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Immunological and kinetic properties of pyruvate kinase in rat pancreatic islets

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Pyruvate kinase in rat pancreatic islets was characterized immunologically and kinetically. It is concluded that this activity is predominantly if not totally of the M_2 type.

Properties of the four isoenzymes of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) have been reviewed (Hall & Cottam, 1978). The M_1 isoenzyme found in muscle and brain displays Michaelis–Menten kinetics with respect to PEP (K_m 0.04–0.09 mM), and is not affected by fructose 1,6-bisphosphate or alanine. The L isoenzyme of liver and the R isoenzyme of erythrocytes are immunologically cross-reactive and both exhibit co-operativity in their kinetics towards PEP (K_m 0.3–0.96 mM). They are allosterically activated by fructose 1,6-bisphosphate and inhibited by alanine. The M_2 isoenzymes of kidney, liver and other tissues display Michaelis–Menten kinetics with respect to PEP (K_m 0.2–0.4 mM) and are allosterically activated by fructose 1,6-bisphosphate and inhibited by alanine. They are immunologically cross-reactive with the M_1 isoenzymes.

The properties of the enzymes involved in the utilization and synthesis of PEP in pancreatic islets are of considerable interest. It has been suggested that PEP may function as an intracellular mediator of glucose- and glyceraldehyde-induced insulin secretion in β-cells (Sugden & Ashcroft, 1977). PEP is reported to inhibit $^{45}Ca^{2+}$ uptake into islet mitochondria (Sugden & Ashcroft, 1978) and to stimulate islet adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity (Capito & Hedeskov, 1977) and a protein kinase in the β-cell plasma membrane (Davis & Lazarus, 1975). PEP causes release of insulin from suspensions of isolated granules and plasma membranes (Davis & Lazarus, 1976; Formby & Capito, 1977), and a positive correlation between islet PEP content and concentrations of 3′:5′-cyclic AMP in islets has been demonstrated (Hedeskov & Capito, 1980).

Abbreviation used: PEP, phosphoenolpyruvate.

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Pyruvate kinase is present in islets in appreciable quantities (Sugden & Ashcroft, 1977; Sener *et al.*, 1980), and the presence of pyruvate carboxylase (pyruvate:carbon-dioxide ligase, EC 6.4.1.1; Ashcroft & Randle, 1970) and PEP carboxykinase [GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32; C. J. Hedeskov, personal communication] has also been demonstrated in islets, with activities of the same order of magnitude as the glucose-phosphorylating enzymes (Ashcroft & Randle, 1970). As yet, the enzymes involved in PEP utilization and synthesis have not been characterized. Taking into consideration the observation that, when PEP carboxykinase is present, the isoenzyme of pyruvate kinase in the same tissue or organism is of a regulatory type (Carbonell *et al.*, 1973), we thought that it was important to establish the nature of pyruvate kinase in pancreatic islets.

In this paper the immunological, kinetic and regulatory properties of islet pyruvate kinase are described. They are consistent with the suggestion that the enzyme is substantially of the M_2 type.

Materials and methods

Lactate dehydrogenase, ADP, fructose 1,6-bisphosphate and collagenase were from Boehringer Corp. (London), Lewes, Sussex, U.K. L-Phenylalanine, L-alanine and collagenase type V were from Sigma. Anti-(chicken immunoglobulin G) (titre 2.5 mg of immunoglobulin G precipitated/ml) was from Miles Laboratories, Slough, Bucks., U.K. PEP (monocyclohexylamine salt) was prepared in our laboratory (Clark & Kirby, 1966). All other chemicals, of analytical grade where possible, were obtained from standard suppliers.

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Preparation of islets and islet supernatants

All experiments were performed with islets isolated from fed Sprague–Dawley rats (male, 150–200g) by the method of Lacy & Kostianovsky (1967). Batches of 200–300 islets were harvested in 20μl of buffer (Gey & Gey, 1936) and were disrupted by incubation for 10min at 20°C with 50mM-triethanolamine / HCl / 200mM-KCl / 10mM-$MgSO_4$/0.1mM-EDTA/1mM-dithiothreitol, pH7.2, containing bovine serum albumin (1mg/ml) and 1% (w/v) digitonin. After centrifugation for 5min (4°C, 12000*g*), supernatants were removed and used immediately for studies on pyruvate kinase. The protein content of islets was measured by the method of Böhlen *et al.* (1972).

Assay of pyruvate kinase activity

Pyruvate kinase activity was measured in 5–10μl portions of islet supernatants at 25°C in triethanolamine buffer as above (containing 1mg of bovine serum albumin/ml) by the method of Sugden & Ashcroft (1977), with a Gilford model 250 recording spectrophotometer. The rate of oxidation of NADH was proportional both to the amount of tissue extract and to the time of incubation. Control rates, in the absence of PEP or ADP, were zero in all cases. Enzyme activity is expressed as nmol of substrate utilized/min per islet.

Addition of dithiothreitol (1mM) to homogenization medium protected against loss of enzyme activity, such that 96% of initial activity was retained after 16h at 4°C (compared with 10% retained in the absence of dithiothreitol).

Pyruvate kinase antisera titrations

Antisera (Hopkirk & Bloxham, 1979) were titrated against supernatants derived from rat liver, kidney cortex and femoral muscle: 1ml of anti-(liver pyruvate kinase) serum neutralized 11 units of liver pyruvate kinase (where 1 unit equals 1μmol/min at 25°C), and 1ml of anti-(muscle pyruvate kinase) neutralized 23 units of kidney cortex pyruvate kinase and 30 units of muscle pyruvate kinase. Pyruvate kinase from liver was inhibited to a small extent by anti-(muscle pyruvate kinase) serum (4 units/ml of antiserum); there was, however, no cross-reaction between anti-(liver pyruvate kinase) serum and the enzyme in muscle or kidney. No reaction was seen when extracts were incubated with rabbit or chicken preimmune sera.

Antisera were diluted 100-fold with 0.9% (w/v) NaCl/50mM-potassium phosphate, pH7.4, before use in titrations with islet extracts.

Titrations were performed by adding different amounts of antisera (0–90μl) to a constant volume of islet supernatant (10μl) and buffer (0.9% NaCl/50mM-potassium phosphate, pH7.4) to a total volume of 100μl. Incubations were for 60min at 4°C. Addition of anti-(chicken immunoglobulin G) (20μl) was followed by incubation for 30min at 37°C and then for 60min at 4°C. After centrifugation for 5min (4°C, 15000*g*), the resulting supernatants were assayed for activity. Control incubations in the presence of preimmune sera and anti-(chicken immunoglobulin G) were included; results were identical with values found in the absence of any additions.

Results and discussion

The mean total activity of pyruvate kinase in islet supernatants was 1.35±0.05nmol/min per islet (mean±S.E.M. from 42 independent observations) at 5.0mM-PEP and 1.0mM-ADP; this value is similar to others reported (Sugden & Ashcroft, 1977; Sener *et al.*, 1980). The protein content was 0.39±0.05μg per islet (mean±S.E.M. for six independent observations).

Islet pyruvate kinase was neutralized by antisera directed against M_1- and M_2-type isoenzymes (Fig. 1). Antisera directed against L-type pyruvate kinase had no effect, suggesting that the enzyme in islets is unlikely to be of the L-type (Hall & Cottam, 1978).

The enzyme displayed Michaelis–Menten kinetics with respect to PEP and ADP (Fig. 2). The K_m for PEP was 0.75±0.05mM (mean±S.E.M. from three independent observations) as determined from

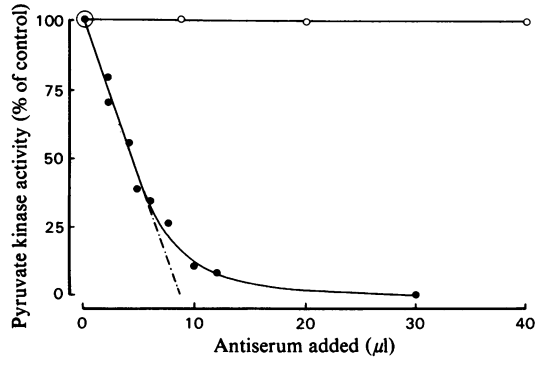

Fig. 1. *Immunotitration of islet pyruvate kinase with anti-(muscle pyruvate kinase) and anti-(liver pyruvate kinase)*

A constant volume of islet supernatant was mixed with different amounts of anti-(muscle pyruvate kinase) (●) or anti-(liver pyruvate kinase) (○) sera and incubated under the conditions described in the text. After centrifugation, enzyme activity was measured in the supernatant. Values were taken from two separate islet preparations; each point represents the mean of duplicate determinations from a single preparation expressed as a percentage of the control value measured at 5mM-PEP and 1.0mM-ADP in the absence of antisera.

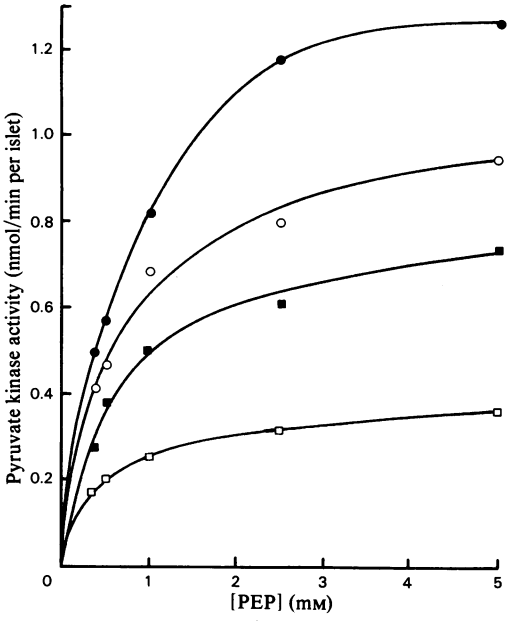

Fig. 2. *Activity of islet pyruvate kinase with various concentrations of PEP and ADP*

Islet preparation (10 μl) was assayed for pyruvate kinase activity as described in the text at various ADP concentrations: ●, 1.5 mM; ○, 0.6 mM; ■, 0.3 mM; □, 0.1 mM. Results are the means of duplicate determinations taken from a single experiment, which was typical of three separate experiments.

double-reciprocal plots and secondary plots of the intercept of the ordinate versus 1/[PEP]. This value is similar to those reported for the M_2 isoenzyme (Eigenbrodt & Schoner, 1977; Berglund & Humble, 1979), and is an order of magnitude greater than those found for the M_1 isoenzyme (Tanaka *et al.*, 1967). The K_m for ADP was 0.36 ± 0.01 mM (mean ± S.E.M. from three independent observations); this agrees well with published data (Hall & Cottam, 1978).

Further evidence supporting the sugestion of an M_2 isoenzyme in islets is gained from inhibition of enzymic activity by physiological concentrations of L-alanine and L-phenylalanine (Ishikawa, 1976) (Fig. 3). Under the conditions described, half-maximal inhibition occurs at 0.05 mM for both amino acids. Values are similar to those reported with the M_2 isoenzyme; incomplete inhibition by L-alanine was also observed, as predicted (Jimenez De Asua *et al.*, 1971; Carbonell *et al.*, 1973). The M_1 isoenzyme is not inhibited by L-alanine and requires a 10-fold higher concentration of L-phenylalanine for half-maximal inhibition (Berglund & Humble, 1979).

Fructose 1,6-bisphosphate activates the enzyme at low PEP concentrations (Table 1). It is, however, apparent from these experiments that the major effect of fructose 1,6-bisphosphate is likely to be as an antagonist of the inhibition by the amino acids; the relief of inhibition occurs at very low concentrations, well within the physiological range (Greenbaum *et al.*, 1971).

These experiments indicate that, when PEP carboxykinase is present in a tissue, the pyruvate kinase is of a potentially regulatory type, a finding well characterized in liver, kidney and adipose tissue. The heterogeneity of the islet makes it conceivable that PEP carboxylase and the M_2 isoenzyme of pyruvate kinase are in different cells; this seems unlikely, however, in view of the rather high enzyme activity reported here and elsewhere (Sugden & Ashcroft, 1977; Sener *et al.*, 1980). It is clear from immunological data that there is no L isoenzyme present in islets. It is, however, more difficult to show the total absence of the M_1 isoenzyme. Nevertheless, the experiments involving inhibition by L-alanine and L-phenylalanine indicate that such an activity could not contribute more than 10% of the total.

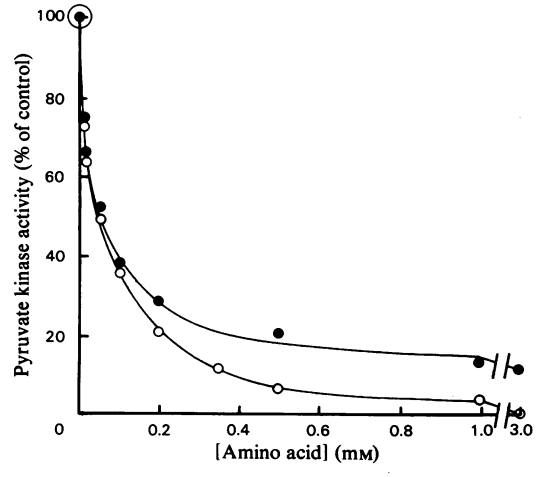

Fig. 3. *Effects of L-alanine and L-phenylalanine on islet pyruvate kinase activity*

Islet supernatant (10 μl) was assayed for pyruvate kinase activity as described in the text with PEP (0.05 mM), ADP (1 mM) and different concentrations of L-alanine (●) or L-phenylalanine (○). Results, as percentages of control values in the absence of inhibitors, represent the mean of three separate experiments; S.E.M. values were less than 10% in all cases and are omitted for clarity.

Table 1. *Effects of PEP on pyruvate kinase activity in the presence of fructose 1,6-bisphosphate, L-phenylalanine and L-alanine*

Islet supernatants were assayed for pyruvate kinase activity as described in the text. Rates were determined sequentially after the addition of L-phenylalanine or L-alanine and then fructose 1,6-bisphosphate to each cuvette, and are expressed as percentages of control values (at 1 mM-ADP) measured before the addition of effectors. Results are means ± S.E.M. for the numbers of separate experiments given in parentheses.

	Pyruvate kinase activity (% of control value)				
Addition ...	—	Alanine (1 mM)	Alanine (1 mM)	Phenylalanine (1 mM)	Phenylalanine (1 mM)
[PEP] (mM)	Fructose 1,6-bisphosphate (0.15 mM)	—	Fructose 1,6-bisphosphate (0.01 mM)	—	Fructose 1,6-bisphosphate (0.01 mM)
0.02	143 ± 5 (6)	—	—	—	—
0.04	139 ± 6 (4)	16.6 ± 2.7 (5)	112, 114 (2)	4 ± 4 (3)	93.3 ± 16.8 (3)
0.10	100, 100 (2)	21.4 ± 1.6 (5)	109, 113 (2)	5.0 ± 0.6 (3)	83, 86 (2)
0.50	—	33.7 ± 6.5 (3)	43.7 ± 6.5 (3)	10, 17 (2)	38, 43 (2)
1.00	—	28.7 ± 1.0 (5)	28, 32 (2)	10.0 ± 1.4 (4)	19, 34 (2)

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