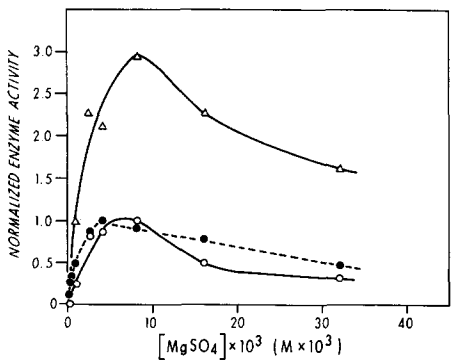


Fig. 2. Plot of the effect of increasing concentrations of ADP on the activity of purified pyruvate kinase from the propositus, assayed with (△—△) and without (○—○) 1 mM Fru-1,6-P_2. All values are normalized to the activity at 0.40 mM ADP in the absence of Fru-1,6-P_2. All other reagents were present at the concentrations of the standard assay. In this particular experiment Fru-1,6-P_2 produced only a maximum of 3.5-fold activation; other experiments with 10-fold activation produced a similarly shaped curve and similar values for the apparent K_m(ADP).

Fig. 3. Plot of the effect of increasing concentrations of $MgSO_4$ on the activity of purified pyruvate kinase from the propositus, assayed with (△—△) and without (○—○) 1 mM Fru-1,6-P_2. All values for the propositus enzyme are normalized to the activity at 8.2 mM $MgSO_4$ in the absence of Fru-1,6-P_2. All other reagents were present at the concentrations of the standard assay. For comparison a curve for the normal enzyme (●— — —●) is included. This curve was unchanged by addition of Fru-1,6-P_2.

Similar kinetic studies were done with ADP (Fig. 2). The normal enzyme had an apparent K_m (ADP) of 104 μM which was unaltered by the presence of Fru-1,6-P_2. The apparent K_m (ADP) of the mutant enzyme (86 μM) was not substantially altered by addition of Fru-1,6-P_2 (apparent K_m (ADP) = 96 μM). Thus, Fru-1,6-P_2 has a specific effect on the mutant enzyme; it alters the enzyme affinity for PEP without changing the affinity for ADP.

Fig. 3 shows the effects of varying the concentration of $MgSO_4$ on the activity of normal and mutant pyruvate kinase. The normal enzyme was maximally active at 4.2 mM $MgSO_4$, but maximal activity for the mutant occurred at 8.2 mM $MgSO_4$, nearly double the normal value. For the mutant enzyme Fru-1,6-P_2 did not change the concentration of maximal activity but did reverse the inhibition of activity at relatively high concentrations of $MgSO_4$.

Studies were done to determine if ATP were inhibitory to the enzyme. Addition of neutralized 1 mM ATP (slightly lower than physiologic concentration) produced a 58% inhibition of the normal enzyme and inhibited the mutant enzyme by 90%. The elevated apparent K_m (Mg^{2+}) for the mutant enzyme is consistent with the augmented inhibition of the mutant enzyme by ATP. Formation of $MgATP^{2-}$ complexes[17,18] would deprive both normal and mutant enzymes of Mg^{2+}. Since the mutant requires a higher concentration of Mg^{2+} for maximal activity, it should be inhibited to a greater extent by ATP than the normal enzyme. Furthermore, the ATP content of red cells from the propositus is normal, whereas in classical pyruvate kinase deficiency, ATP is decreased[7]. An alternate explanation is that the inhibition could be caused by direct binding of ATP at either the site for ADP or PEP[17,18].

Other potential inhibitors and activators of pyruvate kinase were evaluated.

When assayed with 1.5 mM PEP, 1 mM dihydroxyacetone-*P* did not have any effect on production of NAD^+. This demonstrates that glycerophosphate dehydrogenase (L-glycerol-3-phosphate:NAD oxidoreductase, EC 1.1.1.8) was not responsible for the enhanced production of NAD^+ with Fru-1,6-P_2 present. Also, glycerophosphate dehydrogenase has never been demonstrated in mature red cells[19].

The possible activating role of 2,3-P_2-glycerate was also examined. This glycolytic intermediate was selected because of its known high concentration in normal erythrocytes and because of its markedly increased concentration in pyruvate kinase-deficient erythrocytes[20]. However, when assayed with 1.5 mM PEP, 1 mM 2,3-P_2-glycerate failed to activate the mutant enzyme. Physiologic concentration of 2,3-P_2-glycerate is about 4 mM (D. R. MILLER, unpublished observations).

When assayed with either 0.1 or 1.5 mM PEP, 0.5 mM AMP did not alter the enzyme activity. Thus, in contrast to the pyruvate kinase of *Escherichia coli* (ref. 21), AMP does not activate pyruvate kinase from human red cells.

To estimate the effects of sulfhydryl-reducing agents, mutant pyruvate kinase was incubated at 37° for 2 h with 2 mM dithiothreitol in KCl–potassium phosphate–$MgSO_4$–triethanolamine buffer. The resulting solution had the same activity as another aliquot of enzyme which had been incubated at the same enzyme concentration and under the same conditions in KCl–potassium phosphate–$MgSO_4$–triethanolamine buffer without dithiothreitol. Thus, reversible disulfide bond formation and cleavage are not important to the activity of fresh preparations of enzyme. Reducing agents are, however, important for the long-term storage of pyruvate kinase[22].

Interactions of the ADP and PEP sites on mutant pyruvate kinase

CAMPOS *et al.*[2] first demonstrated that, in purified normal pyruvate kinase from red cells, the apparent K_m(ADP) was a dependent variable of the PEP concentration. They also showed that the apparent K_m(PEP) was dependent on the ADP concentration. We confirmed this fact with our purified preparation of normal enzyme.

Fig. 4 illustrates that at high concentrations of ADP and PEP, a similar substrate dependence is present in the purified mutant preparation. However, at concentrations of ADP less than 80 μM or at PEP concentrations less than 2 mM, mutant enzyme activity falls off markedly with decreasing ADP or PEP concentrations. Both substrates must be present in low concentrations to achieve this rapid loss of activity. Thus, a low concentration of ADP (or PEP) is a necessary but not sufficient cause for rapid loss of mutant enzyme activity with decreasing PEP (or ADP) concentration. These results may be interpreted to mean that, in the mutant enzyme, the ADP and PEP sites show much more cooperative interaction than in the normal enzyme.

The same experiments were repeated in the presence of 1 mM Fru-1,6-P_2. In this case all curves were linear, even at the lowest concentrations of ADP and PEP. The Michaelis constants for each substrate were still dependent variables of the concentration of the other substrate.

Effects of dilution and heating on normal and mutant enzymes

4-fold dilution of the normal enzyme (Fig. 5a) caused a 50% reduction in activity; this activity was regained by the addition of Fru-1,6-P_2. Thus, the degree of Fru-1,6-P_2 activation varies with the enzyme concentration. In the mutant enzyme (Fig. 5b), dilution caused a 65% loss of activity; however, Fru-1,6-P_2 augmented the

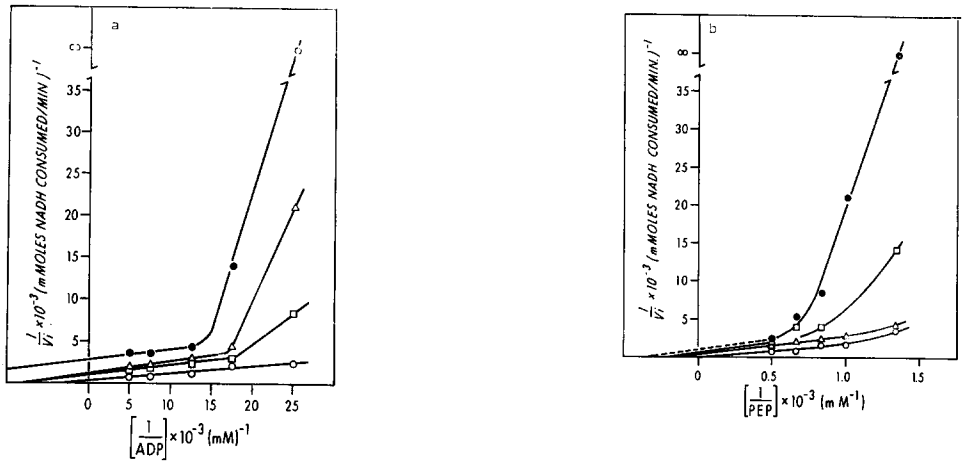


Fig. 4. Double reciprocal plots of enzymatic activity *vs.* concentration of ADP (a) and PEP (b) for the mutant enzyme. In a, concentrations of PEP were 0.75 (●—●), 1.0 (△—△), 1.2 (□—□), and 2.0 mM (○—○). In b, concentrations of ADP were 40 (●—●), 57 (□—□), 80 (△—△), and 200 μM (○—○) .All other reagents were present at the concentrations of the standard assay.

activities of dilute and concentrated solutions to the same extent.

During the first hour, heating the concentrated solution of normal enzyme (Fig. 5a) enhanced the sensitivity to Fru-1,6-P_2. Later, heating did not further augment Fru-1,6-P_2 sensitivity, *i.e.* the ratio of activity with Fru-1,6-P_2 to that without Fru-1,6-P_2 had a constant value of about 1.6. Heating the dilute normal enzyme did

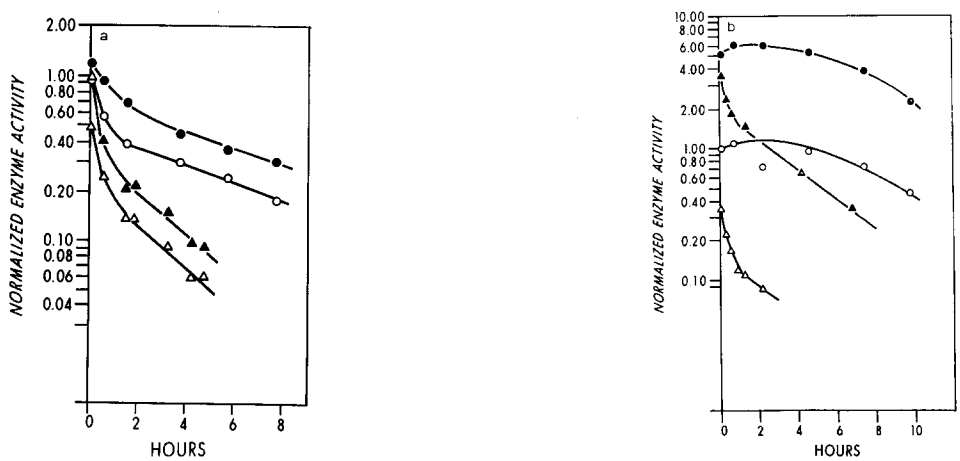


Fig. 5. Plot of the logarithm of the enzyme activity *vs.* duration of incubation at 37° for purified normal enzyme (a) and mutant enzyme (b). In each graph concentrated solutions (0.227 mg protein/ml) were assayed in the absence (○—○) or presence (●—●) of 1 mM Fru-1,6-P_2. Dilute solutions (0.0568 mg protein/ml) were prepared from the concentrated solutions and assayed in the absence (△—△) or presence (▲—▲) of 1 mM Fru-1,6-P_2. All values on each graph are normalized to the value of the concentrated, unheated enzyme assayed in the absence of Fru-1,6-P_2. Activities were measured in the standard assay mixture, containing Fru-1,6-P_2 where appropriate. Equal quantities of protein were present in the cuvettes for all assays.

not at any time change the sensitivity to Fru-1,6-P_2. Fig. 5a also demonstrates that both concentrated and dilute solutions decay in two steps, with the final decay rate being dependent on the protein concentration.

Heating had a different effect on the concentrated mutant enzyme (Fig. 5b). Over the first 2 h heating increased activity. This may represent solubilization of suspended mutant enzyme[23]. This increase in activity through solubilization may obscure a rapid initial decline in activity of mutant enzyme already in solution. The dilute solution (presumably containing only soluble enzyme) decays in two steps and thus resembles the decay of the dilute normal enzyme. In very dilute solutions (not shown) both normal and mutant enzymes decay to zero activity entirely as the rapid component; in concentrated solutions the normal enzyme decays with either a small amount of the rapid component or none at all. These results are considered in the following discussion.

DISCUSSION

Pyruvate kinase from liver[24–26], adipose tissue[27], and human red cells[28] is activated by Fru-1,6-P_2. Most saturation curves for PEP are similar to our results for the purified normal pyruvate kinase (Fig. 1a). The pyruvate kinase from adipose tissue exists in two interconvertible forms, PK-A, which has a sigmoid saturation curve for PEP and is activated by Fru-1,6-P_2, and PK-B, which is insensitive to Fru-1,6-P_2 and exhibits normal Michaelis kinetics for PEP[27]. CARTIER *et al.*[28] found Fru-1,6-P_2-insensitive pyruvate kinase in some preparations of red cells and Fru-1,6-P_2-sensitive pyruvate kinase in others. They claimed that heating PK-A at 25° for 2 h converted the enzyme to PK-B. Their method of purification, which was similar to ours, yielded a concentrate of pyruvate kinase which lacked phosphopyruvate hydratase, fructose diphosphatase, aldolase, and NADH oxidase activities.

All of our preparations of pyruvate kinase were sensitive to Fru-1,6-P_2. Heating or dilution of the concentrated normal enzyme even further increased the sensitivity to Fru-1,6-P_2. Thus, our results are in disagreement with those of CARTIER *et al.*[28]. The curves for normal enzyme show an initial rapid decay phase followed by a slower, terminal phase. In the dilute preparations the degree of Fru-1,6-P_2 activation is almost constant at all times. This must mean that the two-step decay is not related to Fru-1,6-P_2 activation. Further, the two-step decay is consistent with the existence of two enzyme conformations. The fact that the terminal decay of the dilute enzyme is more rapid than the decay of the concentrated enzyme may mean that the terminal conformations of the enzyme in dilute and concentrated solutions are different.

The other experiments reported here provide information about the mechanism of Fru-1,6-P_2 activation. Our original description of this mutant enzyme, done with crude hemolysates, demonstrated that the mutant pyruvate kinase had an apparent K_m (PEP) 10-fold higher than normal[7]. In similar studies, BOIVIN *et al.*[29] later described another mutant which has an elevated apparent K_m (PEP). In crude hemolysates, this mutant was not sensitive to Fru-1,6-P_2, but addition of Fru-1,6-P_2 to a mutant preparation, purified by DEAE-cellulose extraction and $(NH_4)_2SO_4$ precipitation, produced an elevation of v_{max} to normal levels and a reduction of the apparent K_m (PEP) to the range of normal.

Our mutant behaves differently. Purified preparations have the same sigmoid

shape and apparent K_m(PEP) as the purified normal enzyme (Figs. 1a and 1b and Table II). When assayed in the presence of Fru-1,6-P_2, our mutant had normal activity (Table I); however, Fru-1,6-P_2 produced a bizarre saturation curve for PEP (Fig. 1a and Table II). Both v_{max} and the apparent K_m (PEP) were greatly elevated. This activator elevated the v_{max} for ADP but did not change the apparent K_m (ADP) (Fig. 2). Also, Fru-1,6-P_2 effected the ADP–PEP interaction (Figs. 4a and 4b) and could replace either PEP or ADP in maintaining high rates of catalysis. This activator did not affect the Mg^{2+} concentration producing maximal activity (Fig. 3) or the heat denaturation curves (Fig. 5b).

These data indicate that the Fru-1,6-P_2 effect on the mutant is specific. Since Fru-1,6-P_2 principally alters the kinetics for PEP, it is reasonable that Fru-1,6-P_2 binds specifically at the PEP site. This activator may be lost during $(NH_4)_2SO_4$ precipitation with subsequent collapse of some sites for PEP and loss of enzymatic activity (Table I). This loss of cofactor would be similar to the loss of tightly bound $NADP^+$ from glucose-6-phosphate dehydrogenase after $(NH_4)_2SO_4$ precipitation[30]. Addition of exogenous Fru-1,6-P_2 may reopen collapsed sites, allow binding of PEP, and elevate v_{max}. Because Fru-1,6-P_2 also elevates the apparent K_m (PEP), the reopened sites probably do not achieve a normal configuration. The fact that the PEP site influences the ADP site (Figs. 4a and 4b; ref. 2) may allow Fru-1,6-P_2 bound near the PEP site to alter the conformation of the ADP site indirectly. Thus, binding of Fru-1,6-P_2 near the PEP site would explain the alterations in the affinities of both PEP and ADP for the mutant enzyme. This same arrangement of sites should also be present in the normal enzyme.

These studies define new parameters which are required to characterize the heterogeneous group of pyruvate kinase hemolytic anemias. In addition to the apparent Michaelis constants for ADP and PEP, these parameters include the effects of heating and dilution, Fru-1,6-P_2 and other activators, ATP and other inhibitors, and the Mg^{2+} concentration.

ACKNOWLEDGMENTS

G.F.M. is a Medical Science Fellow of the Life Insurance Medical Research Fund. We wish to acknowledge Dr. E. H. Stotz, Chairman of the Department of Biochemistry, University of Rochester, for useful discussions during the course of these experiments. This work was supported by U.S. Public Health Service Grant AM-13947-01.

REFERENCES

1 U. Wiesmann and O. Tönz, *Nature*, 209 (1966) 612.
2 J. O. Campos, R. D. Koler and R. H. Bigley, *Nature*, 208 (1965) 194.
3 P. Boivin and C. Galand, *Rev. Franç. Etudes Clin. Biol.*, 12 (1967) 372.
4 I. A. Rose and J. V. B. Warms, *J. Biol. Chem.*, 241 (1966) 4848.
5 H. K. Hanel and N. J. Brandt, *Lancet*, ii (1968) 113.
6 F. A. Oski and H. Bowman, *Brit. J. Haematol.*, 17 (1969) 289.
7 D. E. Paglia, W. N. Valentine, M. A. Baughan, D. R. Miller, C. F. Reed and O. R. McIntyre, *J. Clin. Invest.*, 47 (1968) 1929.
8 J. R. Sachs, D. J. Wicker, R. O. Gilcher, M. E. Conrad and R. J. Cohen, *J. Lab. Clin. Med.*, 72 (1968) 359.

9 M. A. HENNESSEY, A. M. WALTERSDORPH, F. M. HUENNEKENS AND B. W. GABRIO, *J. Clin Invest.*, 41(1962) 1257.
10 R. D. KOLER, R. H. BIGLEY, R. T. JONES, D. A. RIGGS, P. VANBELLINGHEN AND P. THOMPSON, *Cold Spring Harbor Symp. Quant. Biol.*, 29 (1964) 213.
11 M. KUNITZ, *J. Gen. Physiol.*, 35 (1952) 423.
12 T. BÜCHER AND G. PFLEIDERER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 435.
13 K. R. TANAKA, W. N. VALENTINE AND S. MIWA, *Blood*, 19 (1962) 267.
14 W. N. VALENTINE AND K. R. TANAKA, in S. P. COLOWICK, N. O. KAPLAN AND W. A. WOOD, *Methods in Enzymology*, Vol. 9, Academic Press, New York, 1966, p. 468.
15 G. BEISENHERZ, T. BÜCHER AND K-H. GARBADE, in S.P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 391.
16 E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1967, p. 447.
17 H. HOLMSEN AND E. STORM, *Biochem. J.*, 112 (1969) 303.
18 L. A. KERSON, D. GARFINKEL AND A. S. MILDVAN, *J. Biol. Chem.*, 242 (1967) 2124.
19 M. A. ROBINSON, P. B. LODER AND G. C. DEGRUCHY, *Brit. J. Haematol.*, 7 (1961) 327.
20 K. K. TSUBOI AND K. FUKUNAGA, *J. Biol. Chem.*, 240 (1965) 2806.
21 P. MAEBA AND B. D. SANWAL, *J. Biol. Chem.*, 243 (1968) 448.
22 K. H. IBSEN, K. W. SCHILLER AND E. A. VENN-WATSON, *Arch. Biochem. Biophys.*, 128 (1968) 583.
23 M. D. PRAGER AND W. R. WHIGHAM, *Biochim. Biophys. Acta*, 132 (1967) 181.
24 W. A. SUSOR AND W. J. RUTTER, *Biochem. Biophys. Res. Commun.*, 30 (1968) 14.
25 S. PASSERON, L. JIMÉNEZ DE ASÚA AND H. CARMINATTI, *Biochem. Biophys. Res. Commun.*, 27 (1967) 33.
26 C. B. TAYLOR AND E. BAILEY, *Biochem. J.*, 102 (1967) 32C.
27 C. I. POGSON, *Biochem. Biophys. Res. Commun.*, 30 (1968) 297.
28 P. CARTIER, A. NAJMAN, J. P. LEROUX AND H. TEMKINE, *Clin. Chim. Acta*, 22 (1968) 165.
29 P. BOIVIN, C. GALAND, J. MALLARME AND R. PERROT, *Pathol. Biol. Semaine Hop.*, 17 (1969) 597.
30 A. YOSHIDA, *J. Biol. Chem.*, 241 (1966) 4966.