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ADIPOSE GLYCEROL KINASE: LOW MOLECULAR WEIGHT PROTEIN HAS TWO MICHAELIS CONSTANTS FOR GLYCEROL

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Summary: Using the improved methods, it was found that glycerol kinase activity is not only higher in adipose tissue than previously reported, but more importantly, the enzyme shows two Kms with respect to glycerol.

One of the Kms is in the micromolar range, while the other is in the millimolar range. The different distribution of the two Km activities in ammonium sulfate fractions, and the preferential inactivation of the high Km enzyme by heat and acid pH, suggest that the two Km activities may correspond to two different molecular species. The apparent molecular weight of the enzyme is 54,000 - 58,000 as determined by gel filtration.

Introduction: Glycerol kinase (2·7·1·30) was reported to be present in negligible amount or to be absent in white adipose tissue of animals and humans (1). Newsholme et al. (2) believed that the Km of this enzyme in adipose tissue in respect to glycerol may be similar to that of the liver enzyme, which is in the range of 10^{-5} M. Perisico et al. reported a Km of glycerol kinase of rat epididymal fat pad, without showing the kinetic analysis, to be 10^{-3} M (3). The corresponding Km of glycerol kinase of chicken and human adipose tissue was believed to be 10^{-4} M (4,5). No molecular weight of glycerol kinase of animal source has yet been reported (1). Using a modified and convenient radiochemical assay (6), more information regarding adipose tissue glycerol kinase has been obtained. It is a low molecular weight protein and possesses two discrete affinity constants for glycerol. Portion of this work has been reported as an abstract.

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Abbreviations: G3P, glycerol-3-phosphate; Km, the Michaelis constant: M_r the relative molecular weight; Vmax, the maximal velocity: QAE-sephadex, diethyl (z-hydropropyl) aminoethyl sephadex.

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Materials and methods: Male Sprague-Dawley rats (200-350 grams), Swiss mice (35-40 grams), and Leghorn non-laying hens were used throughout the study.

Preparation of glycerol kinase: Animals were killed by decapitation. Epididymal fat pads of rat and mouse, and perigizard fat of chicken were carefully dissected. Tissue was placed in two volumes of a homogenizing medium contianing Tris-HCl buffer, 10 mM, pH 6.6, sucrose, 0.25 M, and EDTA 1 mM. It was quickly and thoroughly minced, and then homogenized with an Ultraturax homogenizer (small probe) for 10 seconds. The homogenate was centrifuged in the cold (3°C) at 25,000 x g for 20 minutes. The floating fat cake and the pellet were discarded. The infranatant layer (below the fat cake) was referred to as the extract.

Ammonium sulfate fractionation: The glycerol kinase was first precipitated with ammonium sulfate (60% saturation). The sample was allowed to stand at 0°C for 30 minutes, and was then centrifuged at 25,000 x g for 20 minutes. The supernatant was collected. The precipitate was then dissolved in the same homogenizing medium. This fraction was defined as fraction I. The supernatant was then brought up to 93% saturation by adding more ammonium sulfate. After centrifugation, the precipitate was dissolved to provide fraction II. Glycerol kinase in both fractions was stable when stored at -70°C for several months.

In some experiments, the tissue extracts were fractionated with 40% saturated ammonium sulfate (fraction I-a), followed by 60% saturation (fraction I-b).

Assay system for glycerol kinase: The enzyme activity is measured by the isotopic method using ion-exchange column chromatography (6) to separate [^{14}C]-G-3-P from [^{14}C]-glycerol. The radioactivity of [^{14}C]-U-glycerol was 60-100 x 10^3 cpm, non-labelled glycerol was added to give the desired radiospecific activity. The final volume of the assay mixture was 120 µl (6). The desired protein content and time of incubation at 30°C were determined in preliminary protein-activity and time course experiments. At the desired protein concentration, glycerol kinase from rat and mouse epididymal fat pad was linear up to 20 and 30 minutes respectively at 30°C. The method for stopping the reaction, separating the reaction product from [^{14}C] glycerol, and determining radioactivity have been previously reported (6). The activity of the same enzyme preparation was independent of the radiospecific activity of glycerol in the assay system.

The reaction product was identical with G-3-P in two chromatographic systems as well as by QAE-Sephadex column chromatography (6). Protein content was determined by a dye binding method (7). Fat cells were isolated by the method of Rodbell (8).

The kinetic parameters were obtained graphically using the Hofstee plot and the double reciprocal plot (9), and also calculated using the computer programs of J.F. Woessner (University of Miami) as described by Hanson et al. (10) for fitting a single hyperbola by the maximum likelihood, and by Kowalik and Morrison (11) for fitting a double hyperbola by the method of gradient minimization.

Materials: [^{14}C]-U-glycerol (lots 1054-171 and 907-2110, 100-131 mc/mmole) was purchased from New England Nuclear Corp., Boston, Mass. The initial radiochemical purity was greater than 98.6 $\pm$ 1.3%. It was purified on QAE-Sephadex column before use. QAE-Sephadex A-25, particle size 40-120 µ was obtained from Pharmacia Fine Chemicals A.B. Sweden. Bio-Gel P-100, P-200 and P-300, 100-200 mesh were purchased from Bio-Rad Laboratories, Richmond, Calif.

Results: Apparent two Km values for adipose glycerol kinase of chicken, mouse and rat. Fig. 1 A, B, and C are the double reciprocal plot which show the effect of varying [U-^{14}C]-glycerol concentration on the velocity of the reaction catalyzed

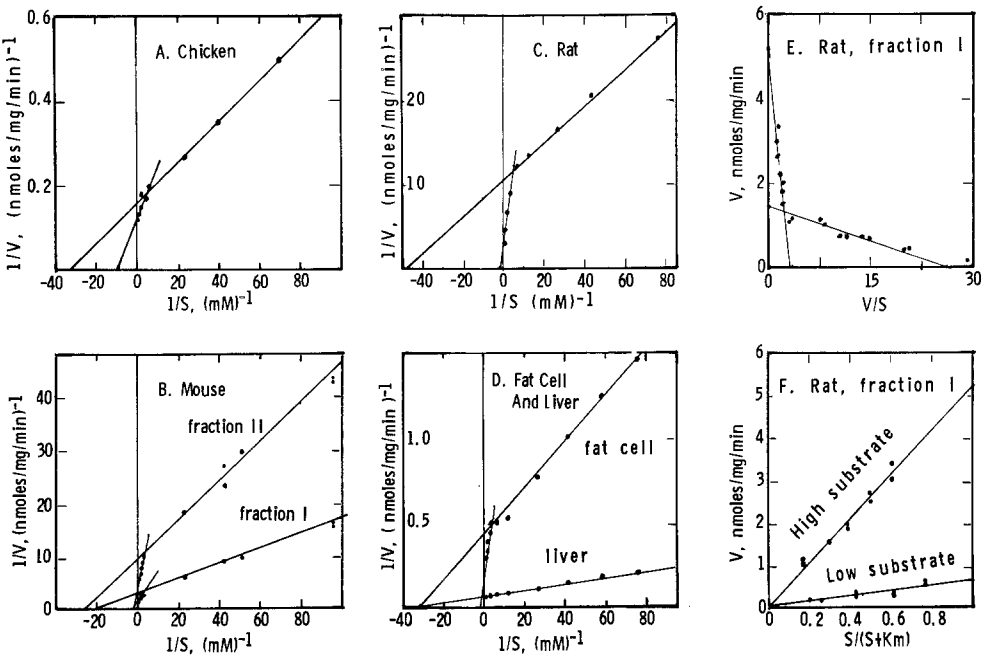

Figure 1. Kinetic anaylses of glycerol substrate-activity relationship of glycerol kinase of adipose tissue of chicken, mouse and rat.

Figure 1-A.B.C. Shows the double reciprocal plot of glycerol concentration vs. glycerol-3-phosphate produced by glycerol kinase from adipose tissue of chicken, mouse and rat respectively.

Figure 1-D. Shows the double reciprocal plot of glycerol concentration vs. glycerol-3-phosphate produced by glycerol kinase of rat liver and rat fat cells.

Figure 1-E. Hofstee plot of the same data as Figure 1-C.

Figure 1-F. Computer generated plot, V vs. S/(S + Km). Data as figure 1-C. Assays were conducted under linear range with respect to protein concentration and time. Glycerol concentrations were 0.01----1.67mM.

by the enzymes of chicken, mouse and rat respectively. All show two different slopes which extrapolate to give two different Km values with approximate values of 0.025 and 0.220 mM for the chicken enzyme, 0.040 and 1.50 mM for the mouse enzyme, 0.020 and 1.26 mM for the rat enzyme.

The maximum velocity (Vmax) for G-3-P formation was found to be o.58, 0.2-0.6, and 7-8 μmole/g/hr for chicken, mouse and rat enzyme respectively.

The same sets of data analyzed with the Hoftsee plot and with computer-generated lines, gave similar values of Km and Vmax. (Only the results of rat enzyme are shown, Fig. 1 D and Fig. 1 E.)

Table 1. SPECIFIC ACTIVITIES OF GLYCEROL KINASE IN FRACTION I AND II FROM MOUSE AND RAT ADIPOSE TISSUE.

Animal	Enzyme Preparation	Glycerol Kinase Activity nmoles/mg/min		Activity Ratio
		A at 0.02 mM Glycerol	B at 0.83 mM Glycerol	B/A
Mouse	Fraction I	0.21 ± 0.115 (n = 10)	0.84 ± 0.047 (n = 10)	4.09 ± 0.34 (n = 10)
	Fraction II	0.071 ± 0.026 (n = 10)	0.135 ± 0.16 (n = 10)	1.84 ± 0.158 (n = 10)
Rat	Fraction I-a	0.05	0.59	11.7
	Fraction I-b	0.07	0.38	5.4
	Fraction II	0.01	0.04	4.0

Activity is expressed as nmoles glycerol 3-phosphate produced per min. per mg of protein ± SEM. n represents number of glycerol kinase preparations. Each preparation was done using adipose tissue from a single mouse or several rats.

Glycerol kinase from rat fat cells: Fig. 1 F shows kinetic analyses of the fat cell enzyme activity obtained at different concentrations of glycerol. As can be seen from the double reciprocal plot, the glycerol kinase activity isolated from rat fat cells exhibited two Km values. This indicates that two activities of glycerol kinase originate from a single cell type, i.e. fat cells.

Glycerol kinase from rat liver: Rat liver glycerol kinase prepared in a manner identical to that of fat tissue exhibits only one apparent Km for glycerol of 0.03 mM (Fig. 1 F). The total activity was 98 μmole/g/hr. Both Km and Vmax are in agreement with previous values reported in the literature.

Differential distribution of high and low Km adipose glycerol kinase in ammonium sulfate fractions. Table 1 shows the difference of distribution of glycerol kinase activity between ammonium sulfate fractions I and II. This difference can be clearly expressed as a change of activity ratio when the enzyme was assayed in the presence of high (0.83 mM) and low (0.02 mM) concentrations of glycerol (Table I, last column).

Table 2 INACTIVATION OF GLYCEROL KINASE AT ROOM TEMPERATURE AND 50° C.

Enzyme	Room Temp. (hours)	50° C min.	G-3-P Formed, nmoles/mg/min Glycerol* 0.02 mM	Glycerol* 0.83 mM	Activity Ratio
Rat	0	-	0.37 ± 0.02 (10)	1.71 ± 0.06 (10)	4.6
	3	-	0.39 ± 0.01 (8)	1.98 ± 0.13 (8)	5.1
	6	-	0.28 ± 0.01 (8)	1.55 ± 0.05 (8)	5.5
	6	5	0.23 ± 0.01 (8)	0.59 ± 0.06 (8)	2.6
	6	30	0.29 ± 0.01 (8)	0.44 ± 0.04 (8)	1.5
Mouse	0	-	0.05	0.44	8.5
	6	-	0.05	0.25	4.5
	6	30	0.06	0.15	2.4

Glycerol kinase in fraction I of adipose tissue of rat and mouse was pre-treated at room temperature and/or 50°C in homogenizing medium, Tris buffer, 10mM, pH 6.6, an aliquot of 10 µl was used for each assay. Results shown are the mean ± SEM of 8-10 separate incubation tubes (rat) or mean of duplicates (mouse).
*Concentrations of glycerol used in each assay.

Thermal sensitivity of adipose glycerol kinase: When adipose glycerol kinase of mouse and rat were left standing at room temperature for 6 hours and then assayed at low (0.02 mM) and high (0.83 mM) glycerol concentration, no substantial loss of either activity was observed (Table 1). When enzyme was incubated at 50°C for 30 minutes, the activity of rat enzyme at high substrate decreased from 1.55 to 0.4 nmoles/mg protein/min (72% loss), wherease only 22% of the low Km activity was lost. The thermal sensitivity of mouse enzyme was similar to that of rat enzyme. The selective loss of high Km activity can be expressed as a decrease in high-to-low substrate activity ratio (Table 2, last column).

Fig. 2, left shows only one apparent Km (0.024 mM) of the heat-inactivated rat enzyme.

Inactivation of glycerol kinase at low pH: As indicated in Fig. 2, right, the "high Km" activity at high (0.83 mM) glycerol concentration was preferentially inactivated after treatment of the enzyme at pH 3.6.

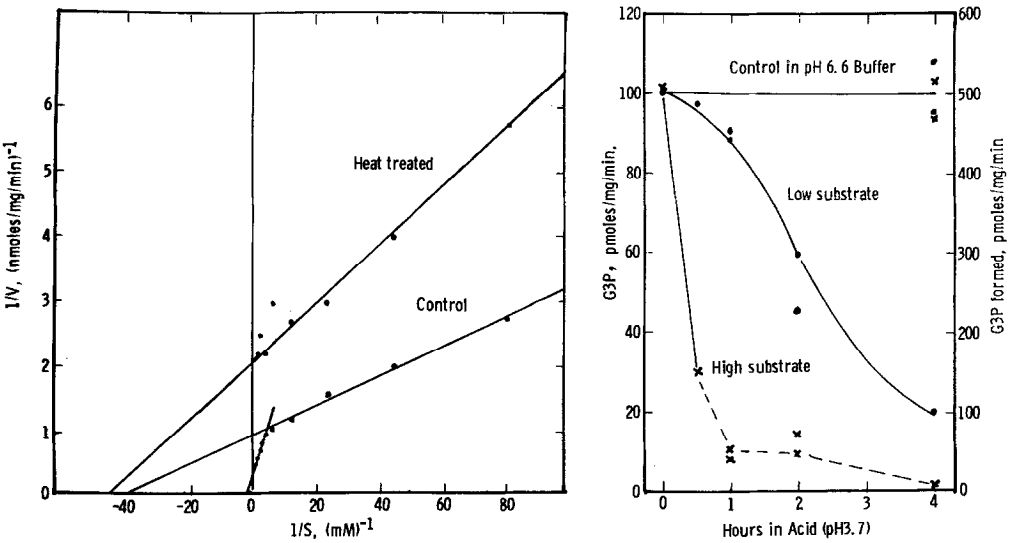

Figure 2. Thermal sensitivity and acid lability of high Km glycerol kinase of whole adipose tissue of rat.

Figure 2 left, Double reciprocal plot of enzyme activity vs. glycerol concentration of the enzyme before and after heating at the enzyme 50^{0}C for 60 minutes.

Figure 2 right, Time course of inactivation of glycerol kinase in acetic acid at pH 3.6. Left scale, (0----0), glycerol 20nmoles/ml, right scale, (x----x), concentration 833nmole/ml.

Determination of molecular weight of rat adipose glycerol kinase by gel filtration: Fig. 3 shows the elution pattern of glycerol kinase of rat adipose tissue of three separate preparations on Bio-Gel P-100, P-200, and P-300 columns. A molecular weight of 54,000 - 58,000 was calculated on the elution volume of four standard proteins. The enzyme obtained from rat liver by precipitation in 60% ammonium sulphate was similarly chromatographed on Bio-Gel P-300 and was eluted in almost the same volume as the adipose enzyme, suggesting approximately the same molecular weight for both enzymes (Fig. 3).

Discussion: It has frequently been reported that adipose tissue of mammalian species possesses low amounts of or no glycerol kinase (1). The apparently low amounts of enzyme were usually detected with a radiochemical assay of Newsholme et al. (2) in which a low glycerol substrate concentration was usually used in order to maintain a high radiospecific activity. Using a sensitive modification

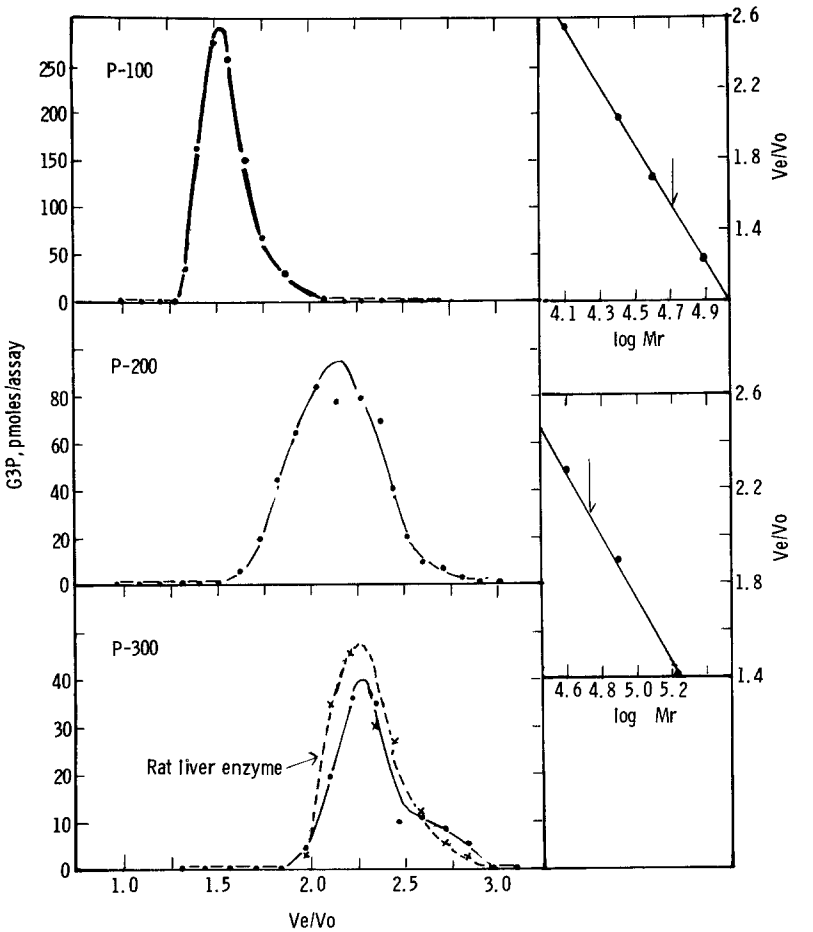

Figure 3. Determination of molecular weight of glycerol kinase by gel filtration. Glycerol kinase of rat adipose tissue was column chromatographed (1x15 cm column) on Biol-Gel p-100 (top panel), p-200 (middle panel), and p-300 (lower panel). Columns were eluted with the homogenizing medium and the enzyme activity immediately determined. The substrate concentration was 2.5 nmoles/assay. Top insert shows the molecular weight determination with Bio-Gel p-100 using transferin, oval albumin, chymotrypsin A and ribonuclease as standards. Lower insert shows M_r determination with Bio-Gel p-200. Rat liver glycerol kinase was also chromatograph on Bio-Gel p-300 column.

of the radiochemical assay (6), however, we are able to demonstrate significant amounts of enzyme possessing a high and a low Km value in chicken, mouse and rat tissue.

Using ammonium sulphate precipited enzyme, rather than the whole homongenate used by others, allows a better determination of the enzyme activity. High glycerol concentration with low radiospecific activity may be used in the present study.

The inhibition by known inhibitors such as G-3-P, AMP and FDP as well as endogenous glycerol (12-14) has been minimized.

The activity of adipose glycerol kinase of rat and mouse are considerably higher than values previously reported. Using the analytical method of Kowalike and Morrison (11), the high Km and Vmax were even higher than that obtained from the double reciprocal plot. The calculated Vmax (8 μmoles/g/hr) is 40 times higher than the values (0.2 μmoles/g/hr) reported by Robinson et al. (15).

The different distribution of the two Km activities in ammonium sulphate fractions and the preferential inactivation of the high Km activity at 50°C and at low pH may point to the presence of two different molecular species. However, attempts to separate the molecules responsible for the two Km activities from rat adipose tissue by physical means have not been successful and the possibility remains that the two Km values may be derived from two different interconvertable forms of the same molecule.

The molecular weight estimated as 54,000 - 58,000 in the present study is the first report concerning the molecular weight of the mammalian glycerol kinase. The enzyme extracted from rat liver under identical conditions may have a molecular weight as similar to that of the adipose glycerol kinase.

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