Lack of Temperature-Sensitivity of Rat Liver Pyruvate Kinase

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Rat liver pyruvate kinase, prepared by Sephadex treatment of a 10^5g supernatant in phosphate buffer, is quite stable and gives reproducible results when a variety of parameters are altered in the enzyme assay. Incubation of this preparation at 25°C or 0–2°C has no effect on the activation by fructose 1,6-diphosphate or inhibition by ATP or alanine.

Pyruvate kinase (EC 2.7.1.40) catalyses the essentially irreversible conversion of phosphoenolpyruvate into pyruvate. Of the two isoenzymes found in liver, the concentration of the major component, type I (termed 'type L'), is under hormonal and dietary control (Tanaka *et al.*, 1965, 1967*a*). Pyruvate kinase type I, which may play an important role in the control of glycolysis and gluconeogenesis, exhibits sigmoidal kinetics with phosphoenolpyruvate, allosteric activation with fructose 1,6-diphosphate and inhibition by ATP (Taylor & Bailey, 1967; Carminatti *et al.*, 1968; Susor & Rutter, 1968; Tanaka *et al.*, 1967*b*). Whereas fructose 1,6-diphosphate or lower pH transforms the sigmoidal response to phosphoenolpyruvate into a hyperbolic relationship, they increase the co-operativity of the ATP inhibition (Tanaka *et al.*, 1967*b*; Rozengurt *et al.*, 1969). Inhibition by L-alanine resembles that of ATP (Llorente *et al.*, 1970). Liver pyruvate kinase type II ('type M') resembles the muscle enzyme but has a different amino acid inhibition spectrum (Jimenez de Asua *et al.*, 1971).

The stability of pyruvate kinase has been a problem, and reproducibility of preparations, particularly with respect to fructose 1,6-diphosphate activation, is sometimes unsatisfactory.

We have developed a preparation, in phosphate buffer, of pyruvate kinase from rat liver that is quite stable and gives reproducible results when a variety of parameters are altered in the enzyme assay. We have been unable to obtain any results that support the claim that incubation at 0–2°C leads to a desensitization of liver pyruvate kinase to 'the homotropic co-operativity of the phosphoenolpyruvate substrate and to the allosteric inhibition by alanine and ATP and the activation by fructose 1,6-diphosphate' (Llorente *et al.*, 1970).

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Materials and methods

Buffers. These were as follows: buffer A contained 5 mM-phosphate (pH 7.5), 159 mM-K⁺, 150 mM-Cl⁻, 5 mM-MgSO₄ and 1 mM-2-mercaptoethanol; buffer B contained 50 mM-phosphate (pH 7.5), 90 mM-K⁺ and 1 mM-2-mercaptoethanol.

Chemicals. The following were purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A.: NADH, disodium salt, grade III; phosphoenolpyruvate, crystalline potassium salt; ADP, sodium salt, grade I; fructose 1,6-diphosphate, tetrasodium salt, Sigma grade; lactate dehydrogenase, crystalline from rabbit muscle in (NH₄)₂SO₄ suspension.

10^5g supernatant. Male albino rats, obtained from the Holtzman Co., Madison, Wis., U.S.A., were maintained on standard laboratory chow and water *ad libitum*. The rats were decapitated and the livers were rapidly removed and homogenized in a Waring blender with buffer A equivalent in volume to twice the liver weight. The homogenate was centrifuged at 10^5g at 4°C for 30 min. The supernatant solution usually had about 75 % of the pyruvate kinase activity of the homogenate.

Sephadex-treated enzyme. The entire operation was performed at 4°C. A 3 ml portion of 10^5g supernatant was passed on to a 21 mm × 205 mm Sephadex G-25 column previously equilibrated with buffer A. The column, whose void volume was about 35 ml, was eluted with buffer A at about 5 ml/min. The enzyme was in the pooled red fractions (approx. 10 ml) that are eluted just behind the void volume. Thus passage over Sephadex resulted in an approximately threefold dilution of the enzyme. When desired, the Sephadex G-25 effluent was concentrated by the collodion-bag technique (Bulletin no. 145; Schleicher and Schuell, Keene, N.H., U.S.A.) to a volume approximately equal to the weight of liver used. This preparation contains both liver pyruvate

kinase types I and II, presumably devoid of small molecules except those in the eluting buffer and those tightly bound to macromolecules. The yield of pyruvate kinase was 65–70% of the initial homogenate. We have found that pyruvate kinase is much more stable in phosphate than in Tris buffers; the Sephadex treated enzyme lost 10–20% of its activity in 1 day at 4°C, whereas type I enzyme, 20–30% pure according to Tanaka *et al.* (1967a), loses a similar portion of activity in 1 week.

Enzyme assays. These were done at 25°C in a 2 ml final volume in buffer B with NADH at 0.14 mM. The buffer was 1 mM with respect to 2-mercaptoethanol and did not contain EDTA. All assay media were 5–10 mM with respect to Na⁺, since NaOH was used for pH adjustments and the sodium salts of ADP, ATP and phosphoenolpyruvate were used. Na⁺ at these concentrations was shown not to affect enzyme activities. Approx. 50 units of lactate dehydrogenase were added per assay, providing a 500–1000-fold excess over pyruvate kinase.

Because most assay mixtures contained undialysed lactate dehydrogenase they were approx. 30 mM with respect to (NH₄)₂SO₄. When dialysed lactate dehydrogenase was used, an additional 50 mM-KCl was added to the buffer. The milliosmolarities of the various assay solutions varied only from 280 to 320.

The reactions were started by addition of a source of pyruvate kinase and the initial rates of decrease in absorbance (shown to depend on phosphoenolpyruvate) at 340 nm were measured.

The use of 5 mM-Mg²⁺, 50 mM-phosphate and 30 mM-(NH₄)₂SO₄ (from undialysed lactate dehydrogenase) creates a situation in which the solubility product of MgNH₄PO₄ is greatly exceeded. However, seldom was precipitation observed during the assay. If precipitation was a problem, the lactate dehydrogenase was dialysed before use.

Fructose 1,6-diphosphate assay. Fructose 1,6-diphosphate in HClO₄ filtrates of the various enzyme preparations was determined fluorimetrically essentially as described by Williamson (1965). In all cases internal standards were used and recoveries were determined. Recoveries ranged from about 45 to 85%, depending on the amount of fructose 1,6-diphosphate present.

Discussion of results

It should be noted that the enzyme preparations contained both pyruvate kinase type I (L) and pyruvate kinase type II (M). However, since the major component in liver is pyruvate kinase type I (Tanaka *et al.*, 1965, 1967a), kinetic results with this preparation are qualitatively similar to those obtained with a highly purified preparation of pyruvate kinase type I (W. Flory, B. D. Peczon, H. O. Spivey & R. E. Koeppe, unpublished work).

Fructose 1,6-diphosphate stimulation (Table 1). We have never observed fructose 1,6-diphosphate activation of the initial homogenate, the 10⁵g supernatant or the latter diluted threefold. Moreover, we have often noted a lack of fructose 1,6-diphosphate stimulation of the undialysed 25–45%-saturation-(NH₄)₂SO₄ precipitate (from 10⁵g supernatant). Dialysis of the precipitate, incubation of the 10⁵g supernatant at room temperature or passage through Sephadex always yielded a preparation whose pyruvate kinase activity was increased by added fructose 1,6-diphosphate. Typical results are presented in Table 1.

In Table 2 are included the concentrations of fructose 1,6-diphosphate in the original supernatant and the changes caused by Sephadex treatment, warming and cooling. Incubation at room temperature of the 10⁵g supernatant destroyed fructose 1,6-diphosphate

Table 1. *Effect of 15 min incubation at room temperature on pyruvate kinase activity in 10⁵g rat liver supernatant fraction before and after passage through Sephadex G-25*

The standard assay in buffer B, pH 7.5, with 5 mM-MgSO₄, 0.6 mM-phosphoenolpyruvate and 1.5 mM-ADP was used; fructose 1,6-diphosphate was added as indicated. Usually 0.01 ml of supernatant was used per assay. In some experiments the 10⁵g supernatant was diluted to correspond to the volume change due to Sephadex treatment.

Enzyme source	Incubation	Activity (μmol/min per mg of protein)		
		No fructose diphosphate	10 μM-Fructose diphosphate	1 mM-Fructose diphosphate
10 ⁵ g supernatant	No	0.32	—	0.29
	Yes	0.08	0.32	0.30
10 ⁵ g supernatant diluted	No	0.26	0.31	—
	Yes	0.06	0.31	0.34
10 ⁵ g supernatant through Sephadex	No	0.08	0.29	—
	Yes	0.09	0.28	0.26

Table 2. *Effect of warming and cooling on pyruvate kinase activity in 10⁵g rat liver supernatant fraction*

For assay conditions see Table 1.

Enzyme treatment	Activity ($\mu\text{mol/min per mg of protein}$)		Concn. of fructose diphosphate (μM)
	No fructose diphosphate	1 mM-Fructose diphosphate	
Kept at 0°C	0.37	0.36	200
At 24°C for 5 min	0.40	0.39	35
At 25°C for 10 min	0.38	0.40	<4
At 25°C for 30 min	0.16	0.35	<3
At 25°C for 45 min	0.20	0.39	<3
Back to 0°C for 30 min*	0.23	0.37	<1
Back to 0°C for 1 h*	0.24	0.35	<3
Back to 0°C for 3 h*	0.31	0.39	<2
Through Sephadex G-25 at 4°C	0.21	0.43	0

* Supernatant incubated at 25°C for 45 min before being returned to 0°C.

enzymically and passage through Sephadex removed it by filtration. Although there was variation from one preparation to another, in each case fructose 1,6-diphosphate stimulation was observed after sufficient incubation at 25°C (usually 15 min). The concentration of fructose 1,6-diphosphate needed to maintain maximal enzymic activity was very low. Thus after 10 min incubation little fructose 1,6-diphosphate stimulation was noted (Table 2), even though the concentration of fructose 1,6-diphosphate in the 10⁵g supernatant had fallen from 200 μM to less than 4 μM . Since the enzyme was diluted 200-fold when added to the standard assay solution, the fructose 1,6-diphosphate in the assay mixture was about 0.02 μM or less. The Sephadex treatment provided a simpler method of getting reproducible results with and without fructose 1,6-diphosphate and simultaneously removes other small molecules (ATP etc.) not very tightly bound to protein.

Bailey *et al.* (1968) have reported that preincubation at room temperature of the 25–45%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction results in decreased pyruvate kinase activity, instantaneously restored by fructose 1,6-diphosphate. They suggested a temperature-dependent conformational change to explain these results, but observed quantitative variation from day to day. Our results (Tables 1 and 2) indicate it is much more likely that incubation of many of the crude enzyme preparations from liver results in destruction of endogenous fructose 1,6-diphosphate, thus enhancing the subsequent activation by added fructose 1,6-diphosphate. In contrast, incubation of Sephadex-treated supernatant at room temperature had no effect on pyruvate kinase activity (Table 1).

Llorente *et al.* (1970) have reported studies on liver and kidney pyruvate kinase (a 30000g supernatant)

that suggested that at low temperature (0–2°C) pyruvate kinase type I loses its allosteric responses to alanine, ATP and fructose 1,6-diphosphate. With Sephadex-treated enzyme (or highly purified type I pyruvate kinase; results not shown) we have never been able to obtain results in support of the conclusion by Llorente *et al.* (1970) that pyruvate kinase activity of liver is reversibly affected by preincubation at low (0–2°C) or higher (25–35°C) temperature. Our Sephadex-treated enzyme, which was prepared in the cold, was always activated by fructose 1,6-diphosphate (Tables 1 and 2). Warming at room temperature had no effect (Table 1), nor did recooling to 0°C. In contrast with these results, Llorente *et al.* (1970) state that the 'removal of the metabolites by passing the extracts through a G-25 Sephadex column did not affect the susceptibility to cold desensitization'. However, no details of the Sephadex treatment were presented.

Although we found that incubation of the 10⁵g supernatant at room temperature invariably resulted in the appearance of fructose 1,6-diphosphate activation and loss of endogenous fructose 1,6-diphosphate, the reincubation of these supernatants at 0–2°C sometimes led to partial loss of the fructose 1,6-diphosphate effect (Table 2), as reported by Llorente *et al.* (1970) with a 30000g supernatant. Although we were unable to demonstrate an increased concentration of fructose 1,6-diphosphate in the recooled supernatant, the Sephadex results make it seem likely that the results reported by Llorente *et al.* (1970) can be explained by subtle changes in fructose 1,6-diphosphate concentrations during the various incubations. Since the amounts of fructose 1,6-diphosphate required for the activation of pyruvate kinase type I are extremely small, lack of fructose 1,6-diphosphate activation in

crude preparations must be attributed to small amounts of fructose 1,6-diphosphate unless this possibility is rigorously disproved.

Kinetic results obtained with Sephadex-treated enzyme and various concentrations of H^+ , phosphoenolpyruvate, MgADP, the inhibitors L-alanine and ATP, and fructose 1,6-diphosphate were qualitatively the same as those obtained from similar experiments with more highly purified type I enzyme (W. Flory, B. D. Peczon, H. O. Spivey & R. E. Koeppe, unpublished work). In none of these experiments have we obtained any evidence suggesting a temperature-dependent conformational change of type I rat liver pyruvate kinase relative to its allosteric activation or inhibition.

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