KINETIC PROPERTIES OF PYRUVATE KINASE ISOLATED FROM RAT HEPATIC TUMOURS

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SUMMARY: The results demonstrate the existence of L and M forms of pyruvate kinase in rat hepatomas. Tumours were induced by feeding N-Nitrosodiethylamine. The kinetic properties of the L-type tumour enzyme was markedly different from the L-enzyme form found in normal liver. The L-form of tumour enzyme was purified by DEAE cellulose-Sephadex G200 chromatography (Sp. activity 41 units/mg). $MgADP^-/ADP^{2-}$ of 20/1 gave optimum activity for both the intrinsic and F1,6di-P stimulated reactions. ATP did not inhibit the enzyme. Alanine (2.5 mM) caused 60% inhibition at low PEP concentrations (0.25 mM). The homotropic effector (PEP) exhibited a complex allosteric pattern and saturation kinetics were not observed for either the intrinsic or F1,6di-P stimulated reactions with PEP concentrations as high as 10 mM.

INTRODUCTION: Following studies of pyruvate kinase from Novikoff hepatomas, it was suggested (1) that this enzyme may be a new isozyme, different from either the liver or muscle forms of the enzyme. Criss (2) using isoelectric focusing observed a unique pyruvate kinase isozyme in poorly differentiated hepatomas. A number of investigators (3,4,5) have shown by means of gel electrophoresis that an isozyme of pyruvate kinase was present in certain hepatomas that migrated like the form of this enzyme found in foetal rat liver.

Few studies have been reported of the kinetic properties of pyruvate kinase isolated from liver tumours. It was reported (6) that the pyruvate kinase of the rapidly growing hepatoma 3924A had kinetic properties different from the L-form enzyme found in normal liver and also from the

normal muscle enzyme. Because of the instability of the tumour enzyme it was not possible to demonstrate conclusively that this tumour enzyme was not allosterically activated by fructose 1,6-diphosphate.

Tumours induced in rats by the hepatic carcinogen N-Nitrosodiethylamine have been shown to have a high rate of glycolysis (7). In preneoplastic rats administered this carcinogen, marked decreases in the hepatic concentrations of phosphoenolpyruvate and triose-phosphates and increases in the hepatic concentration of lactate occurred (8), suggesting the activation of an enzyme or enzymes in the lower segment of the glycolytic pathway. This paper reports the isolation of two forms of pyruvate kinase from hepatic tumours induced in rats administered N-Nitrosodiethylamine. The unusual kinetic response of the L-form of the rat liver tumour pyruvate kinase to increasing concentrations of phosphoenolpyruvate suggests that the altered kinetic properties of this enzyme may increase flux in the lower segment of the glycolytic pathway.

METHODS: Male Wistar rats (initial body weight 150-200g) were administered N-Nitrosodiethylamine at a level of 50 mg/l in the drinking water for 16 weeks, followed by 6 weeks on a carcinogen-free diet, before the animals were sacrificed. Hepatic tumours were dissected from non-involved liver at $0^{o}C$ and freed of necrotic tissue before preparation of tumour homogenate. Histological features of tumours were examined by the procedures of Schmähl *et al.* (9). All of the experimental procedures used in these studies were as described by Irving & Williams (10).

RESULTS AND DISCUSSION: Two forms of rat hepatic tumour pyruvate kinase were isolated using the procedures described by Irving & Williams (10). The results in Table 1 show that there was a 320-fold increase in the specific activity of the L-form of rat liver tumour pyruvate kinase following DEAE-cullulose and Sephadex G-200 chromatography. It had been reported (6) that the L-form of pyruvate kinase isolated from the trans-

Table 1. Purification of Rat Liver Tumour Pyruvate Kinase

Extraction, isolation and assay procedures were as described by Irving & Williams (1973). The enzyme reaction was started with the addition of phosphoenolpyruvate to a final concentration of 1.0 mM. Values are for an extract obtained from 28 g of tumour tissue.

Step	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units /mg)	Yield %	Purification
Crude extract	130	520	4000	0.13	100%	1
0-45%-satd. $(NH_4)_2SO_4$ fraction	20	375	536	0.7	72%	5.4
$(NH_4)_2SO_4$ fractionation	3.0	305	105	2.9	59%	22.3
DEAE-cellulose	17	163	5	32.6	31%	251
Sephadex G-200	9	105	2.53	41.5	20%	319
45-55%-satd. $(NH_4)_2SO_4$ fraction	5	30	60	0.5	6%	3.8
55-65%-satd. $(NH_4)_2SO_4$ fraction	5	90	64.2	1.4	17%	10.8

plantable Morris hepatoma 3924A was very unstable in solution ($t_{½}$ = 4 min at 37°C), and it was for this reason that heat treatment of the $(NH_4)_2SO_4$ fraction was deleted from the procedure used here (10). However, the addition of sucrose (0.25 M) and dithiothreitol (0.25 mM) to all buffer solutions appeared to afford some protection to the enzyme because the loss of enzyme activity was not as marked as was seen in the partial purification of the rabbit liver enzyme (10).

The M-fraction of the enzyme sedimented in the 45-65%-satd. $(NH_4)_2SO_4$ fractions. This enzyme fraction showed similar properties to the M-form of the enzyme isolated from rabbit liver in that it did not absorb to DEAE-cellulose until the pH was raised to 8.5, and it exhibited Michaelis-Menten kinetics with phosphoenolpyruvate as the variable substrate.

The activation of the L-form of rat liver tumour pyruvate kinase by

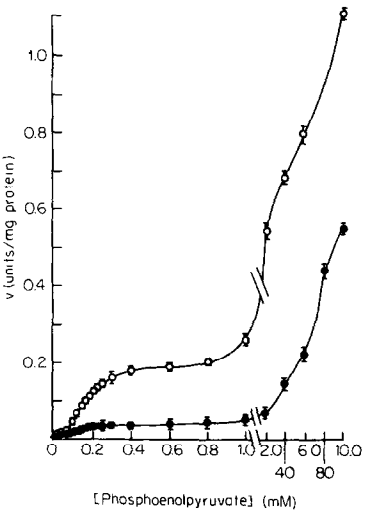

Fig. 1. Influence of Fructose 1,6-Diphosphate on the Relationship Between the L-Form of Rat Liver Tumour Pyruvate Kinase Activity and Phosphoenolpyruvate Concentrations at a Constant $MgADP^-$ Concentration (4 mM-$MgADP^-$, $MgADP^-/ADP^{2-}$ = 50:1)
The reaction mixture including enzyme was incubated at 30°C for 10 min, and the reaction was started by the addition of phosphoenolpyruvate and fructose 1,6-diphosphate. All concentrations of phosphoenolpyruvate were studied separately, and were not due to incremental additions of phosphoenolpyruvate to the assay mixture. Each experimental point represents quadruplicate determinations for each phosphoenolpyruvate concentration. ●, no fructose 1,6-diphosphate; ○, 0.1 mM-fructose 1,6-diphosphate. Standard errors for each experimental point are shown.

Mg^{2+} and ADP^{2-} was studied using the method of Irving & Williams (10). A $MgADP^-/ADP^{2-}$ ratio of 20:1 equivalent to a total Mg^{2+} concentration of 6.0 mM gave optimum activity for both the intrinsic and fructose 1,6-diphosphate-stimulated reactions. These experiments were carried out using $MgADP^-$ concentrations of 1 mM and 4 mM. The effect of increasing the concentration of $MgADP^-$ at a fixed $MgADP^-/MgADP^{2-}$ ratio of 50:1 was also studied. Saturation curves were obtained for both the intrinsic and fructose 1,6-diphosphate-stimulated reactions, with K_m values of 0.4 and 0.36 mM respectively, being recorded. All experiments were carried out using a final concentration of phosphoenolpyruvate of 0.25 mM.

The effect of increasing concentrations of phosphoenolpyruvate on the velocity of the L-form of rat liver tumour pyruvate kinase is shown

in Fig. 1. A sigmoid response was obtained for the L-form of the enzyme at low concentrations of phosphoenolpyruvate. However unlike the L-form of the rabbit liver enzyme, the activity of both the intrinsic and fructose 1,6-diphosphate-stimulated reactions continued to increase and saturation kinetics were not observed with 10 mM-phosphoenolpyruvate. This kinetic pattern was obtained with four different preparations of the enzyme. Approximate K_m values for the intrinsic reaction were in excess of 5 mM as was the K_m value for the fructose 1,6-diphosphate-stimulated reaction. Hill coefficients between 3 and 4 were obtained for the intrinsic reaction, and between 1 and 2.5 for the fructose 1,6-diphosphate-stimulated reaction. Reinvestigation of the kinetic parameters of the L-form of pyruvate kinase from normal rat liver revealed that saturation kinetics occurred with both the intrinsic and fructose 1,6-diphosphate-stimulated reactions; with K_m values of 0.7 mM and 0.15 mM and Hill coefficients of 2.9 and 1.1 respectively being recorded. These results indicate that there is a marked difference in the kinetic response of the L-form of the rat liver tumour enzyme to increasing concentrations of phosphoenolpyruvate in comparison with the kinetic properties of the L-form of the enzyme isolated from normal rat liver. It would appear that the tumour enzyme has a greatly increased capacity to catabolize phosphoenolpyruvate and this may be reflected in activation of the lower segment of the glycolytic pathway in rat liver tumours.

The response of the M-form of the tumour enzyme to increasing concentrations of phosphoenolpyruvate gave Michaelis-Menten kinetics with a K_m value of 0.54 mM. No activation by fructose 1,6-diphosphate was observed.

Study of the effect of pH on the activity of the L and M forms of the rat liver tumour pyruvate kinase at a constant phosphenolpyruvate concentration of 0.25 mM revealed that for both enzyme forms the maximum acitivity was recorded at pH 6.5, and the addition of fructose 1,6-diphosphate

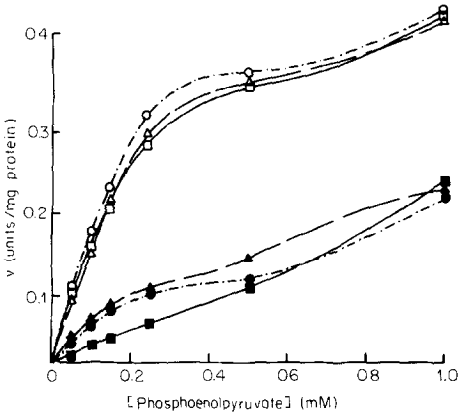

Fig. 2. Effect of ATP and Alanine on the Relationship Between the L-Form of Rat Liver Tumour Pyruvate Kinase Activity and Phosphoenolpyruvate Concentrations in the Presence and Absence of Fructose 1,6-Diphosphate
The reaction mixture, including enzyme, was preincubated at 30°C for 5 min, and then ATP or alanine was added to the cuvette and allowed to incubate at 30°C for a further 5 min before the reaction was commenced by the addition of phosphoenolpyruvate or phosphoenolpyruvate and fructose 1,6-diphosphate. All concentrations of phosphoenolpyruvate were studied separately and were not due to incremental additions of phosphoenolpyruvate to the assay mixture. Each experimental point represents triplicate determinations for each phosphoenolpyruvate concentration. ●, phosphoenolpyruvate with no fructose 1,6-diphosphate; ○, phosphoenolpyruvate plus 0.1 mM-fructose 1,6-diphosphate; ▲, phosphoenolpyruvate plus 2.5 mM-ATP; △, phosphoenolpyruvate, 2.5 mM-ATP plus 0.1 mM-fructose 1,6-diphosphate; ■, phosphoenolpyruvate plus 2.5 mM-alanine; □, phosphoenolpyruvate, 2.5 mM-alanine plus 0.1 mM-fructose 1,6-diphosphate. Reaction conditions were as described in the Experimental Section except that the Mg^{2+} concentration was doubled to give a total value of 33 mM.

did not affect the pH optimum of the L-form of the enzyme. As the pH was raised, the shapes of the curves for the intrinsic reaction of the L-form became increasingly sigmoidal (n_H = 1.9 at pH 6.5; n_H = 2.2 at pH 7.7) but the enzyme did not become more susceptible to fructose 1,6-diphosphate activation at low phosphoenolpyruvate concentrations. These results differed from those observed with normal rat liver (11) and normal rabbit liver (10) where it was observed that as the pH was raised, the value of the Hill coefficient for the intrinsic reaction increased, and there was increased fructose 1,6-diphosphate-stimulation as the pH increased. Furthermore, there was marked fructose 1,6-diphosphate-stimulation at pH 6.5 which was not observed with the L-form of pyruvate kinase isolated either from normal

rat or rabbit liver. These two kinetic properties which also distinguished the tumour enzyme from the normal enzyme are probably related to the unique response of the tumour enzyme to increasing phosphoenolpyruvate concentrations with non-saturating kinetics being observed with 10 mM-phosphoenolpyruvate.

Wood (12) reported that inhibition of pyruvate kinase by ATP may be attributed to chelation of Mg^{2+} by ATP, together with an inadequate control of pH. An equivalent amount of Mg^{2+} was added to prevent inhibition by ATP caused solely by the chelation of Mg^{2+}, and solutions of all effectors were adjusted to the experimental pH before assay. The results in Fig. 2 show that ATP (2.5 mM) in no way inhibited the intrinsic reaction for the L-form of rat liver tumour pyruvate kinase, and in fact some activation occurred at high phosphoenolpyruvate concentrations (1 mM). A slight inhibition (4%) of the fructose 1,6-diphosphate-stimulated reaction occurred in the presence of ATP, a feature not observed with the L-form of normal rabbit liver (10). Alanine (2.5 mM) was an effective inhibitor of the L-form of the tumour enzyme causing a 60% inhibition at low phosphoenolpyruvate concentrations (0.25 mM), although this inhibition was relieved with some activation occurring at higher phosphoenolpyruvate concentration (1 mM). Alanine also caused a 10% inhibition of the fructose 1,6-diphosphate-stimulated reaction. It would appear that the presence of either ATP or alanine in the assay mixture caused some steric hindrance to the L-form of the tumour enzyme preventing a full expression of the fructose 1,6-diphosphate-stimulated enzyme activity.

The kinetic patterns caused by interactions of ATP and alanine with the M-form of the rat liver tumour pyruvate kinase were identical to those found in muscle pyruvate kinase (13) and the M-form of rabbit liver pyruvate kinase (10). Even in the presence of additional Mg^{2+} to counteract any inhibition caused by chelation of Mg^{2+}, the M-form of rat liver tumour pyruvate kinase was inhibited by ATP (26% at 1 mM-phosphoenolpyruvate). Alanine was still the more effective inhibitor causing a 43% inhibition

in the presence of 1 mM-phosphoenolpyruvate. Fructose 1,6-diphosphate did not alleviate the inhibition by either alanine or ATP.

The unpublished report referred to by Weinhouse (14) that the three isozymes of pyruvate kinase could be separated by DEAE-cellulose chromatography was not observed in any of the preparations of tumour enzyme studied here. However the kinetic properties of the rat liver tumour enzyme are consistent with those of a new isozyme which has an increased capacity to catabolize phosphoenolpyruvate, and is not as sensitive to regulation of its activity by changes in the concentration of effectors.

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