SOME DISTINCTIVE PROPERTIES OF PYRUVATE KINASE PURIFIED FROM RAT LIVER*

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Pyruvate kinase (PyK) occupies a key branch point in the glycolytic and gluconeogenic sequences; thus, it seems possible that the PyK from the primarily glycolytic muscle tissue might have different regulatory characteristics from that derived from the actively gluconeogenic liver tissue. Tanaka *et al.* (1965, 1967) have shown that rat liver contains a PyK (here termed PyK-B) which can be distinguished from the muscle enzyme (PyK-A) by its distinctive electrophoretic, immunological, and kinetic properties. Furthermore, the activity of liver but not muscle PyK is influenced by diet, diabetes, and insulin treatment (Krebs and Eggleston, 1965; Tanaka *et al.*, 1965, 1967; Weber *et al.*, 1965; Yudkin and Krauss, 1967). Recent studies of Passeron *et al.* (1967) and Taylor and Bailey (1967) have shown that liver PyK, like yeast PyK (Gancedo *et al.*, 1967; Hess *et al.*, 1966, 1967; Hunsley and Suelter, 1967) is activated by fructose diphosphate (FDP) *in vitro*. On the other hand, muscle PyK is indifferent to FDP. We have independently made similar observations. In this paper, we report the stabilization and purification of PyK-B from rat liver and describe some of its molecular and catalytic properties. PyK-B was activated by FDP with K_m (apparent) for FDP of 0.95×10^{-7} M or by high concentrations of PEP. The K_m (app.) for PEP ranged from 8.7×10^{-4} M in the presence of FDP to 1.9×10^{-2} M in its absence. The enzyme was inhibited by ATP with K_i (app.) = 10^{-4} M. In addition, partially purified PyK-B was converted into a form which was essentially independent of PEP or FDP activation and less sensitive to inhibition by ATP. The K (app.) of this insensitive form were 1.5×10^{-4} M, $>10^{-2}$ M and 10^{-3} M for PEP, FDP and ATP, respectively. We also report here the presence in various tissues of a third electrophoretically distinct form of PyK that is

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immunologically similar to PyK-A derived from skeletal muscle. In preliminary studies, no effect of FDP on the kinetics of this PyK has been detected.

Methods. PyK was assayed by coupling with lactic dehydrogenase (LDH) according to the method of Bücher and Pfleiderer (1955). NADH oxidation was monitored continuously at 340 mμ with a Gilford spectrophotometer at 25°. The routine assay mixture contained 0.05 M Tris-Cl (pH 7.5), 0.005 M EDTA, 0.01 M MgCl, 0.06 M KCl, 0.001 M tricyclohexylamine PEP, 0.001 M NaADP, 0.0003 M NaNADH, and approximately 1 unit LDH/ml (Calbiochem). Modifications of the assay conditions are noted in the text. One unit of enzyme activity refers to the oxidation of 1 μmole NADH per min under assay conditions; specific activity is in units/mg/protein. Protein was estimated by absorbance at 280 mμ, assuming $E^{1\%}_{cm} \approx 10$.

Initial attempts to purify PyK-B from rat liver were hampered by the lability of the enzyme activity in fairly dilute solutions and under conditions of fractionation. We found subsequently that sucrose stabilized the enzyme activity. This allowed us to develop the following purification procedure: Livers from Sprague-Dawley rats, fed *ad libitum* on Purina Lab Chow, were homogenized in two volumes of a solution containing 0.25 M sucrose, 0.025 M Tris-Cl (pH 7.5), and 0.0025 M EDTA and centrifuged at 30,000 x *g* for 20 min. The protein from the supernatant fluid was precipitated between 25 and 45% saturated ammonium sulfate. It was then dissolved in the above buffer solution and passed through a Sephadex G-25 column equilibrated with 0.5 M sucrose, 0.02 M Tris-Cl (pH 7.5), and 0.002 M EDTA. The enzyme-containing fractions were combined and placed on a DEAE cellulose column equilibrated with the same buffer. PyK activity was eluted with a linear gradient of 0.02 M Tris-Cl (pH 7.5) and 0.002 M EDTA in 0.5 M sucrose to 0.3 M Tris-Cl (pH 7.5) in 0.5 M sucrose. The activity-rich fractions were pooled and brought to 45% ammonium sulfate saturation. The resulting precipitate was dissolved in 0.5 M sucrose , 0.02 M Tris-Cl (pH 7.5) and passed through a Sephadex G-25 column equilibrated with the same buffer. The solution was then placed on a second DEAE cellulose column and eluted with an 0.02 M - 0.30 M Tris-Cl (pH 7.5) gradient in 0.5 M sucrose. A purification of approximately 100-fold was achieved by this procedure. There was no aldolase activity detectable in the final preparation.

Experimental. The distribution of pyruvate kinase in various tissues was investigated by zone electrophoresis using an activity stain and by precipitation with antibodies prepared against the skeletal muscle enzyme. As shown

in Fig. 1, electrophoresis of crude liver extracts resulted in two bands of enzymatic activity: a dense anionic and a faint cationic band. PyK activity from skeletal muscle and heart extracts (PyK-A) migrated to a point midway between the two liver bands. Lung, spleen and testis extracts exhibited a single band with the same mobility as the minor cationic liver band. Brain and kidney extracts produced both cationic bands. All three bands are resolved from mixtures of appropriate tissue extracts. The PyK isolated from liver migrated as a single band corresponding to the major anionic band found in crude liver extracts.

Antibodies prepared against purified rat muscle PyK (anti-PyK-A) quantitatively precipitated PyK from rat skeletal muscle, heart, lung, spleen, duodenum, testis, brain, and kidney homogenates. Thus, the two cationic bands of PyK activity exhibit very similar immunological properties. Anti-PyK-A had

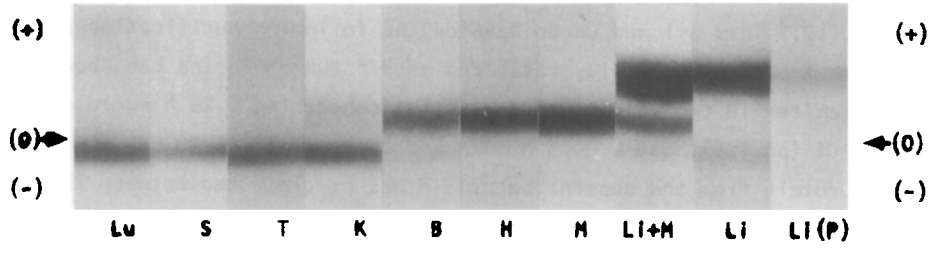

FIGURE 1. *Electrophoretic Resolution of Rat PyK-A and PyK-B Activity.*

Lu, lung; S, spleen; T, testis; K, kidney; B, brain; H, heart; M, skeletal muscle; Li+M, mixed liver and muscle; Li, liver; Li(P), purified liver. Tissue was homogenized in 2 volumes of 0.25 M sucrose, 0.025 M Tris-Cl (pH 7.5) and 0.0025 M EDTA and centrifuged at 30,000 x *g* for 20 min.

Electrophoresis was carried out on cellulose polyacetate strips at a potential gradient of 17 v/cm for 2 hours at 4° in a buffer containing 0.5 M sucrose, 0.01 M Tris-Cl (pH 7.5), and 0.001 M NaFDP. Samples were applied to the center of the strips.

Pyruvate kinase activity was detected by coupling with the lactic dehydrogenase system. The region producing NADH oxidation was visually observed by the loss of fluorescence. It was recorded by contact printing with 340 mμ light. The cellulose acetate strips to be assayed were placed on an agar film containing 8 mg/ml Nobel agar, 5×10^{-2} M Tris-Cl (pH 7.5), 5×10^{-3} M EDTA, 10^{-2} M MgCl, 6×10^{-2} M KCl, 2×10^{-3} M PEP, 10^{-3} M ADP, 10^{-3} M NADH, 2×10^{-5} M FDP and 30 units LDH/ml. Development was allowed to proceed for 1-5 min at 37°. PyK activity was recorded by contact printing on photographic enlarging paper using a 340 mμ light source similar to the method independently developed by von Fellenberg *et al.* (1966). Areas of enzymatic activity (low NADH) transmit the incident light and expose the paper while other areas (high NADH) do not.

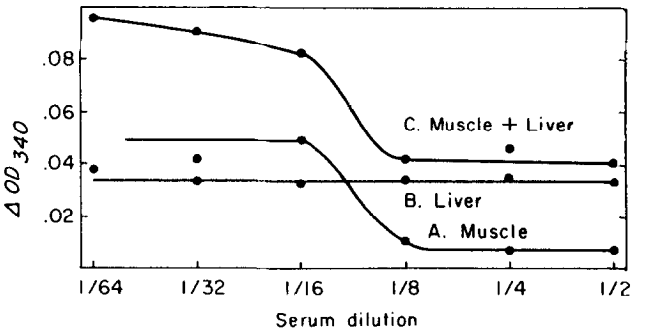

FIGURE 2. *Immunological Distinction of PyK-A and PyK-B.*

PyK-A (rat skeletal muscle) was purified by the method of Tietz and Ochoa (1958). Antiserum to the purified enzyme of specific activity 400 was prepared in rabbits according to Campbell *et al.* (1963). One ml anti-PyK-A serum quantitatively precipitated 125 units PyK-A activity from a rat muscle homogenate. Constant aliquots of the 30,000 x *g* supernate of rat muscle and liver homogenates were incubated with dilutions of anti-PyK-A serum in a medium containing (final concentration) 0.125 M sucrose, 0.012 M Tris-Cl (pH 7.5), 0.00125 M EDTA, 1% bovine serum albumin, and 0.85% NaCl. After incubation for 3 hours at 25°, the samples were centrifuged for 10 min at 15,000 x *g* and the supernate was assayed for PyK activity.

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no observable effect on the PyK-B activity in liver homogenates. Fig. 2 presents the precipitation curves obtained by mixing a constant amount of muscle or liver extracts or a mixture of both with serial dilutions of anti-PyK-A. Eighty percent of muscle PyK activity precipitated (curve A) while there was no significant effect on liver PyK activity (curve B). A mixture of muscle and liver homogenates gave an additive response (curve C). In these experiments, short incubation times and high viscosity prevented complete removal of the PyK-A-antibody complex; other experiments have indicated that PyK-A is completely precipitated by anti-PyK-A.

The instability of PyK activity in crude liver homogenates led to a systematic study of the effects of various metabolic intermediates which might stabilize or enhance PyK activity. It was found that PyK activity in liver homogenates was slightly stimulated (1.5-fold) by FDP at 2×10^{-4} M PEP; but no effect was observed at 10^{-3} M PEP. At intermediate stages of purification, liver PyK was stimulated 2- to 3-fold by the addition of 10^{-4} M FDP at the low (2×10^{-4} M) PEP concentrations. After the second DEAE cellulose step, the enzyme required FDP for catalytic activity at low PEP concentrations. With purification, there was usually an increased effect of FDP on enzyme activity. An occasional preparation, however, did not exhibit this greatly increased sensitivity toward FDP. In addition, other sensitive preparations

sometimes lost their FDP requirement during storage at -20° C. The factors that effect these transitions are unknown. PyK activity in extracts of muscle, spleen, testis and kidney was unaffected by FDP.

Fig. 3 presents Hill plots (log $\frac{v}{V-v}$ vs. log [M]) obtained with one of the more sensitive liver PyK-B preparations and a plot obtained with a preparation which had lost its sensitivity on storage at -20°. K_m (app.) was taken as the concentration of M at log $\frac{v}{V-v} = 0$. The slope of the curve, n, may be taken, with reservation, as the number of binding sites of M per enzyme molecule (Atkinson *et al.*, 1965). The sensitive preparation was

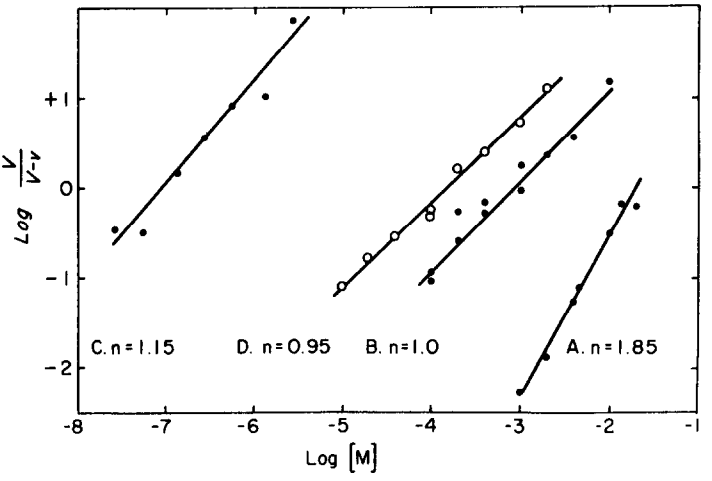

FIGURE 3. *Effect of FDP and PEP on PyK-B Activity.*

Assay conditions were defined under *Methods* except as noted below. Curves A, B and C were obtained in the absence of Na^+ and NH_4^+ (the salts were present in the tris or the cyclohexylamine form). PyK-B was prepared as described under *Methods*.

A. Sensitive PyK-B, M = PEP, K_m (app.) = 1.9×10^{-2} M.
B. Sensitive PyK-B, M = PEP, K_m (app.) = 8.7×10^{-4} M at 10^{-5} M FDP.
C. Sensitive PyK-B, M = FDP, K_m (app.) = 0.95×10^{-7} M at 2×10^{-3} M PEP
D. Insensitive PyK-B, M = PEP, K_m (app.) = 1.5×10^{-4} M.

found to have a K_m (app.) for PEP of 1.9×10^{-2} M with n = 1.85 which decreased to 8.7×10^{-4} M and n = 1.0 in the presence of 10^{-5} M FDP. FDP activated this preparation of PyK-B with K_m (app.) for FDP of 0.95×10^{-7} M and n = 0.95. The loss of FDP sensitivity was recognized as a decrease in K_m (app.) for PEP to 1.5×10^{-4} M in the absence of FDP. The K_m (app.) for PEP in the presence of

FDP also dropped to 7.1×10^{-5} M (10^{-4} M FDP). At the same time, K_m (app.) for FDP (2×10^{-4} M PEP) increased from 10^{-7} M to $>10^{-2}$ M. The slope of the Hill plot of log (PEP) in the absence of FDP also changed from n = 1.85 (FDP-sensitive) to n = 0.95 (FDP-insensitive). Other preparations have yielded a range of values intermediate to those presented here. Both the sensitive and insensitive forms of PyK-B were inhibited by ATP with a Hill plot of n = -1.0. The K_i (app.) of the sensitive form was 10^{-4} (at both 4×10^{-3} M and 10^{-2} M PEP) while K_i (app.) of the insensitive form was 10^{-3} M (at 2×10^{-4} M PEP). The K_i (app.) for ATP of the sensitive form of PyK-B in the presence of 10^{-5} or 10^{-4} M FDP was 10^{-3} M (at 10^{-3} M PEP). The pH-activity curve of purified PyK-B in the presence of FDP has a broad maximum of 6.8 - 7.6 with very low activity in the absence of FDP through this region.

The variations in the sensitivity of PyK-B to FDP could be a result of varying degrees of association of the molecule. This possibility was tested by filtering several pyruvate kinase preparations through Sephadex G-200 columns equilibrated in 0.5 M sucrose and 0.02 M Tris (pH 7.5), using catalase m.w. "250,000," commercial rabbit muscle PyK-A m.w. 237,000, aldolase m.w. 150-160,000, and lactic dehydrogenase m.w. 140,000 as molecular weight standards. The elution profiles of PyK-A (rat muscle) or PyK-B (rat liver) in sensitive or insensitive forms and in the presence and absence of added 10^{-4} M FDP were identical to those of rabbit muscle PyK-A. All were eluted prior to liver catalase, thus these enzymes behave as if they have a larger molecular size than catalase under these conditions.

Summary and Discussion. The present experiments indicate that the purified liver pyruvate kinase, PyK-B, is distinct from the extensively studied muscle enzyme, PyK-A, by electrophoretic, chromatographic, and immunological criteria. These studies thus confirm the recent findings of Tanaka *et al.* (1967). In addition, and most significantly, PyK-B exhibits remarkable regulatory characteristics. This enzyme can be isolated in a state which requires added FDP or high concentrations of PEP for catalytic activity and is inhibited by ATP. Curiously, the enzyme also appears able to exist in another state(s) which is independent or much less dependent on these effectors and is 1/10 as sensitive to inhibition by ATP.

The regulative characteristics of PyK-B appear similar to those reported for yeast PyK by Hess *et al.* (1966, 1967), Hunsley and Suelter (1967), and Gancedo *et al.* (1967). There has been no report, however, of an FDP-insensitive state of yeast PyK.

The modulations of PyK-B activity by FDP, PEP and ATP are of considerable

interest since they suggest means of controlling the glycolytic and gluconeogenic flux in liver tissue. Of as much interest, however, is the molecular mechanism of action of the effectors, the basis for the transition of PyK-B from an effector-sensitive to insensitive state(s), and the specific molecular relationships of the three forms of PyK detected here.

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