THE KINETIC QUANTITATION OF ATP : D-GLUCOSE 6-PHOSPHOTRANSFERASES

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1. Introduction

It is currently held that the cytosolic fraction of rat liver contains four isozymes of ATP : D-glucose 6-phosphotransferase, three of which exhibit high affinities for D-glucose and are designated hexokinases [EC 2.7.1.1] and the fourth, namely glucokinase [EC 2.7.1.2], has a high K_m for glucose.

Sols et al. [1] postulated that glucokinase is unique to the liver parenchymal cell and that the hexokinase activity of liver extracts represents a contamination with non-parenchymal cells. Evidence for the existence of more than one form of ATP : D-glucose 6-phosphotransferase in rat liver extracts was furnished by Walker [2] who in a later report [3] found that the isozyme with the high K_m for glucose was highly adaptive, and decreased significantly in liver extracts from fasted and diabetic rats. Sols et al. [1] could not confirm the existence of residual glucokinase activity in livers from alloxan diabetic rats and attributed this to the interference by the unmodified hexokinase activity.

Fractionation of tissue extracts by chromatography on DEAE-cellulose columns [4–7] and by starch-gel electrophoresis [6–9] confirmed the kinetic evidence for the existence of high and low K_m-glucose ATP : D-glucose 6-phosphotransferases [2] and, in addition, resolved the dilemma around the Michaelis constants of the hexokinases obtained from various sources, which was due to the presence of various proportions of the isozymes in the different tissues. The isozymes were designated Types I–IV in accordance with their rates of mobility towards the anode upon electrophoresis, type IV being the fastest [7].

In non-hepatic tissues isozyme II is highly adaptive [10–15] and appears to parallel the sensitivity of the tissue to insulin [7] as does glucokinase [16].

The study of adaptive changes of the ATP : D-glucose 6-phosphotransferases in various tissues has, to date, been qualitative through the electrophoretic separation on starch-gels or on cellulose acetate membranes [17], or at most, semiquantitative where electropherograms were optically scanned. The quantitative approach of Hansen et al. [8] to the study of the adaptive changes in isozymes I and II of rat epididymal fat pads is the first kinetic approach to the problem, and the present study extends the procedure of Hansen et al. [8] to cover a range of tissues with diverse isozymic patterns. The differing kinetic properties of the four isozymes of ATP : D-glucose 6-phosphotransferase, in addition to their varying stabilities to heating [6] makes it possible to quantitate each of the four isozymes separately from their mixture in crude tissues extracts.

2. Materials and methods

D-glucose and D(–) fructose, glucose-free, were purchased from B.D.H. Tris (hydroxylmethyl)-aminoethane, ATP, $NADP^+$ and glucose 6-phosphate dehydrogenase were from Boehringer. 6-Phosphogluconate dehydrogenase was partially purified from rat liver according to the method of Glock and McLean [18].

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2.1. *Preparation of tissue extracts*

Adult albino rats of the Wistar strain were sacrificed by cervical dislocation and the appropriate tissues dissected out, weighed, finely minced with scissors and homogenized in 9 vol (v/w) of an ice-cold medium of the following composition: 150 mM KCl, 6.6 mM $MgCl_2$, 5.6 mM EDTA, 0.13 mM dithiothreitol, adjusted to pH 7.4 with $KHCO_3$. The homogenates were centrifuged at 105,000 *g* for 45 min in a Spinco model L centrifuge at 3°, and the clear supernatants were dialyzed for 1 hr against 100 vol of fresh homogenizing medium at 2°–4°. A portion of the dialyzed extract was heated in a water-bath at 45° for 60 min [6, 10] and subsequently kept on ice till assayed. All assays were carried on fresh extracts.

2.2. *Assay procedure*

The procedure is a modification of that of Sharma et al. [19]. The reaction mixture contained the following components in a final volume of 1.35 ml; 40 mM Tris-HCl pH 7.6, 15 mM $MgCl_2$, 7.5 mM ATP, 0.35 mM $NADP^+$, 2.5 units glucose 6-phosphate dehydrogenase and about 0.34 unit 6-phosphogluconate dehydrogenase. Glucose was added to a final concentration of 0.5 or 100 mM and fructose, when used, was 25 mM. The inclusion of 6-phosphogluconate dehydrogenase in the reaction mixture doubles the sensitivity of the method and allows accurate correction for the contribution of the endogenous enzyme to the reduction of $NADP^+$ [20]. The relative excess of D-glucose-6-phosphate ketol-isomerase [EC 5.3.1.9] over the ATP : D-glucose 6-phosphotransferases in crude extracts makes it unnecessary to add extra D-glucose-6-phosphate ketol isomerase to the reaction mixture when fructose is the substrate, but it must be added when purified enzymes are studied.

The reaction mixture is kept at 25° and the reaction is initiated by the addition of enough tissue extract to give a rate of not more than 0.10 absorbance unit/min. The rate of reduction of $NADP^+$ was continuously monitored in a Unicam SP 800 recording spectrophotometer fitted with a constant temperature cell housing. Rates were followed for at least 5 min but were linear for no less than 15 min. Blank cuvettes contained the entire reaction mixture, with water in place of the extract. These blanks are essential to correct for the "glucose dehydrogenase" activity of glucose 6-phosphate dehydrogenase, which is particularly significant at the high glucose concentration used.

A unit of enzyme activity is defined as that amount which produces 1 μmole of glucose 6-phosphate or 2 μmoles of NADPH per min at 25° [2] in the above assay system.

3. Results

The units of activity of ATP : D-glucose 6-phosphotransferase in the 105,000 *g* supernatant fraction of some rat tissues are given in table 1. The tissues were selected to encompass the four isozymes as well as the change in their profile during adaption as in the lactation cycle of the mammary gland.

Table 1
Activity of ATP : D-glucose 6-phosphotransferase in some rat tissues.

Tissue	A	B	C	D
Liver	0.217	0.174	0.370	1.750
Epididymal fat pad	0.068	0.043	0.340	0.305
Lung	0.250	0.217	0.391	0.325
Brain (whole)	1.520	1.500	1.806	1.770
Leucocytes (human)	0.140	0.095	0.194	0.115
Mammary gland:				
20 days pregnant	1.684	1.584	3.790	3.790
1 day lactating	1.565	1.525	5.647	5.593
14 days lactating	2.052	2.046	13.355	12.134
16 hr weaned[a]	2.231	2.175	12.513	8.371
2 hr resuckled[b]	2.113	2.113	13.169	11.630

[a] 14 days lactating rats were weaned by removing the litters 16 hr prior to sacrificing the mothers.

[b] Rats weaned as described above were replaced with their litters for 2 hr prior to sacrifice.

All results are the means of at least four animals and are expressed in units/g tissue except for mammary gland where they represent units/total gland to allow for the increase in weight of the tissue in weaning due to the accumulation of milk. Leucocyte activity is in units/mg protein, and are the means of 24 specimens. A, B, C and D are explained in the Results section.

The heating of tissue extracts of 45° for 60 min in the absence of substrates results in almost complete

Table 2
Some kinetic properties of rat ATP : D-glucose 6-phosphotransferase.

Isozyme	K_m Glucose	K_m Fructose	V fructose/ V glucose
I	5.0×10^{-5}	3.4×10^{-3}	1.15
II	2.5×10^{-4}	3.4×10^{-3}	1.15
III	7.0×10^{-6}	3.4×10^{-3}	1.15
IV	1.0×10^{-2}	2.0×10^{-1}	–

All constants are from published works, especially [6].

Table 3
Theoretical velocities of ATP : D-glucose 6-phosphotransferases.

Isozyme	Glucose 0.5 mM	100 mM	Fructose 25 mM
I	0.91	1.00	0.88
II	0.67	1.00	0.88
III	0.99	0.00	0.88
IV	0.05	0.83	0.01

The values given are the fractions of the corresponding V_{max} with the substrate indicated, and represent theoretical observed velocities (v_x).

loss of type II isozyme [6], 50% of type III isozyme [6, 11] and almost the entire activity of type IV isozyme [21]. In addition, type III isozyme has the distinguishing property of being inhibited at 100 mM glucose [6].

The ATP : D-glucose 6-phophotransferases obey normal Michaelis–Menten kinetics [6, 22] and it may therefore be expected that the following relationship would hold;

$$\frac{1}{v_x} = \frac{1}{V_x} \cdot \frac{K_m x}{[S]} + 1 \qquad (1)$$

where v_x is the observed velocity of isozyme x, V_x is its maximal velocity, $K_m x$ is its Michaelis constant for substrate S and [S[is the molar concentration of the substrate. From the above relationship the maximal velocity of any of the four isozymes ar any of the substrate concentrations used may be computed. As an example, the observed velocity of pure isozyme I at 0.5 mM glucose would be

$$\frac{1}{v_1} = \frac{1}{V_1} \cdot \frac{5 \times 10^{-5}}{5 \times 10^{-4}} + 1 \qquad (2)$$

$$\frac{1}{v_1} = \frac{1.1}{V_1} \quad \text{i.e. } V_1 = 1.1\, v_1 \qquad (3, 4, 5)$$

The above procedure was carried through for all four isozymes of ATP : D-glucose 6-phosphotransferase at 0.5 and 100 mM glucose and 25 mM fructose and the computed velocities are given in table 3. All the necessary kinetic data are compiled in table 2.

The observed velocities of isozymes I, II and III with 25 mM fructose as the substrate are 88% of the corresponding V_{max} (table 3), and since the V_{max} with fructose is 1.15 times higher than the V_{max} with glucose, it follows that the observed velocities with fructose closely approximate the theoretical V_{max} with glucose since $1.15 \times 0.88 = 1.01$.

Extending the above procedure to crude tissue extracts as presented in table 1 gives the following isozyme patterns:

A = Heated extract with 0.5 mM glucose
$= 0.91\, V_1 + 0.5\, V_3$ (6)

B = Heated extract with 100 mM glucose
$= 0.91\, V_1$ (7)

C = Unheated extract with 25 mM fructose
$= V_1 + V_2 + V_3$ (8)

D = Unheated extract with 100 mM glucose
$= V_1 + V_2 + 0.83\, V_4$ (9)

In order to calculate the activities of the four isozymes in the crude extracts manipulations of equations 6 to 9 would provide the required data. Thus,

$V_1 = 1.1\, B$ (10)
$V_3 = 2\,(A-B)$ (11)
$V_2 = C - (V_1 + V_3)$ (12)
$V_4 = \frac{D - (V_1 + V_2)}{0.83}$ (13)

Applying the above procedure to the data presented in table 1 yields the isozymic profiles given in table 4.

4. Discussion

The present approach to the quantitation of the isozymes of ATP : D-glucose 6-phosphotransferase in crude extracts of rat tissues completes the span of previous procedures [8, 9] by taking into consideration all four isozymes. The use of fructose for the assay of the total hexokinase [EC 2.7.1.1] activity is more advantageous than the use of glucose since the K_m for the former substrate is almost identical for all three isozymes, none of which is inhibited by fructose. The assumption of fixed heat-stable fractions of the isozymes is not unequivocal yet the error introduced is not greater than sampling errors, which makes the present procedure more sensitive in detecting minor isozymic changes than is the electrophoretic technique. Indeed table 4 indicates that activities as low as 1% or less of the total ATP : D-glucose 6-phophotransferase may be detected by the above procedure. This would not be possible with ionophoretic techniques since the time necessary to saturate one zone of activity with the stain may not be adequate to stain a zone of minor activity, and where one aims at staining a low activity zone, the more active zones would be underestimated upon optical scanning due to the saturation limit of the stained zone.

The study of Walters and McLean [10] on the changes in pattern of the isozymes of ATP : D-glucose 6-phosphotranferase in the cytosol of rat mammary gland during the lactation cycle indicated the presence of isozymes I and II. Isozyme I maintained its activity from pregnancy to the peak of lactation while isozyme II increased throughout in parallel with the increasing sensitivity of the tissue to insulin. These findings are confirmed in the present study (table 4) and contrast with the electrophoretic results of Shatton et al. [23] who demonstrated the predominance of isozyme I in the cytosol of both pregnant and lactating mammary glands with no change in isozyme II. However, the present study confirms the presence of isozymes III and IV in pregnant rat mammary gland extracts, the latter isozyme disappearing upon the establishment of lactation [23].

Table 4
The molecular species of rat tissue ATP : D-glucose 6-phosphotransferase.

Tissue	V_I	V_{II}	V_{III}	V_{IV}
Liver	0.191	0.093	0.086	1.766
Epididymal fat pad	0.047	0.243	0.050	0.018
Lung	0.239	0.086	0.066	0.000
Brain (whole)	1.650	0.116	0.044	0.000
Leucocytes (human)	0.105	0.000	0.089	0.013
Mammary gland:				
20 days pregnant	1.742	1.906	0.200	0.241
1 day lactating	1.678	3.889	0.080	0.031
14 days lactating	2.251	11.092	0.012	0.000
16 hr weaned	2.393	10.008	0.112	0.000
2 hr resuckled	2.324	10.845	0.000	0.000

For details see footnote to table 1. V denotes maximal velocity of the indicated isozyme.

The presence of glucokinase [EC 2.7.1.2] activity in non-hepatic tissues (table 4) does not support the view that glucokinase is unique to liver parenchymal cells [1], and implies that the corresponding gene may be as "common" as that of hexokinase. The higher glucokinase activity of hepatic tissue may only be a reflection of its high intracellular concentration of glucose with adequate supplies of insulin. Since hepatocytes are freely permeable to glucose [24] the intracellular glucose concentration may be as high as 20 mM postprandial, while in contrast, non-hepatic tissues with limited permeability to glucose may have an intracellular concentration of only a fraction of that of the extracellular fluid [25] so that even though insulin is available, only minor rates of glucokinase synthesis take place. This supports the view of Sato et al. [17] that where no glucokinase activity is detectable, it is due to lack of expression rather then deletion of the appropriate gene.

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