Original Articles

Distinguishing the Type I and Type II Isozymes of Hexokinase

The Need for a Reexamination of Past Practice

John E. Wilson

The type I and type II isozymes of hexokinase coexist in insulin-sensitive tissues, such as cardiac and skeletal muscle and adipose tissue. Based on an early report that the purified type I isozyme was stable at 45°C whereas the purified type II isozyme was not, investigators in a number of studies have used heat lability as a criterion for distinguishing these isozymes in crude tissue extracts or subcellular fractions; that is, activity lost after incubation at 45°C was believed to be type II while remaining activity was considered type I. This extrapolation is dangerous because thermal lability can be markedly affected by the solvent environment, including the presence or absence of other proteins. In the present study, the rate of thermal inactivation of the type I isozyme has been shown to vary by at least an order of magnitude in soluble and particulate fractions prepared from rat heart and brain. Thus, the use of thermal stability as a general criterion for identifying the type I isozyme is invalid, and conclusions based on thermal inactivation as a means for distinguishing the type I and type II isozymes need to be reconsidered.
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The type I isozyme of hexokinase (ATP:D-hexose 6-phosphotransferase [EC 2.7.1.1]) can be described as a "housekeeping" enzyme, i.e., one that is widely expressed and catalyzes a reaction of fundamental metabolic importance in virtually all tissues (1,2). Expression of the type II isozyme is much more restricted in that it is found primarily in insulin-sensitive tissues, such as heart, skeletal muscle, and adipose tissue (1,2). Both isozymes exhibit a capacity for binding to mitochondria (1); this capacity is mediated by a hydrophobic NH_2-terminal sequence that is present, with slight differences, on both isozymes and is known to be both necessary (3,4) and sufficient (5,6) for targeting of these isozymes to mitochondria. Binding to mitochondria is thought to be of regulatory importance (1). Thus, there is general interest in assessing not only the relative amounts of the type I and type II isozymes, but also their distribution between mitochondrially bound (particulate) and nonbound (soluble) forms in response to altered metabolic or hormonal status.

Accurate quantitation of the type I and type II isozymes requires the ability to distinguish these isozymes in a reliable manner. Physical separation by ion-exchange chromatography or electrophoresis has proved convenient in some cases (7–9). Other investigators (10–18) have used a thermal inactivation method, attempting to distinguish the isozymes based on differences in heat stability. Total hexokinase activity in tissue extracts, or in soluble and particulate subfractions therefrom, is considered to represent the sum of type I and type II activities, whereas activity remaining after heating at 45°C for 45 min (11) or 60 min (10,12–18) is considered to represent the "heat-stable" type I isozyme; hence, the amount of activity corresponding to the "heat-labile" type II isozyme is calculated by the difference. This approach is based on the early observations of Grossbard and Schimke (7) that the purified type I and type II isozymes differed in thermal stability. In extrapolating the work of Grossbard and Schimke, the assumption is made that thermal stability or lability is an intrinsic property of these isozymes. However, thermal stability can be markedly affected by solvent conditions and other components that may be present, including ligands, other proteins, or subcellular structures with which the protein of interest may interact. Indeed, in work that has apparently been overlooked by those (10–18) citing Grossbard and Schimke (7) as support for the thermal denaturation method, Okazaki et al. (19) reported that under their conditions, both the type I and type II isozymes were inactivated to a similar extent by incubation at 37°C, that these isozymes were stabilized to quite different extents by addition of bovine serum albumin to the solutions, and that both forms were stabilized by interaction with mitochondria. Other studies apparently not considered were those demonstrating that the purified type I isozyme is rapidly inactivated by heating at 48°C (20), a temperature just slightly higher than the 45°C at which the type I isozyme is presumed to be stable (10–18), and that the type I isozyme expressed in COS-1 cells is inactivated to a significant degree (25% inactivation in 20 min) by incubation of crude cell extracts at 40°C (21).

In the present work, the thermal stability of the type I and type II isozymes was examined by the same method of thermal inactivation, and under the same conditions, used in several previous studies (10–18). The results demonstrate that thermal inactivation cannot be used as a general and reliable method for distinguishing the type I and type II isozymes in crude extracts. Thus, conclusions based on the use of the thermal inactivation method must be reconsidered.

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RESEARCH DESIGN AND METHODS

Preparation of tissue extracts and subcellular fractions. The procedure was based on that used by Mandarino and colleagues (14,16–18). Rat heart, which contains both type I and type II isozymes (8), was minced and homogenized using a Teflon-glass homogenizer in a buffer (5 ml/g fresh tissue) containing 50 mmol/l potassium phosphate, 2 mmol/l dithiothreitol, 2 mmol/l EDTA, and 20 mmol/l sodium fluoride, pH 7.4. This buffer (hereafter referred to as M buffer) was identical to that used by Mandarino and colleagues in their previous studies, except for deletion of several protease inhibitors, which proved unnecessary because proteolysis was not a problem under the conditions of these experiments (see below). Homogenates were centrifuged at 14,000*g* for 30 min at 4°C. The supernatant (soluble fraction) was carefully removed by pipette, and the pellet (particulate fraction) was resuspended in the original volume of M buffer containing 0.1% (vol/vol) Triton X-100. For some experiments, a soluble fraction containing almost exclusively the type I isozyme (1,2,7) was prepared from rat brain following this same procedure.

Assay of hexokinase activity. Hexokinase activity was determined spectrophotometrically, with glucose-6-phosphate formation coupled to NADPH formation, monitored at 340 nm, in the presence of excess glucose-6-phosphate dehydrogenase (22).

For thermal inactivation experiments, samples contained in 12 × 75 mm glass test tubes were immersed in a water bath maintained at 45°C. At indicated times, aliquots were removed for immediate assay of activity or were transferred to an ice water bath for subsequent analysis by electrophoresis.

Electrophoretic and immunoblotting procedures. Isozymes were separated by nondenaturing electrophoresis on cellulose acetate plates and were detected by staining for hexokinase activity as previously described (21). The latter was done by overlaying the cellulose acetate plate with an agar layer containing components of the standard spectrophotometric assay (see above) plus phenazine methosulfate and nitro blue tetrazolium, so that NADPH formation was coupled to deposition of the insoluble purple formazan at the regions containing hexokinase activity. Because prolonged incubation resulted in increased background staining and the removal of the agar layer resulted in loss of much of the formazan deposit, the plates were photographed by video camera with the agar layer intact immediately after specific color development and thermal prints were obtained, using the GDS-2000 gel documentation system from UVP, Inc. (San Gabriel, CA). The video image was also captured on disk for subsequent densitometric analysis of the staining pattern, using software associated with the gel documentation system. Electrophoresis was performed under denaturing conditions and in the presence of SDS, and immunoblotting was done as in earlier work (23). SDS gels were stained with Coomassie blue. Immunoblots were probed with a polyclonal (rabbit) antiserum against the type I isozyme purified from rat brain (22), diluted 1:1,500 in 1% gelatin in Tris-buffered saline. The secondary antibody was horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin (Bio-Rad, Hercules, CA), with detection using the tetrazolium method of Taketa et al. (24).

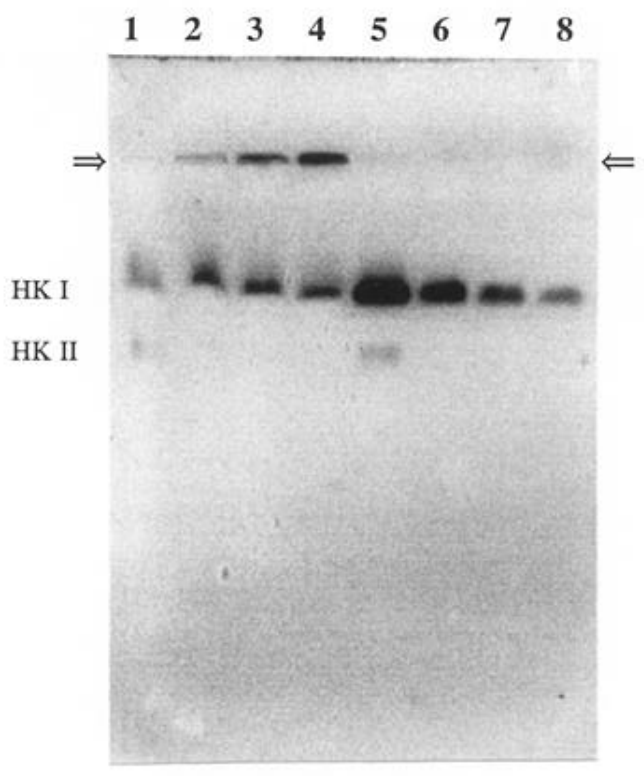

FIG. 1. Effect of heating at 45°C on type I and type II isozymes of hexokinase (HK) present in soluble and particulate fractions from rat heart, evaluated by nondenaturing electrophoresis on cellulose acetate. *Lanes 1–4* represent the soluble fraction from rat heart after heating at 45°C for 0, 30, 60, and 120 min, respectively. *Lanes 5–8* represent the particulate fraction after heating at these same respective times. Hexokinase activity in the unheated soluble and particulate fractions was 0.56 and 0.48 U/ml, respectively. The position of the origin is marked by arrows at right and left, and the bands corresponding to type I and type II isozymes are indicated at the left. For reasons explained in the text (see METHODS), the electrophoretogram was photographed immediately after color development, with the overlying agar layer containing staining reagents still present. This image is a photograph of the resulting thermal print; the latter accurately reflects the essential features of the electrophoretogram itself, although with some degradation of image quality.

RESULTS

Separation of type I and type II isozymes by cellulose acetate electrophoresis. As in the previous work of Katzen et al. (8), the type I and type II isozymes were found in both soluble and particulate fractions from rat heart (Fig. 1, *lanes 1* and *5*). In the experiment shown in Fig. 1, total similar activity was present in the unheated soluble and particulate fractions (0.56 and 0.48 U/ml, respectively). Yet, it is readily evident that the intensity of staining after electrophoresis was markedly less with the soluble fraction. This observation has been made in each of several experiments and is reasonably interpreted as indicating a difference in stability under the electrophoretic conditions. Why the type I isozyme in the soluble fraction should be less stable than the same isozyme in the particulate fraction is not known, but this discrepancy clearly indicates that stability is not determined solely by isozyme type.

Heating the soluble fraction at 45°C resulted in time-dependent formation of precipitated proteins and gave rise to increased staining at the origin because of adsorption of hexokinase to the denatured aggregate remaining at the origin (Fig. 1). Removal of the aggregate and adsorbed hexokinase by centrifugation abolished staining at the origin (results not shown). Adsorption stabilized the activity against inactivation during electrophoresis, with progressive increase in overall staining intensity (i.e., decreased loss of activity during electrophoresis) as the time at 45°C, and thus amount of aggregate with adsorbed hexokinase, increased (Fig. 1, *lanes 1–4*). Direct assay of the hexokinase activity present showed no increased activity (Fig. 2). It is evident that these complications make cellulose acetate electrophoresis, at least as performed in this study, unsatisfactory as a method of quantitating the type I and type II isozymes present in the soluble fraction from heart tissue. Nonetheless, it is clear from a qualitative perspective that the unheated soluble fraction contained both isozymes and, in accord with a greater thermal instability of the type II isozyme (7,19), that the band corresponding to type II hexokinase quickly diminished in intensity, being virtually undetectable after 30 min, whereas the band of type I activity remained readily detectable even after prolonged incubation at 45°C.

Quite different results were obtained with the particulate fraction from rat heart. Heating at 45°C again resulted in a

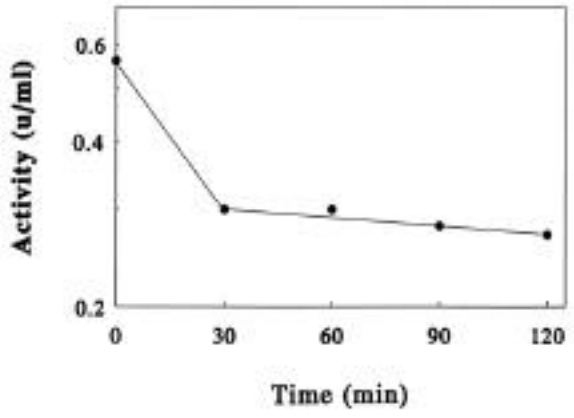

FIG. 2. Effect of heating at 45°C on hexokinase activity in soluble fraction from rat heart. Results are expressed in units per milliliter and are plotted in semilogarithmic format to demonstrate concordance with first-order kinetics of inactivation. Total activity present was determined by spectrophotometric assay as described in the text (see METHODS).

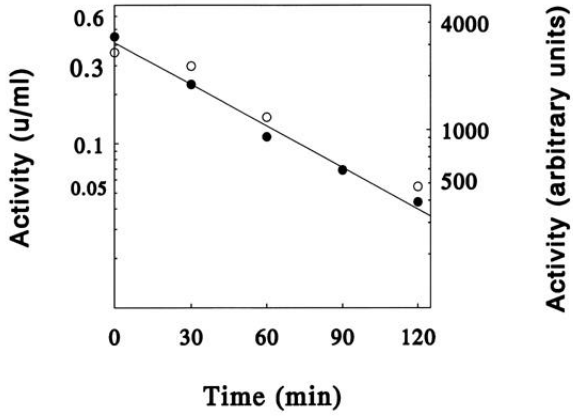

FIG. 3. Effect of heating at 45°C on hexokinase activity in particulate fraction from rat heart. Results are expressed in units per milliliter and are plotted in semilogarithmic format to demonstrate concordance with first-order kinetics of inactivation. Total activity (●) was determined by spectrophotometric assay, and activity of the type I isozyme (○) was determined by densitometric analysis after cellulose acetate electrophoresis, as described in the text (see METHODS).

rapid loss of the small amount of type II activity present and in a progressive and substantial decrease in type I activity with increasing time of incubation. These changes were readily apparent from visual examination of the electrophoretogram (Fig. 1) and were documented by densitometric analysis of the image (see below). Although precipitation also occurred during heating of the particulate fraction, in contrast to the situation with the soluble fraction, it did not interfere with the electrophoretic analysis.

Kinetic analysis of thermal inactivation. Loss of activity during incubation at 45°C was monitored by direct spectrophotometric assay. Results with the soluble fraction are shown in Fig. 2. There was a rapid initial decline in activity, likely corresponding to inactivation of the type II isozyme (Fig. 1). This rapid decline was followed by a much slower phase of activity loss corresponding to inactivation of the type I isozyme and consistent with the first-order kinetics ($r = 0.95$) previously seen in thermal denaturation of this enzyme (20); from the slope, the first-order rate constant for inactivation of type I hexokinase in the soluble fraction was determined to be 5.6×10^{-4} min^{-1}. In two other experiments, the slope was not distinguishable from zero. It is thus evident that thermal inactivation of the type I isozyme in the soluble fraction from heart tissue is extremely slow.

Results were again quite different when inactivation of hexokinase in the particulate fraction from heart was examined (Fig. 3). Loss of activity associated with rapid inactivation of the type II isozyme was not apparent, reflecting the overwhelming predominance of the type I isozyme in this fraction (8) (Fig. 1). Inactivation kinetics were consistent with a single first-order process ($r = 0.994$ for linear fit of the data shown), with a rate constant of 8.5×10^{-3} min^{-1}; the mean ± SD from three experiments was $5.3 \pm 2.8 \times 10^{-3}$ min^{-1}. Moreover, densitometric analysis of the type I band after cellulose acetate electrophoresis (i.e., area under peak obtained by scanning an electrophoretogram as shown in Fig. 1) also gave results consistent with first-order inactivation; linear fit ($r = 0.988$) of the densitometric data (Fig. 3) produced a rate constant of 6.6×10^{-3} min^{-1}, in reasonable agreement with the rate constant determined by direct assay of activity in this same experiment (8.5×10^{-3} min^{-1}).

Inactivation of type I hexokinase in the soluble fraction from rat brain was determined under these same conditions. In each of two experiments, first-order inactivation kinetics were observed with $r = 0.83$ and $r = 0.91$, and rate constants of 1.2×10^{-3} min^{-1} and 1.9×10^{-3} min^{-1}, respectively.

Inactivation, not degradation. Analysis by denaturing gel electrophoresis (Fig. 4*A*) demonstrated no change in the protein composition of either soluble or particulate fractions from heart tissue as a result of heating at 45°C, nor was there a decrease in intensity of the band of type I hexokinase seen on immunoblots (Fig. 4*B*). These results clearly demonstrate that no significant proteolysis occurred under these conditions and that the activity loss was truly thermal denaturation, not proteolytic degradation of the isozymes.

Effect of Triton X-100 on thermal stability of type I hexokinase. As noted in METHODS, the soluble fraction was prepared in M buffer alone, whereas the particulate fraction contained M buffer plus 0.1% Triton X-100. The obvious difference in the stability of type I hexokinase in the soluble and particulate fractions from heart tissue raised the question of whether the greater lability of the type I hexokinase in the particulate fraction could be attributed to the presence of Triton X-100. This was not the case (Table 1). Thus, approximately half of the activity was lost after incubation of the soluble fraction for 1 h at 45°C; much of this loss was attributable to inactivation of the type II isozyme (see above). Addition of 0.1 or 0.25% Triton X-100 did not increase the inactivation beyond that seen in the absence of Triton. Surprisingly, quite different results were seen with the type I isozyme in the soluble fraction from brain tissue. Thus, addition of Triton X-100 to the soluble fraction resulted in marked increases in inactivation (Table 1).

DISCUSSION

These results demonstrate the unreliability of the thermal inactivation method for differentiating type I from type II activities in soluble or particulate fractions from tissue extracts. This study and others demonstrate that the type II isozyme is more heat labile than the type I isozyme (7,19,21).

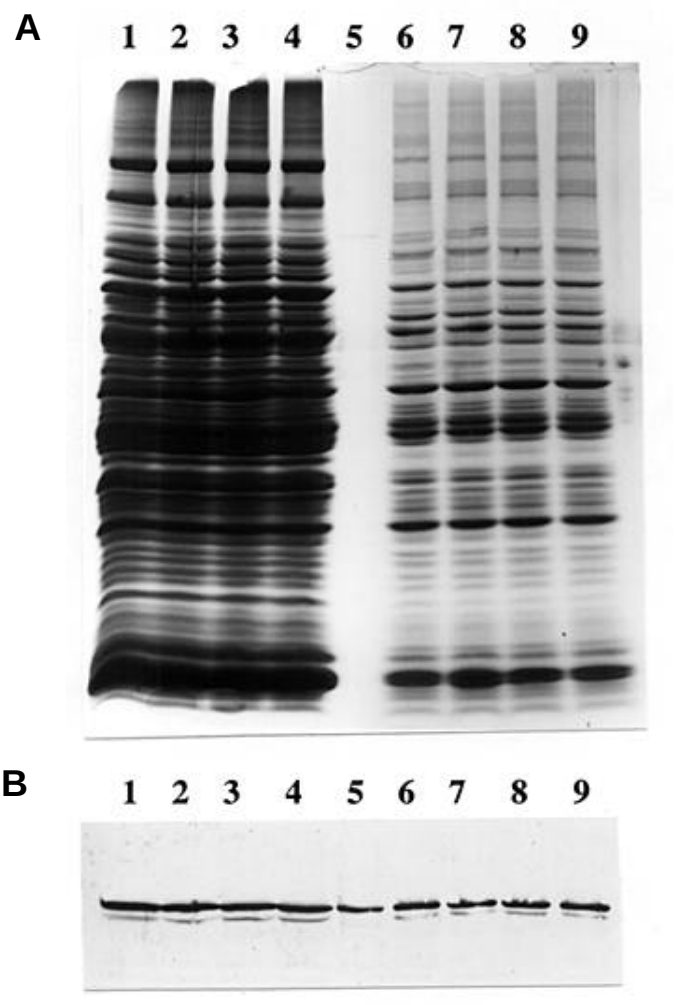

FIG. 4. Effect of heating at 45°C on protein composition and type I hexokinase content of soluble and particulate fractions from rat heart. *A*: Proteins present in soluble fraction (*lanes 1–4*) and particulate fraction (*lanes 6–9*) were analyzed by SDS gel electrophoresis after heating at 45°C for 0 (*lanes 1* and *6*), 30 (*lanes 2* and *7*), 60 (*lanes 3* and *8*), and 120 (*lanes 4* and *9*) min. *Lane 5* contains purified type I hexokinase (22) as a marker. *B*: Immunoblot was prepared from gel shown in *A* and probed with antiserum against purified type I hexokinase. The faint band just below the major band corresponds to a minor proteolysis product that may occur in vivo (22); clearly, it is not produced during the incubation at 45°C because it is present at equal intensity at all times of incubation.

However, the assumption that the type I isozyme is heat stable or is inactivated at a negligible rate is invalid. Furthermore, the rate of inactivation of the type I isozyme has been shown to vary by at least an order of magnitude in the soluble and particulate fractions from rat heart, and there is a severalfold difference between the rates of inactivation of the type I isozyme in soluble fractions from heart and brain. It should be emphasized that these differences were seen under otherwise identical conditions. It is thus apparent that the stability of the type I isozyme, and the effect of Triton X-100 on that stability, depend on other factors present in these crude extracts. Although we cannot yet identify these factors, we can say that they are highly unlikely to be proteases. Rather, the point is that the assumption that type I hexokinase represents an intrinsically heat-stable form is demonstrably false, with the rate of inactivation (in crude extracts) varying

TABLE 1
Effect of Triton X-100 on rate of inactivation of hexokinase in soluble fractions from rat heart and brain

	% Original activity remaining after 1 h at 45°C	
% Triton X-100 (vol/vol)	Heart	Brain
0	51	78
0.25	51	28
0.5	45	16

significantly with tissue of origin or subcellular fraction used. The amount of type II hexokinase present, assuming that it is the only heat-labile form, will be overestimated, with the error depending on the inactivation rate of type I hexokinase in the particular fraction under study; in the case of the particulate fraction from heart tissue (Fig. 3), a gross overestimation would result and lead to the conclusion that the amount of type II isozyme present had increased with increasing time of inactivation, an obvious absurdity.

Noting that the type II isozyme has a somewhat higher K_m for glucose than does the type I isozyme, Mandarino et al. (14) showed that heating a "muscle mitochondrial" extract, thought to contain 83% of the activity as type II, resulted in a decrease of the apparent K_m for glucose into the range expected for the type I isozyme. This finding is consistent with the greater thermal lability of the type II isozyme, but that point is not in dispute and is, in fact, confirmed by the results of the present study. However, the fact that residual hexokinase activity (presumably, mostly the type I isozyme) has the expected K_m reveals nothing about how much of the original type I activity remained (i.e., its thermal stability) and cannot be considered validation of the heat inactivation method for distinguishing these isozymes.

A correlation (r = 0.53) was claimed to exist between changes in type II hexokinase activity, determined by the thermal inactivation method, and changes in type II hexokinase protein, determined by densitometric analysis of immunoblots (16). However, there is wide scatter in the data, and with a single exception, all points for the soluble fraction lie well below those for the particulate fraction. If indeed there is a correlation between activity and protein, why should there be this clustering of points obtained with the different fractions? The explanation may lie in the observations presented here, that is, that the stability of the type I isozyme differs in the soluble and particulate fractions and, therefore, that estimates of the amount of type II hexokinase by the thermal inactivation method are in error to different extents in these fractions.

While these results bring into question the general validity of the thermal inactivation method, they also indicate that cellulose acetate electrophoresis, at least as performed in the present study, is not a universally applicable method for quantitation of these isozymes. Although the method seems to work well for the particulate fraction from heart, it clearly does not perform satisfactorily with the soluble fraction from this same tissue. These results thus emphasize the need for documenting the reliability of the chosen technique, whatever it may be, with the particular tissue and subcellular fractions of interest. This documentation is particularly important for

studies using human biopsy material because such studies are associated with special cost and ethical considerations.

ACKNOWLEDGMENTS

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Q3: Is “Hexokinase activity...monitored at 340 nm” correct as edited, i.e., it is hexokinase activity that is being monitored? (Original text was unclear.)>
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