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CONCENTRATION OF SKELETAL AND CARDIAC MUSCLE PYRUVATE KINASE DETERMINED BY SPECIFIC RADIOIMMUNOASSAY

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Summary

A specific radioimmunoassay has been developed for rabbit skeletal muscle pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) and applied to extracts of skeletal and cardiac muscle from rabbits subjected to differing dietary states and alloxan diabetes. In animals fed Purina Checkers ® rabbit chow the concentration was 11.1 ± 3.3 μM in skeletal and 3.1 ± 0.6 μM (μmol/1000 g wet weight) in heart muscle. Compared to the chow-fed animals the enzyme concentration increased approximately 2-fold in both tissues after a 4 day fast or after fat feeding, without any change in enzyme activity. Using slab gel electrophoresis, we demonstrated that fat feeding and fasting evidently cause the muscle cell to accumulate inactive or less active, tetrameric pyruvate kinase. Alloxan diabetes had no influence on the concentration of the enzyme in chow-fed animals.

Pyruvate kinase activity was measured in the same extracts by a conventional assay. From the data specific activities were calculated: for example, 40 units/nmol skeletal muscle enzyme and 19 units/nmol heart muscle enzyme in chow-fed animals. It is only by the combined application of activity and chemical concentration assays to tissue extracts could such data be obtained. Thus, in addition to measuring enzyme concentration an approach is provided to detect differences in the catalytic state of the enzyme.

Our data show that assayable activity of this enzyme does not always reflect enzyme concentration and that regulation of enzyme concentration and enzyme activity can occur independently.

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Abbreviation: SDS, sodium dodecyl sulfate.

Introduction

Little is known about the intracellular concentration of tissue enzymes. Although assayable activity is often offered as a measure of concentration hardly ever is the enzyme concentration measured or a precise quantitative relationship established between activity and concentration [1]. We have been adapting radioimmunoassay techniques to the quantitative analysis of multiple enzyme proteins. In this paper we report on the development and application of a radioimmunoassay to rabbit muscle pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40), an enzyme whose activity has been reported previously not to be under hormonal or dietary control [2]. Our work was undertaken to answer three basic questions. What is the intracellular concentration of pyruvate kinase in rabbit skeletal muscle and cardiac muscle? Does the concentration of the enzyme change in response to dietary state or diabetes? Does assayable activity as measured by a conventional assay always reflect enzyme concentration?

Methods and Results

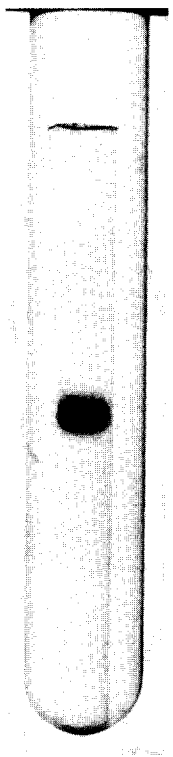
All methods not specifically described in this paper were identical to those discussed in the preceding paper [1].

Rabbit muscle pyruvate kinase was purchased from Boehringer Mannheim Biochemicals of Indianapolis, IN. The enzyme was further purified by preparative polyacrylamide gel electrophoresis [3] and when examined on dodecyl sulfate (5.6%, pH 7.4) gel electrophoresis (Fig. 1) was homogeneous. The enzyme had a specific activity of 240 units/mg and was used as antigen for preparation of antibody, as enzyme for ^{125}I labeling and as standard in competitive binding assays.

Pyruvate kinase activity was determined spectrophotometrically by modification of previously described assay [4] using 50 mM Tris-HCl (pH 7.6), 2.5 mM ADP, 7.5 mM $MgSO_4$, 75 mM KCl, 0.31 mM NADH, 5 units lactate dehydrogenase and 0.005—0.30 ml of enzyme solution. Phospho*enol*pyruvate (0.75 mM) was added to start the reaction. The final reaction volume was 2.0 ml and the assay was performed at 25°C.

Antibody to pyruvate kinase, raised in goats, was evaluated for ability to inhibit the enzyme in various tissues. 27 000 $\times g$ supernatants of 20% homogenates of skeletal muscle (anterior tibialis), heart (left ventricle), kidney, and liver were examined (Fig. 2). All of the enzyme activity in skeletal muscle and heart was inhibited by the antibody. Kidney and liver pyruvate kinase activity was not completely inhibited, indicating the presence of other isozymes of pyruvate kinase in those tissues. Double-diffusion studies revealed only a single precipitin line between antibody, crude or purified enzyme and extracts of the studied tissues.

The purified enzyme (15 μg) was iodinated using the chloramine T method [5]. Fig. 3 shows the results of gel filtration chromatography of ^{125}I-labeled pyruvate kinase on BioGel P-300 to separate fragmented enzyme generated by the iodination procedure from intact ^{125}I-labeled enzyme. The radioactivity in fractions 30—45 had identical mobility to native pyruvate kinase and when an



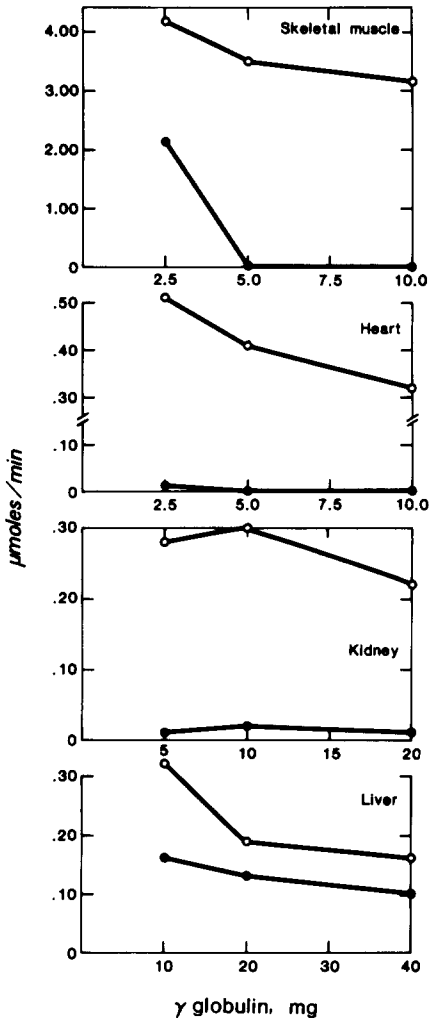


Fig. 1. SDS gel electrophoresis of purified pyruvate kinase. 3 μg of purified enzyme was boiled in 10 M urea/1% SDS for 10 min and then applied to the SDS gel. Electrophoresis was for 2 h at 2 mA/gel.

Fig. 2. Tissue enzyme inhibition studies. 25-μl aliquots of muscle, 50 μl of heart and kidney and 100 μl of liver extracts were incubated with increasing amounts of immune γ-globulin (●———●) or non-immune γ-globulin (○———○). The incubations were buffered at pH 7.6 with 100 μl of 0.4 M Tris-HCl and volumes adjusted to 610 μl with 0.154 M NaCl. Incubation was for 24 h at 4°C. 10 μl of 5000 × *g* supernatants were then assayed for pyruvate kinase activity.

aliquot (10 μl) from peak fraction 38 was electrophoresed in our SDS gel system identical mobility to unlabeled pyruvate kinase was observed (upper insert). An aliquot (20 μl) from fraction 53 was also electrophoresed and showed a slightly faster mobility (lower insert) indicating a smaller molecular weight protein. Fractions 30—45 were pooled and diluted to 25 000 cpm/50 μl of solution.

The immunologic integrity of the ^{125}I-labeled enzyme was evaluated by

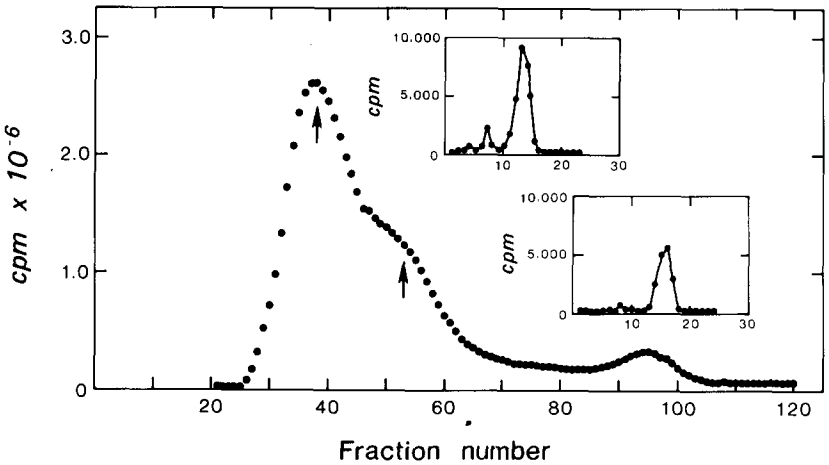


Fig. 3. BioGel P-300 chromatography of ^{125}I-labeled pyruvate kinase. 44 μCi of ^{125}I-labeled pyruvate kinase in 3.0 ml was applied to the column (1 X 100 cm). 1.0 ml fractions were collected. 10 μl of fraction 38 (upper insert) and 20 μl of fraction 53 (lower insert) were electrophoresed using our SDS gel system. The gels were then frozen and sectioned into 2.5-mm slices which were counted for radioactivity.

means of an antibody saturation curve. Increasing amounts of γ-globulin were incubated with 25 000 cpm of ^{125}I-labeled enzyme (10 ng). After a 24 h first-stage incubation a maximum of 87% of the ^{125}I-labeled pyruvate kinase was precipitated. Furthermore, 1.5 μg γ-globulin was needed to precipitate 40% of the label. Based on a time dependence study 48 h first-stage incubation was chosen as optimal and was, therefore, used in the radioimmunoassay. In all

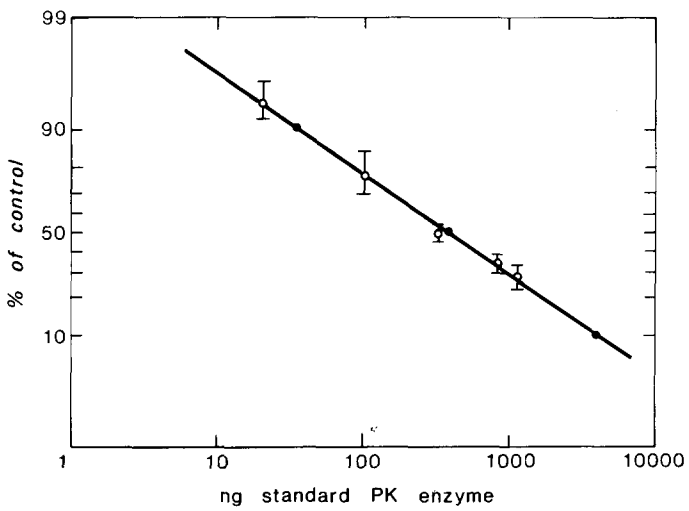


Fig. 4. Standard competitive binding curve. 25 000 cpm of ^{125}I-labeled pyruvate kinase was incubated with increasing amounts of unlabeled standard enzyme (0—1000 ng) and enough specific γ-globulin to precipitate 40% of labeled enzyme (no unlabeled enzyme present). ○, mean of eight values with S.D. shown as error bars. •, linearized computer-generated points.

TABLE I

CONCENTRATION, ASSAYABLE ACTIVITY AND SPECIFIC ACTIVITY OF SKELETAL MUSCLE PYRUVATE KINASE

Rabbits were subjected to differing dietary states as indicated by animal group. Diabetic and fasted rabbits prior to fasting were maintained on Purina Rabbit Chow. N, number of animals in each group. Values are given as mean ± S.D. The Student's t-test was used for statistical analysis. Carbohydrate animal group served as basis for comparison. See Methods and Results for description of diets. Total protein was determined by a modified Lowry method [14,15]. Concentration (μM) μmol/1000 g wet weight. Total protein is mg cytosol protein/g wet weight tissue.

Animal group	Activity (units/g wet wt.)	Concn. (μM)	Specific activity (units/nmol)	Animal weight	Total protein
Carbohydrate (N = 7)	421.37 ± 94.61	11.06 ± 3.31	40.03 ± 9.13	2165 ± 556	50.21 ± 1.71
Fat (N = 6)	489.82 ± 55.90	20.85 * ± 7.46	25.10 * ± 5.93	2088 ± 345	48.74 ± 2.76
Protein (N = 6)	461.69 ± 63.83	9.43 ± 2.26	50.18 ± 8.55	1907 ± 394	56.02 ± 8.49
4 day fast (N = 5)	441.31 ± 69.68	23.90 * ± 6.49	19.63 * ± 6.26	2031 ± 530	46.64 ± 4.01
Diabetic (N = 8)	472.23 ± 86.62	11.18 ± 2.44	43.29 ± 8.21	1978 ± 593	48.50 ± 4.91

* $P < 0.005$

studies a second-stage donkey anti-goat γ-globulin was used to insure complete precipitation of antigen-antibody complexes formed in the first stage.

A representative standard curve is shown in Fig. 4. As little as 30 ng of unlabeled pyruvate kinase could be measured with the radioimmunoassay assay. For tissue radioimmunoassay dilutions of 114 000 × g supernatants of

TABLE II

CONCENTRATION, ASSAYABLE ACTIVITY AND SPECIFIC ACTIVITY OF HEART MUSCLE PYRUVATE KINASE

Heart muscle was obtained from the same rabbits from which skeletal muscle was taken (Table I). N, number of animals in each group. Values are given as mean ± S.D. The Student's t-test was used for statistical analysis. Carbohydrate animal group served as basis for comparison. Total protein was determined by a modified Lowry method [14,15]. Concentration (μM) is μmol/1000 g wet weight. Total protein is mg cytosol protein/g wet weight tissue.

Animal group	Activity (units/g wet wt.)	Concn. (μM)	Specific activity (units/nmol)	Animal weight	Total protein
Carbohydrate (N = 7)	57.44 ± 8.67	3.13 ± 0.58	18.98 ± 4.66	2165 ± 556	42.67 ± 4.17
Fat (N = 6)	52.12 ± 9.90	4.10 *** ± 0.69	12.74 ** ± 1.54	2088 ± 345	41.14 ± 3.36
Protein (N = 6)	48.02 * ± 7.63	2.96 ± 0.51	16.30 ± 1.30	1907 ± 3.94	47.74 ± 7.72
4 day fast (N = 5)	48.31 * ± 5.83	5.15 ** ± 0.94	9.73 ** ± 2.55	2031 ± 530	38.39 ± 1.68
Diabetic (N = 8)	40.50 ** ± 12.62	2.51 ± 0.99	15.25 ± 4.23	1978 ± 593	36.20 ± 4.95

* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.01$.

1 : 100—1 : 2000 for muscle and 1 : 20—1 : 400 for heart were used.

The concentrations of pyruvate kinase for rabbit skeletal muscle and cardiac left ventricle are shown in Tables I and II. Also included are assayable activity measurements which allowed calculation of specific activities (units activity/nmol pyruvate kinase) of the enzyme. Table I shows that the assayable activity of skeletal muscle pyruvate kinase does not change significantly in the various dietary or diabetic states. Surprisingly the concentration of the enzyme did change. In the carbohydrate-fed rabbit the concentration was 11.06 ± 3.31 μM which doubled to 20.85 ± 7.46 μM and 23.90 ± 6.49 μM in the fat-fed and 4 day fasted rabbits, respectively. This resulted in a specific activity for the fat-fed and 4 day fasted rabbits of one-half of the carbohydrate-fed rabbit. The concentration and specific activity of pyruvate kinase in the diabetic rabbit were the same as the carbohydrate-fed normal rabbit.

In contrast the assayable activity of the heart pyruvate kinase (Table II) did show significant changes particularly in the diabetic rabbit. The concentration of the enzymes in heart paralleled changes seen in the skeletal muscle. The enzyme increased in concentration from 3.13 ± 0.58 μM (carbohydrate-fed rabbits) to 4.10 ± 0.69 μM and 5.15 ± 0.94 μM for fat and 4-day-fasted rabbits, respectively, and in each case resulted in a specific activity which was approximately one-half of the carbohydrate-fed rabbit. Thus both tissues showed increases in concentration without associated increases in assayable activity.

Slab gel electrophoresis (3.65% acrylamide solution, pH 8.35, using a Model 220 Vertical Slab Electrophoresis Cell, Bio-Rad Labs, Richmond, CA) was employed to determine if the increase in skeletal muscle pyruvate kinase concentration was due to tetrameric, dimeric or monomeric enzyme. The protein profiles of muscle extracts from fat-fed, 4-day-fasted, and carbohydrate-fed rabbits were identical. A duplicate slab gel was stained for pyruvate kinase activity using a modification of previously described methods [6]. Only one band of activity was observed in all three extracts, at identical position in each extract. Moreover, the position of the activity corresponded to the position of electrophoresed standard tetrameric pyruvate kinase. Finally, another duplicate slab was soaked in an anti-muscle pyruvate kinase antibody solution. The only region of precipitation from each extract corresponded to the position of pyruvate kinase activity. These results demonstrated that only tetrameric pyruvate kinase was being measured by the radioimmunoassay. Dimeric (and monomeric) pyruvate kinase, if present at all, were catalytically inactive and immunologically unreactive with our antibody preparation.

Discussion

Pyruvate kinase is a tetrameric enzyme having at least three distinct isozymes. The kinetic properties and tissue distribution of these isozymes have been well documented for many species. Studies with rat [2,7,8], human [8], bovine [9], and chick [10] tissues have disclosed many interspecies similarities for these isozymes. Current nomenclature is somewhat confusing as multiple systems are in use [9]. We prefer the use of type L, M, and K for isozyme designation. Type L is the major enzyme in liver and a minor component in many other tissues. The L type is allosterically activated by fructose-1,6-bis-

phosphate. Type M, which comprises the exclusive isozyme of skeletal muscle, is also found in heart and brain. The M type does not have any known allosteric activator. The K type is kinetically similar to L type but immunologically cross-reactive only with the M type. Type K is found in almost all tissues. Both the M and L-type isozymes have been reported in rabbit liver [11] and our data support this. In addition we have further evidence (not shown) to suggest the presence of a third isozyme in liver. The data presented in this paper (Fig. 2) clearly demonstrate that rabbit heart and kidney contain an isozyme immunologically similar to the M type. In addition the kidney contains some L-type isozyme (not shown). The nature of the isozyme in heart will be discussed later. In skeletal muscle we find only the M type. Thus, our data agree with the studies of others [8], indicating a tissue distribution of pyruvate kinase isozymes in rabbit similar to other species.

We used a conventional activity assay in this work with substrate concentrations chosen to be at least ten times their K_m values [12]. Furthermore, we demonstrated that the assay was sensitive enough to detect any change in catalytically active enzyme concentration up to a five-fold increase above normal values. Thus our activity measurements were an accurate reflection of the tissue activity of the enzymes.

The main objective of this work, however, was not to measure the tissue activity of pyruvate kinase, but to directly measure the tissue concentration of pyruvate kinase with a specific radioimmunoassay. The validity of our radioimmunoassay was based on five major criteria. (1) Highly purified rabbit muscle pyruvate kinase was used. (2) Antibodies were raised and shown to be specific for the rabbit muscle pyruvate kinase isozyme. (3) The purified enzyme has been radioiodinated without loss of quaternary structure or immunologic integrity. (4) An accurate and precise standard competitive binding curve has been generated. (5) Tissue proteins have been shown not to interfere with the competition between ^{125}I-labeled pyruvate kinase and unlabeled enzyme for antibody. Having successfully developed a precise and sensitive radioimmunoassay, we measured pyruvate kinase concentration in heart and skeletal muscle from rabbits subjected to differing dietary states and alloxan diabetes.

The results of the radioimmunoassay applied to skeletal muscle were surprising. The enzyme activity did not change in response to diet or diabetes, agreeing with the work of Tanaka et al. [2] with rat skeletal muscle pyruvate kinase. However, the enzyme concentration varied from 9.43 to 23.90 μM, depending on the dietary state of the animal. The change in enzyme concentration with fat feeding or 4 day fasting of the rabbits might be considered a function of water redistribution resulting in changes in total protein concentration, therefore, making our comparison on a per 1000 g wet weight invalid. However, no significant weight differences existed between the various animal groups and no diferences in total protein concentration in the 114 000 $\times g$ supernatants were observed (Tables I and II). Thus, the changes in concentration cannot be accounted for by an artifact of our analysis. A logical speculation for the increase in enzyme concentration and one supported by the data is the accumulation of a catalytically inactive tetrameric form which arises as a result of failure to degrade completely the inactivated enzyme or to process

completely an inactive precursor or 'pro' form of the tetrameric pyruvate kinase. Recent work in rat adipose tissue [13] suggests that a protease is synthesized in fasting and diabetic states which facilitates inactivation of pyruvate kinase. Perhaps a similar situation exists in rabbit skeletal muscle in which a protease responsible for pyruvate kinase degradation or activation is under tight metabolic control and is inhibited in the fat-fed and 4-day-fasted rabbit. It is interesting that in the alloxan diabetic rabbit the concentration of the pyruvate kinase did not increase, despite mean blood glucose concentrations of 400 mg%.

The skeletal muscle specific activity data from the carbohydrate-fed, protein-fed and diabetic rabbit correlates closely with that of purified enzyme: 200 units/mg tissue enzyme (averaged) compared to 240 units/mg purified enzyme. The tissue specific activity is probably a minimum since we have demonstrated that inactive enzyme can be measured by our radioimmunoassay. These data indicate that the radioimmunoassay assay is accurately disclosing the tissue enzyme concentration.

The results from the cardiac left ventricle studies differ from the skeletal muscle on two main points. The activity of the enzyme showed changes in response to 4 day fasting, high-protein diet and alloxan diabetes whereas no change in activity occurred in skeletal muscle. Since our inhibition studies show that all of the heart enzyme activity is inhibited with our anti-muscle pyruvate kinase γ-globulin, we initially assumed this would prove to be a reflection of changes in enzyme mass. However, concentrations by radioimmunoassay demonstrated something entirely unexpected: the specific activity of the heart enzyme is about one-half of the skeletal muscle enzyme. Clearly, the heart isozyme is kinetically different from the skeletal muscle. Whether or not the enzyme in these tissues will be distinct isozymes or the same isozyme under different regulation because of the tissue microenvironment remains to be proved. Nonetheless the heart enzyme concentration did change in a parallel fashion to the skeletal muscle enzyme, providing further support for the validity of our observations.

The radioimmunoassay has allowed us to quantitate the concentration of pyruvate kinase in rabbit skeletal muscle and heart. Most importantly, determination of these concentrations has given us the ability to measure changes in enzyme mass even when no changes in activity are observed, clearly demonstrating that the enzyme concentration can change independently of enzyme activity as measured by a conventional assay. The radioimmunoassay has also proven a powerful tool in determining differences in the catalytic nature of an enzyme from one tissue to another where application of standard methodologies revealed no differences. Furthermore, the radioimmunoassay allows calculation of the enzyme's specific activity, a necessary parameter for the study of enzyme and metabolic regulation.

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