TWO INTERCONVERTIBLE FORMS OF PYRUVATE KINASE IN ADIPOSE TISSUE

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Recent reports have indicated that pyruvate kinase (EC 2.7.1.40) from gluconeogenic sources may be regulated by allosteric feed-forward and feed-back mechanisms (Hess, Haeckel & Brand, 1966; Tanaka, Sue & Morimura, 1967; Taylor & Bailey, 1967; Weber, Lea & Stamm, 1967). Although fructose 1,6-diphosphatase is absent in epididymal adipose tissue (Weber, et al, 1965), evidence has been put forward to show that both pyruvate and propionate may act as precursors of α-glycerophosphate in this tissue, particularly under conditions of limited glucose availability (Ballard, Hanson & Leveille, 1967; Reshef, Niv & Shapiro, 1967).

In this paper results are presented which demonstrate that pyruvate kinase in rat epididymal adipose tissue exists in two interconvertible forms. One, designated PK-A[1], exhibits sigmoid steady-state kinetics with PEP, and is activated by $F16P_2$; the other (PK-B) is insensitive to $F16P_2$ and shows normal Michaelis-Menten kinetics with PEP (Pogson, 1968). PK-A is converted to PK-B upon incubation with low levels of $F16P_2$; this process is reversed by incubation with EDTA, which acts competitively with $F16P_2$. Evidence is also presented to show that this interconversion is accompanied by overall change and conformational changes.

[1]Abbreviations: $F16P_2$, fructose 1,6-diphosphate; PEP, Phosphoenolpyruvate; PK, pyruvate kinase; EGTA, ethyleneglycol-bis-(aminoethyl)tetra-acetate.

METHODS AND MATERIALS. $F16P_2$ (tetracyclohexylammonium salt), substrates and assay enzymes were obtained from the Boehringer Corporation (London) Ltd. PK was prepared freshly each day from fat pads of 200 - 250gm. Wistar rats by extraction with 2ml. buffer per gm. tissue at 0°. To obtain PK-A, the buffer was 20mM-imidazole, 5mM-EDTA, 10mM-2-mercaptoethanol pH 6.8; for PK-B, 20mM-imidazole, pH 6.8, unless otherwise stated. 30,000g. supernatants were used throughout.

PK was assayed using a Hilger-Gilford recording spectrophotometer with full scale deflection of 0.1 optical density units. Total activity was measured in a medium containing 0.125M-tris; 12.5mM-$MgCl_2$; 150mM-KCl; 2mM-ADP; 1.5mM-PEP; 0.125mM-NADH, pH 7.4 with 3.6 units of lactate dehydrogenase in 3.0ml. at 25°. One unit of PK catalyses the disappearance of 1µMole PEP/min. at 25°.

The relative concentrations of PK-A and PK-B after incubation experiments were determined using a medium similar to that above but with 2.5×10^{-4}M ADP and 3×10^{-5}M PEP at 30°. Although V_{max} values for PK-A and PK-B are similar, PK-A exhibits sigmoid kinetics with PEP, and has a much higher "apparent K_m" for this substrate than does PK-B (Pogson, 1968). From rates (as µMoles/min./unit of PK) at the low PEP concentration and from known values of the rates of 'pure' PK-A and PK-B under these standard conditions, the proportions of the two forms in a mixture may be calculated.

Sedimentation data were obtained in the Beckman Model E ultracentrifuge using the method of Yphantis & Waugh (1956), as described by Schachman (1957). In these experiments PK-A was extracted in 15mM-imidazole, 5mM-EDTA, 10mM-2-mercaptoethanol pH 6.8; PK-B was stabilized by extraction in 15mM-imidazole, 0.5mM-$F16P_2$ pH 6.8. After preliminary assay for total activity, enzyme was diluted to 0.5 units/ml. Runs were of 30 mins., 60 mins. and 90 mins duration at 39,460 r.p.m. and 5°. Assays for total activity, in triplicate or quadruplicate, were carried out immediately after each run; recovery was quantitative.

RESULTS AND DISCUSSION Extraction expts. In preliminary experiments, sigmoidicity with PEP was noted only when EDTA was present in the extraction medium. Under these conditions the enzyme (PK-A) was also sensitive to activation by $F16P_2$, and showed cooperativity with Mg^{2+} (Pogson, 1968). In an attempt to determine the role of EDTA in this process, PK activity was extracted with various buffers (Table 1). The ratios of rates obtained at

Table 1. Effect of Extraction Medium upon PK Activity

Procedure was as described in the text. Rates were measured at 30° with 2.5×10^{-4} M ADP and two concentrations of PEP - low (3×10^{-5} M), and high (10^{-3} M). All buffers were adjusted to pH 6.8.

Extraction Medium	Rate(μMole/min./unit PK) Low PEP	High PEP	Ratio High/Low
20mM-imidazole	.358	1.094	3.1
20mM-imidazole, 20mM-KCl	.349	1.133	3.2
20mM-imidazole, 10mM-2-mercaptoethanol	.320	1.088	3.4
20mM-imidazole, 5mM-EDTA	.018	.930	52
20mM-imidazole, 5mM-EDTA, 10mM-2-mercaptoethanol	.019	.985	52
20mM-imidazole, 5mM-EDTA, 20mM-$MgCl_2$	.157	1.013	6.4
20mM-imidazole, 5mM-EGTA	.276	.988	3.6
20mM-imidazole, 10mM-NaCitrate	.020	.909	45

high and low PEP concentrations are typical of the two forms; PK-A giving values of 40 - 60, PK-B values of 2.5 - 4. PK-B was extracted with imidazole alone, with KCl or with 2-mercaptoethanol. PK-A resulted only when EDTA or citrate was present. EGTA produced slight conversion to PK-A, but was less effective than EDTA. $MgCl_2$ in excess partially prevented the EDTA-mediated reaction, but had no effect in the absence of EDTA (not shown). These effects were independent of pH in the range of 6.5 to 8.0.

Incubation expts. Extracted PK-A is relatively stable towards incubation at 34°; under these conditions PK-B shows a consistent, although quantitively variable, conversion to PK-A (Table 2).

Table 2. Effect of Incubation on the Interconversion of PK Activities

Enzyme was prepared by extraction in the stated buffer. Incubations were for 10 min. at 34°, and consisted of 0.2ml. of enzyme plus additions in a total volume of 0.3ml. After incubation activity was assayed at 30° with 2.5×10^{-4}M ADP and 3×10^{-5}M PEP. The percentage of PK-A was calculated as described in the text.

Expt.	Extraction Medium	Additions	Time(min.)	% PK-A
1	20mM-imidazole, 5mM-EDTA, 10mM-2-mercaptoethanol pH 6.8	H_2O	0	99
		H_2O	10	91
		16.7mM-EDTA	10	99
		0.5mM-$F16P_2$	10	1
		0.05mM-$F16P_2$	10	33
		33.3mM-EDTA + 0.05mM-$F16P_2$)	10	80
2*	20mM-imidazole, pH 6.8	H_2O	0	64
		H_2O	10	76
		0.05mM-EDTA	10	88
		16.7mM-EDTA	10	98
		0.05mM-$F16P_2$	10	28
		16.7mM-EDTA + 0.05mM-$F16P_2$)	10	58

*Preincubated for 10 min. at 34° to obtain preliminary partial conversion to PK-A.

This conversion, accelerated by EDTA, is reversed by concentrations of $F16P_2$ similar to those in vivo (R.M. Denton, personal communication). EDTA and $F16P_2$ appear to act competitively in mediating the interconversion of the two forms, possibly through a mechanism involving binding to a divalent cation. The process is entirely reversible by suitable addition of EDTA or $F16P_2$. The effect of $F16P_2$ is specific; other sugar phosphates are inactive. Preliminary results indicate that ATP, ADP, PEP amd monovalent cations may substitute for EDTA. Palmitoyl CoA, acetyl CoA and cyclic 3'5'AMP are entirely without effect.

Electrophoresis. Results are shown diagrammatically in Fig.1. Stability of fat pad PK during runs was ensured by the use of the relevant extraction medium as electrophoretic buffer. Although this resulted in high endosmotic flow rates, satisfactory separation of activities was achieved.

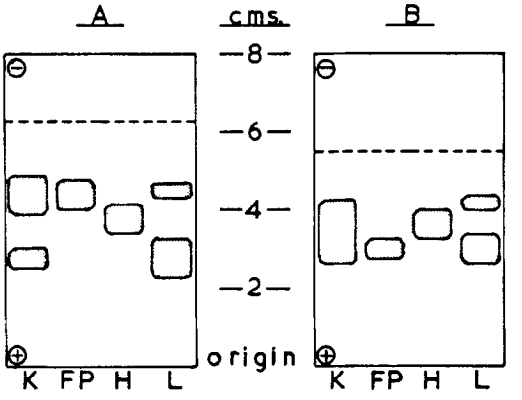

Fig.1. Electrophoresis of Pyruvate Kinase activities of rat kidney (K), fat pad (FP), heart (H) and liver (L), using 10 x 5 cm. cellulose acetate strips. The electrophoretic buffer was of the same composition as the extracting medium. A) Buffer: 15mM-imidazole, 0.5mM-$F16P_2$, pH 7.4; runs were for 1 hr. at 0.15mA/cm. B) Buffer: 15mM-imidazole, 5mM-EDTA, 10mM-2-mercaptoethanol, pH 6.8; runs were for 2 hr. at 0.25mA/cm. Strips were sprayed with a mixture containing 5mM-tris, pH 7.4; 5mM-$MgCl_2$; 150mM-KCl; 6mM-ADP; 4.5mM-PEP; 2mM-NADH, 22.5 units/ml. lactate dehydrogenase; under UV light, PK activity appeared as dark spots on a fluorescent blue background. ADP was omitted for blanks. Dotted lines represent the position of glucose markers; these were developed by spraying successively with 30mM-$AgNO_3$ in acetone and 50mM-NaOH in ethanol.

The relative mobilities of liver and heart PKs are in agreement with those reported by Tanaka et al, (1967). PK-B (strip A) shows a mobility similar to that of the non-allosteric liver PK 'M', whilst PK-A (strip B) moves similarly to the $F16P_2$-sensitive liver PK 'L'. Kidney PK, classified as 'LM' by Tanaka et al (1967), also shows changes in mobility under these conditions.

Ultracentrifuge Data. The sedimentation properties of both forms of fat pad PK have been investigated. Partition-cell ultracentrifugation yielded values of 5.3 - 5.6s and 7.2 - 7.3s for PK-A and PK-B respectively. These compare with reported values of 7.2s for erythrocyte PK (Koler et al, 1963), and of 7.2s for the active dimer of rabbit muscle PK (Steinmetz & Deal, 1966). Since PK-A and PK-B both show similar cooperativity for K^+ binding ('n' values of 2.0, as calculated from Hill plots; Pogson, 1968), the differing sedimentation constants of the two forms may reflect large conformational change rather than alteration in molecular weight.

The physiological importance of the interconversion of the two forms is difficult to assess, partly due to the great dilution of the experimental system. It is conceivable, however, that the enzyme is normally present as PK-B during active glycolysis. In starvation, when the dicarboxylate shuttle is active, a wasteful ATPase cycle might be prevented by conversion of PK-B to PK-A, mediated by alterations in the steady-state levels of $F16P_2$ or other active metabolites.

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