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Purification and Properties of the Pyruvate Kinase of Sturgeon Muscle

By RICHARD F. RANDALL and PETER J. ANDERSON
Department of Biochemistry, University of Ottawa, Ottawa, Ont. K1N 6N5, Canada

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Pyruvate kinase was purified from sturgeon muscle in yields comparable with those obtained from the muscles of other species. In contrast with mammalian muscle pyruvate kinase the enzyme from sturgeon muscle gives a sigmoidal velocity curve with respect to phosphoenolpyruvate saturation, is activated by fructose 1,6-diphosphate, and is inhibited by bivalent copper ions. In these respects it is similar to the enzyme isolated from mammalian liver. The degree of interaction between phosphoenolpyruvate-binding sites is dependent on temperature.

Multiple forms of pyruvate kinase (EC 2.7.1.40) have been isolated from animal tissues (Kayne, 1973), with two major forms, designated the muscle and liver types, being found in mammals. Mammalian skeletal-muscle pyruvate kinase has been purified from rat (Tanaka *et al.*, 1967), ox (Cardenas *et al.*, 1973) and rabbit (Tietz & Ochoa, 1958) muscles. This form of the enzyme exhibits standard Michaelis–Menten kinetics under most conditions and is not activated by fructose 1,6-diphosphate. The liver enzyme has been purified from rat (Tanaka *et al.*, 1967), pig (Kutzbach & Hess, 1970) and ox (Cardenas & Dyson, 1973) liver. This form of the enzyme has a sigmoidal velocity profile with respect to phosphoenolpyruvate concentration and is activated by fructose 1,6-diphosphate.

Comparison of the structures of the two forms of pyruvate kinase, which are presumably homologous, might be expected to yield information useful in the correlation of protein structure and function and might be expected to facilitate X-ray crystallographic studies of pyruvate kinase (McPherson & Rich, 1972; Muirhead & Stammers, 1974). Preliminary structural studies have been carried out on muscle enzyme, which can be isolated in reasonable yield (Kayne, 1973). However, very little information is available about the structure of the liver enzyme, which appears to be unstable and hence has been isolated in very low yield. It would therefore be of value to isolate in reasonable yield a form of pyruvate kinase that exhibits sigmoidal kinetics with respect to phosphoenolpyruvate saturation. The enzyme from yeast can be purified in good yield, exhibits such kinetics, and is activated by fructose 1,6-diphosphate (Roschlau & Hess, 1972; Hunsley & Suelter, 1969). However, the smaller subunit size of this form of the enzyme and the large degree of evolutionary divergence between yeast and mammals suggest that the structural homology between mammalian muscle enzymes and the yeast enzyme may be too low to allow identification of the structural features that lead to the different kinetic behaviour.

Studies on semi-purified preparations of pyruvate kinase from trout muscle indicated that this enzyme exhibited kinetic properties similar to the enzyme from mammalian liver (Somero & Hochachka, 1968). It was therefore decided to purify pyruvate kinase from fish muscle in the hope that a form of the enzyme that showed allosteric saturation curves with phosphoenolpyruvate and yet was highly homologous to mammalian muscle enzyme could be obtained in good yield. The muscle of the river sturgeon (*Acipenser fulvescens*) was chosen as the source of the enzyme, since previous studies had shown that a high degree of homology existed between the glycolytic enzyme aldolase isolated from this source and mammalian liver and muscle aldolases (Gibbons *et al.*, 1970).

Experimental

Materials

Phosphoenolpyruvate, ADP, fructose 1,6-diphosphate, NADH, lactate dehydrogenase and rabbit muscle pyruvate kinase were obtained from BMC Ltd., Montreal, Canada. Whatman DE 23 and CM 23 Advanced Fibrous ion-exchange celluloses were obtained from Mandel Scientific Co., Montreal, Canada. All other reagents were the best grade available. Frozen sturgeon was obtained from Waldman's Fish Store, Montreal, Canada.

Pyruvate kinase assay

Pyruvate kinase activity was determined either directly by following the decrease in E_{230} (Pon & Bondar, 1967) or by using a coupled assay in which the disappearance of NADH is monitored spectrophotometrically at 340nm (Bücher & Pfleiderer, 1955). The assay volume was 0.25ml in a cell of 1mm

light-path for the direct assay and 2.5ml in a cell of 1cm light-path for the coupled assay. Both assays contained 2mM-ADP, 8mM-$MgCl_2$, 88mM-KCl in 50mM-Tris–HCl buffer, pH7.5, and 0.1mg of bovine serum albumin/ml. Phosphoenolpyruvate was used at a concentration of 4mM and assays were carried out at 30°C unless otherwise indicated.

Purification of pyruvate kinase from sturgeon

Extraction. Frozen muscle was ground in a meat grinder and extracted with 2vol. of a solution containing 2% (v/v) glycerol, 2mM-EDTA and 10mM-Tris base for 30min. The suspension was then strained through cheesecloth and the muscle re-extracted with 5mM-EDTA, pH5.8, containing 0.15M-KCl and the suspension strained through cheesecloth again. The extracts were combined and adjusted to pH5.4 by the addition of 5M-acetic acid and then centrifuged at 9000*g* for 15min in a Beckman JA-21 centrifuge. These and all subsequent procedures were carried out at 4°C.

Ammonium sulphate fractionation. $(NH_4)_2SO_4$ (24g/100ml) was added to the clear extract and the solution was stirred for 30min and then centrifuged at 15000*g* for 15min. Further $(NH_4)_2SO_4$ (16g/100ml) was added to the supernatant, which was then stirred for 30min and centrifuged at 15000*g* for 15min. The precipitate was dissolved in as small a volume of 5mM-EDTA, pH5.8, as possible and desalted by passage through a column (5cm×80cm) of Sephadex G-25 equilibrated with 5mM-EDTA, pH5.8, containing 0.1mM-dithiothreitol. All subsequent ion-exchange buffers contained 0.1mM-dithiothreitol.

Ion-exchange cellulose treatments. CM-cellulose CM 23 equilibrated with 5mM-EDTA, pH5.8, was added to the protein solution until pyruvate kinase activity just began to be adsorbed. The mixture was then centrifuged and the protein solution containing pyruvate kinase activity was poured off. The CM-cellulose was washed with an equal volume of 5mM-EDTA, pH5.8, and the washings were combined with the protein solution. The protein was concentrated by the addition of $(NH_4)_2SO_4$ (45g/100ml) and then redissolved in as small a volume of 5mM-Tris–HCl buffer, pH7.8, as possible and desalted on Sephadex G-25 equilibrated with 5mM-Tris–HCl buffer, pH7.8. The desalted protein was applied to a column (2.5cm×80cm) of DE 23 DEAE-cellulose, and the column was washed with 2vol. of 5mM-Tris–HCl, pH7.8, containing 0.04M-KCl. The pyruvate kinase was then eluted with a linear gradient formed from 400ml of 0.04M-KCl in 5mM-Tris–HCl buffer, pH7.8, and 400ml of 0.12M-KCl in 5mM-Tris–HCl, pH7.8. The pyruvate kinase activity eluted in this fashion was precipitated with $(NH_4)_2SO_4$ (45g/100ml) and recrystallized by the slow addition of $(NH_4)_2SO_4$ to the enzyme dissolved to a concentration of 10mg/ml in 0.1M-Tris–HCl, pH7.5, containing 1mM-dithiothreitol.

Protein determination

Protein concentration was measured by a modified biuret procedure (Itzhaki & Gill, 1964). For pure enzyme the absorbance at 280nm was used. An extinction coefficient of 0.55 at 280nm for a 1mg/ml solution and a 1cm light-path was calculated for the sturgeon enzyme.

Results

Table 1 summarizes the purification procedure for pyruvate kinase from 500g of sturgeon muscle. The specific activity of the crystals compares well with that obtained for pyruvate kinase purified from other sources (Kayne, 1973). The purified enzyme was stable for at least 6 months when crystallized from 0.1M-Tris–HCl buffer, pH7.5, containing 1mM-dithiothreitol. The preparation was at least 95% pure as judged from electrophoresis on polyacrylamide gels. For purified preparations of the enzyme it was necessary to add bovine serum albumin to the assay mixture to a concentration of 0.1mg/ml to prevent denaturation during the assay.

Fig. 1 shows the velocity profiles of the pure sturgeon enzyme obtained in the presence and absence of

Table 1. *Summary of the purification procedure for pyruvate kinase from sturgeon muscle*

The enzyme was purified from 500g of frozen sturgeon muscle. Experimental details are given in the text.

Stage	Total activity (1 μM substrate converted/min)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)
1. Supernatant of pH5.4 treatment	186000	14580	12.8	100
2. $(NH_4)_2SO_4$ fraction	137000	2400	57.1	74
3. Supernatant of CM-cellulose treatment	129000	1732	74.5	69
4. Eluate from DEAE-cellulose column	70000	278	251.8	38
5. Crystals	56000	199	281.4	30

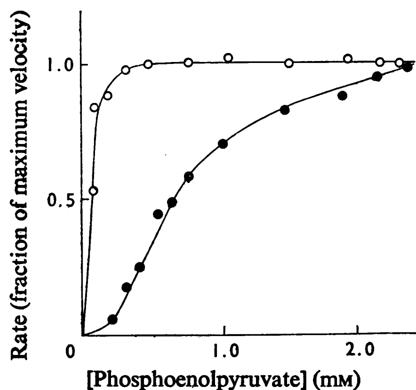

Fig. 1. *Substrate saturation curves for pyruvate kinase*

Sturgeon pyruvate kinase velocity profiles were determined in the absence (●) and presence (○) of fructose 1,6-diphosphate. Assays were carried out at 30°C under conditions described in the text. Bovine serum albumin was present at a concentration of 0.1 mg/ml to prevent denaturation and the concentration of fructose 1,6-diphosphate, when present, was 0.5 mM. Reactions were initiated by the addition of phosphoenolpyruvate to give the desired concentration.

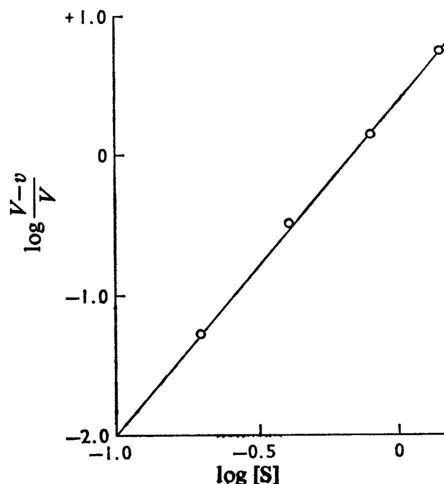

Fig. 2. *Hill plot of the data obtained for sturgeon muscle pyruvate kinase*

Rates were determined at 30°C for various concentrations of phosphoenolpyruvate. No fructose 1,6-diphosphate was present.

0.5 mM-fructose 1,6-diphosphate at 30°C. Under the assay conditions used, half the maximum velocity was obtained at a phosphoenolpyruvate concentration of 0.6 mM in the absence of fructose 1,6-diphosphate and at a concentration of 0.04 mM when fructose 1,6-diphosphate was present. Lowering the temperature of the assay had no effect on the substrate concentration required for half-maximum velocity when fructose 1,6-diphosphate was present at a concentration of 0.5 mM. However, in the absence of fructose 1,6-diphosphate, half-maximum velocities were observed at phosphoenolpyruvate concentrations of 0.25 mM at 20°C and 0.10 mM at 8°C.

Fig. 2 shows a Hill plot of the data for the sturgeon enzyme obtained at 30°C, which indicates that co-operative interactions between the binding sites for phosphoenolpyruvate occur. The Hill coefficient, h, was calculated to be 2.45. Plots of data obtained under the same conditions except for the addition of 0.5 mM-fructose 1,6-diphosphate gave a Hill coefficient of 1.31. Hill plots of data obtained at 20°C and 8°C in the absence of fructose 1,6-diphosphate gave coefficients of 2.11 and 1.27 respectively.

The effects of temperature and pH on the reaction catalysed by pyruvate kinase from rabbit muscle and sturgeon muscle were compared. When phosphoenolpyruvate was present at a concentration of 4 mM the reaction rate increased 3.25-fold over the temperature range 25–50°C for both enzymes. Fig. 3 shows the

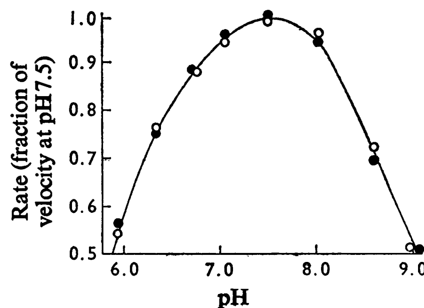

Fig. 3. *Effect of pH on pyruvate kinase activity*

Activity was measured as a function of pH at 30°C for enzyme isolated from rabbit (○) and sturgeon (●) muscle. Imidazole–HCl buffers (0.05 M in imidazole) were used in the pH range 6.0–7.5 and Tris–HCl buffers (0.05 M in Tris) were used in the range 7.5–9.0.

effect of pH on the pyruvate kinase reaction. Imidazole–HCl buffer was used in the pH range 6.0–7.5 and Tris–HCl buffer in the range 7.5–9.0. The curves were identical for both enzymes, with a pH optimum of 7.5 being observed in both cases.

Examination of the effects of two known inhibitors of the pyruvate kinase reaction were carried out. Fig. 4 shows the effect on initial rates of preincubating the enzyme at 30°C for 30 min at a concentration of 0.1 mg/ml in various concentrations of pyridoxal

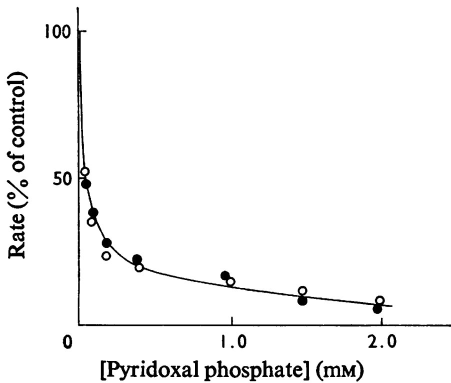

Fig. 4. *Effect of pyridoxal phosphate on pyruvate kinase activity*

Enzyme isolated from rabbit (○) and sturgeon (●) muscle was preincubated for 30 min at 30°C with various concentrations of pyridoxal phosphate. Experimental details are given in the text.

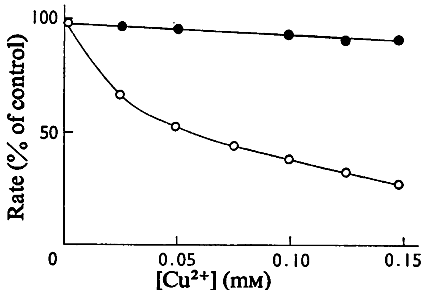

Fig. 5. *Effect of Cu^{2+} on the activity of pyruvate kinase from sturgeon muscle*

Cu^{2+} was present in the assay at the concentrations indicated. Fructose 1,6-diphosphate was either absent (●) or present at a concentration of 0.5 mM (○).

phosphate in 0.05 M-imidazole buffer, pH 7.5, containing 0.1 mg of serum albumin/ml. Preincubation with 2 mM-pyridoxal phosphate decreased the reaction rate to about 5 % of the control value for both rabbit muscle and sturgeon muscle pyruvate kinase. Cu^{2+} ions have been shown to inhibit mouse liver pyruvate kinase, but not the mouse muscle enzyme (Passeron *et al.*, 1967; Bailey *et al.*, 1968). The presence of fructose 1,6-diphosphate protects the liver enzyme from the effects of Cu^{2+}. Fig. 5 shows the effect of Cu^{2+} on the sturgeon muscle pyruvate kinase. About 75% inhibition is observed in 0.15 mM-Cu^{2+}. The presence of fructose 1,6-diphosphate almost completely prevents this effect. Similar experiments with rabbit muscle pyruvate kinase indicated that only about 5 % inhibition could be obtained in the presence of Cu^{2+} and that fructose 1,6-diphosphate had no effect on this.

Discussion

The procedure described allows the purification of pyruvate kinase in sufficient quantities for structural studies. The crystalline preparation was stable for at least 6 months at 4°C. Comparison with procedures that have been used to purify pyruvate kinase from mammalian sources indicates that the sturgeon enzyme is precipitated by concentrations of $(NH_4)_2SO_4$ similar to those that precipitate the mammalian muscle enzyme, but not the mammalian liver enzyme (Tanaka *et al.*, 1967). However, the sturgeon enzyme is adsorbed on DEAE-cellulose under conditions that allow the adsorption of mammalian liver enzyme, but not mammalian muscle enzyme (Tanaka *et al.*, 1967). It therefore appears that the sturgeon enzyme has certain structural features in common with each type of enzyme.

The major difference in kinetic behaviour between mammalian pyruvate kinases is in the velocity profile obtained by varying the concentration of phosphoenolpyruvate and the effect of fructose 1,6-diphosphate on it (Tanaka *et al.*, 1967; Cardenas *et al.*, 1973). The velocity profile for the sturgeon muscle enzyme and the effect of fructose 1,6-diphosphate on it are very similar to the mammalian liver enzyme. However, a dependence of the degree of co-operativity of phosphoenolpyruvate binding on the temperature was observed for the sturgeon enzyme. The decrease in the Hill coefficient and K_m value with decreasing temperature demonstrated in the present study has been shown by others in poikilothermic animals such as trout (*Thematomus bernacchii*) (Somero & Hochachka, 1968) and king crab (*Paralithodes camtshatica*) (Somero, 1969). Studies on the liver pyruvate kinase from the Arctic ground squirrel (*Citellus undulatus*) indicate that the Hill coefficient changes very slightly and that the K_m (substrate concentration giving half-maximum velocity) increases with decreasing temperature (Behrisch & Johnson, 1974). This enzyme shows co-operative binding of phosphoenolpyruvate and is activated by fructose 1,6-diphosphate. Since the behaviour of the enzyme from this source is different from that observed for the sturgeon enzyme under the same experimental conditions, the temperature effects appear to be due to a property of the enzyme rather than to an artifact of the assay system.

Although the enzyme from sturgeon muscle appears to be very similar to the rabbit muscle enzyme with respect to the effects of temperature, pH and pyridoxal phosphate on the reaction rate, the effect of Cu^{2+} is clearly very different. The inhibition by Cu^{2+} and the protection from this inhibition by

fructose 1,6-diphosphate observed for the sturgeon enzyme are very similar to that observed for the mammalian liver enzyme (Passeron *et al.*, 1967; Bailey *et al.*, 1968).

It would therefore appear that although some properties of the pyruvate kinase from sturgeon muscle are similar to those of mammalian muscle pyruvate kinases, the enzyme behaves like the enzyme from mammalian liver with respect to binding of the substrate phosphoenolpyruvate. The physiological reason for this is not clear, since carbohydrate metabolism is presumably more similar between muscles of various species, in that these tissues are primarily glycolytic, than between muscle and the gluconeogenic liver. Perhaps the same mechanism may be used for different purposes in the two species, with the ability to compensate for temperature effects being the reason for an allosteric form of the enzyme in the sturgeon muscle and the ability to control the relative rates of glycolysis and gluconeogenesis being of primary importance in liver.

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