

Inactivation and Reactivation of Hexokinase Type II¹

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Hexokinase isozyme II which loses activity rapidly in the absence of glucose ($t_{1/2} \approx 10$ min) is stabilized in the presence of glucose-6-P, P_i , and ADP when glucose is also present but not by kinetically inert analogs. Enzyme inactivated by incubation in the absence of glucose is fully and rapidly recovered ($t_{1/2} \approx 10$ min) by addition of both glucose and mercaptoethanol, each at 0.1 M. In the presence of 0.1 mM glucose, both glucose-6-P and P_i facilitate the reactivation. Reactivation proceeds in two steps both with unfavorable equilibria: a fast reduction followed by a slow renaturation. Native enzyme is much more resistant to irreversible inactivation by trypsin than is enzyme that has lost its activity by incubation in the absence of glucose. The latter form shows no protection from trypsin action by glucose. Streptozotocin-diabetic rats that have lost hexokinase II preferentially in their insulin-sensitive tissues do not contain an activatable form of hexokinase in at least one of these, heart. The greater sensitivity of inactivated hexokinase to denaturation by trypsin suggests that such a "reservoir" form may be destroyed rapidly *in vivo*. Glucose may be important in determining the steady-state level of hexokinase II by "guiding" the folding of translation product. In this view insulin would act through its effect on glucose permeability.

Mammalian hexokinase [EC 2.7.1.1] isoenzymes distinguished electrophoretically and by ion-exchange chromatography are characterized by a low K_m for glucose and a low K_i for glc-6-P (1, 2). Among the three types observed in the various rat tissues, type II has been reported to be most responsive to varying nutritional and hormonal conditions (3-9). In particular, only the activity of type II is reported to decrease in the epididymal fat pad in fasting (2, 10) and to decrease in drug-induced diabetes (3-6). Many of these comparisons have been only approximate, comparing the intensity of electrophoretic gels developed with the glc-6-P dehydrogenase and NADPH reduction indicators (2). In such studies it has been reported that incorporation of 5 mM mercaptoethanol in the electrophoretic medium tended to prevent the disappearance of type II from diabetic fat

pad and muscle (7, 8). Subsequent studies using ion-exchange separation of the isoenzymes confirmed a definite loss of type II in several insulin-sensitive tissues using streptozotocin-induced destruction of the β -cells of the pancreas (5) and similar experiments reported here indicate that these results do not depend on the presence of mercaptoethanol in the assay. Of interest also are reports of a very rapid recovery of hexokinase II upon insulin treatment of diabetic rats (5) or their tissues (7).

The observation that hexokinase II is much more sensitive than types I and III to thermal denaturation in the absence of glucose (2, 11) suggests that a lowering of glucose in insulin-sensitive tissues of fasting and diabetic animals might be directly related to the lowered steady-state level of this isoenzyme. If such inactivation is due to an unfolding of the enzyme in the absence of glucose, there may be conditions that allow reactivation and hence perhaps explain the rapid recovery of enzyme.

In the present study conditions for inactivation and reactivation of isolated hex-

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okinase II are established and the occurrence of an activatable form of hexokinase in streptozotocin-diabetic rat heart is examined.

EXPERIMENTAL PROCEDURES

Preparation of hexokinase. Hexokinase was solubilized from a particulate fraction (containing mitochondria and nuclei) of Ehrlich-Létré hyperdiploid ascites tumor cells (ELD cells). An equal volume of standard buffer (20 mM triethanolamine, pH 7.5, 2 mM EDTA, 5 mM mercaptoethanol, and 10 mM glucose) was added to the fraction, and the suspension was centrifuged at 20,000g for 20 min. The pellet was rinsed with another portion of the standard buffer and the supernatants were pooled. To prevent proteolytic digestion, phenylmethane sulfonyl fluoride, 18 mg/100 ml, was added. Then, 0.3 ml of 10% streptomycin sulfate per 100 mg of protein was added and the precipitate discarded. From this supernatant, hexokinase was precipitated between 30 and 55% of saturated $(\text{NH}_4)_2\text{SO}_4$, and dialyzed overnight against standard buffer. Then it was applied to DEAE-cellulose column (Whatman DE52) previously equilibrated with standard buffer containing 5 mM KCl. After washing with the same solution, the hexokinase type II was eluted from the column by the use of a five-chamber Buchler Varigrad containing eluant. The concentration of KCl in the standard buffer in the chambers were 5, 5, 200, 5, and 600 mM, respectively. The peak fraction of the hexokinase type II was concentrated by 40–55% saturation of $(\text{NH}_4)_2\text{SO}_4$, and dialyzed against the buffer. This type II had a specific activity of 3.8 units/mg protein.

Assay of hexokinase. Hexokinase was assayed by coupling to glucose-6-P dehydrogenase and measuring NADP reduction. The sample was added to a cuvette containing the following (μmoles) per ml at 26°C: triethanolamine-Cl (100), pH 8.0; MgCl_2 (5); NADP (0.4); glucose (1); ATP (2); and glc-6-P dehydrogenase, 0.5 unit.

Experiments with streptozotocin-treated rats followed in detail the procedures of Katzen *et al.* (5) for Fig. 2 of that publication. The streptozotocin used was generously supplied by Dr. Katzen.

RESULTS AND DISCUSSION

Figure 1 shows the changes of hexokinase type II activity as the result of incubation at 30°C under various conditions. Activity was rapidly lost in the presence of 0.1 mM glucose, approximately its K_m concentration. Almost complete protection was afforded by 10 mM glucose. Grossbard and Schimke (14) reported instability of hexokinase type II of muscle and fat pad of rat in the absence of glucose at 45°C, and

protection by glucose from inactivation by trypsin at 25°C. As will be shown below, the loss of activity of this partially purified enzyme at low glucose is not the result of an irreversible proteolysis.

The presence of effectors of hexokinase: 0.1 mM glc-6-P or its analogs and 5 mM P_i , gave marked protection when they were included with glucose. Such protective effects had the specificity required of substrates and effectors and depended on the presence of glucose (Table I).

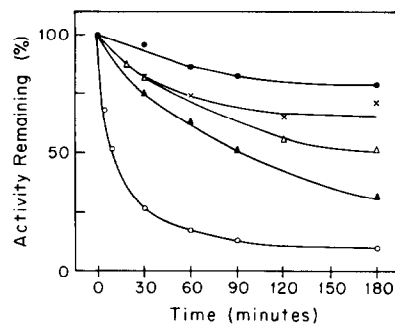


FIG. 1. Loss of hexokinase type II activity by incubation at 30°C. Ascites hexokinase type II was incubated at 30°C in 50 mM triethanolamine-Cl (pH 7.5) containing (1), 10 mM glucose (●); (2), 0.1 mM glucose (○); (3), (2) + 1 mM ADP-Mg (▲); (4), (2) + 5 mM P_i (Δ); and (5), (2) + 0.1 mM glc-6-P (×). Hexokinase was assayed in samples taken at the noted times as described in the Experimental Procedures.

TABLE I
SPECIFICITY FOR PROTECTION OF HEXOKINASE II

Additions (mM)	Activity remaining after 120 min (%)
Glucose (10)	78
Galactose (10)	9
Glucose (0.1)	9
+ Glc-6-P (0.1)	71
+ Glc-1,6- P_2 (0.1)	65
+ 1,5-Anhydroglucitol-6-P (0.1)	63
+ ADP (1.0) + MgCl_2 (1.0)	31
+ P_i (5)	51
+ Man-6-P (0.1)	17
+ 2-Deoxy-glc-6-P (0.1)	18
+ AMPCP (1.0) + MgCl_2 (1.0)	7
Glc-6-P (0.1 mM) alone	12
ADP (1.0) + MgCl_2 (1.0)	28 (60 min)
ATP (1.0) + MgCl_2 (1.0)	19

Incubation of inactivated enzyme in the presence of high concentrations of both mercaptoethanol and glucose resulted in complete reactivation within 30 min (Fig. 2). At 1 mM glucose the rate of activation was stimulated by *glc-6-P* or P_i . Neither of these effectors in the presence of mercaptoethanol was sufficient for reactivation when glucose was omitted (not shown). A good reducing agent such as mercaptoethanol is essential for any activation.

A simple working hypothesis is that the enzyme can be found in three states, native (N), unfolded (U), and oxidized (O) represented by the two equilibria $N \rightleftharpoons U$ and $U \rightleftharpoons O$. The presence of glucose stabilizes the native form by converting it to forms which do not participate in step 1. Other ligands such as ADP (and presumably ATP), P_i , and *glc-6-P* produce additional stable forms in the presence of glucose, perhaps by raising the affinity for glucose.

If reactivation is studied under optimal conditions ($t_{1/2} \approx 10$ min) with 0.1 M mercaptoethanol and 0.1 M glucose, one observes a clear delay of about 2 min before activation begins (Fig. 3). Incubation with glucose prior to addition of mercaptoetha-

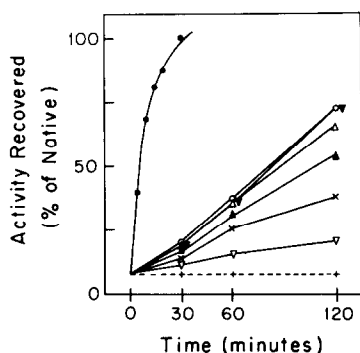


FIG. 2. Reactivation of inactivated hexokinase type II. Inactive enzyme was prepared by incubation in 50 mM triethanolamine-Cl (pH 7.5) containing 0.1 mM glucose at 30°C for 2 hr. Reactivation was observed upon further incubation at 30°C with the following additions in millimolar: (●) Mercaptoethanol (100) + glucose (100); (○) Mercaptoethanol (10) + glucose (100); (×) Mercaptoethanol (10) + glucose (1); (▲) Mercaptoethanol (10) + glucose (1) + *glc-6-P* (0.1); (Δ) Mercaptoethanol (10) + glucose (1) + P_i (5); (▼) Mercaptoethanol (10) + glucose (1) + P_i (5) + *glc-6-P* (0.1); (▽) Glutathione (10) + glucose (1) + P_i (5) + *glc-6-P* (0.1); (---) Glucose (100). Hexokinase was assayed at the noted times.

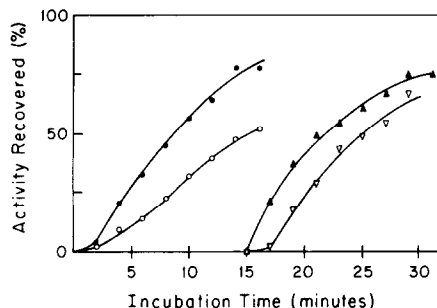


FIG. 3. The reactivation rate of the inactive hexokinase type II. The inactive enzyme was obtained as in Fig. 2 and was immediately adjusted to 100 mM β -mercaptoethanol and 100 mM glucose (●); or 50 mM β -mercaptoethanol and 100 mM glucose (○); and the recovery of activity was followed as in Fig. 2. Alternatively the inactivated enzyme was first incubated in 100 mM β -mercaptoethanol (▲) or 100 mM glucose (Δ) and after 15 min, 100 mM glucose (▲) or 100 mM β -mercaptoethanol (Δ) were added. The "activity recovered" ordinate represents the increase in activity above the zero time baseline, expressed as percentage of the increase upon full recovery at 30 min.

nol does not remove the lag. However, if the enzyme is incubated with mercaptoethanol prior to addition of glucose, there is no lag. Since the activation rates are similar in all cases, the reduction must be rapid compared with the refolding step. When 0.05 M mercaptoethanol is used a similar lag is seen but the activation rate is half that seen with 0.1 M. Therefore, the reactivation of oxidized form proceeds by a rapid but thermodynamically very unfavorable reduction followed by a slow process, probably the refolding.

The ability to reactivate the enzyme with glucose plus mercaptoethanol was destroyed if inactive enzyme was treated with trypsin, 5 μ g/ml at 30°C for 2 min. Contrary to the result with native enzyme, there was no protection by glucose (Table II). It may be that the glucose binding site is no longer present in the unfolded form and that the mode of action of glucose is to interact only with a small amount of form N that exists in unfavorable equilibrium with U.

The results obtained above may be useful for interpreting some of the earlier *in vivo* studies noted in the introduction. In the experiments of Katzen (7), fat pad and heart extracts of normal and diabetic rats

TABLE II
SENSITIVITY OF INACTIVATED HEXOKINASE TO TRYPSIN
IN THE PRESENCE OF GLUCOSE^a

Enzyme	Trypsin (μ g added)	Glucose (mM)	Activity remaining (%)
Inactivated	0	0	100
	0.25	0	34
		100	35
	0.5	0	17
100		21	
Native	0.5	100	96

^a Inactive enzyme, with 10% activity remaining, obtained as in Fig. 2, was treated in 0.1 ml with trypsin in the absence or presence of 0.1 M glucose for 2 min at 30°C followed by soybean trypsin inhibitor (10 \times weight). Activity remaining was determined after reactivation with 0.1 M glucose and 0.1 M mercaptoethanol. Native hexokinase II treated with trypsin was assayed directly.

were made and run in starch gel electrophoresis without added glucose. The complete absence of type II from the diabetic samples may reflect both the lower *in vivo* level and a greater instability of surviving hexokinase II in these extracts. Such tissues would have a much lower glucose level and perhaps also a lowered —SH content due to the lowered glucose available for the phosphogluconate pathway. When mercaptoethanol was present in the electrophoresis medium, the prolonged development with glucose and ATP would have reactivated the residual hexokinase II activity present in the gel, thus leading to an apparent reversal of the diabetic effect.

However, not all of the loss of hexokinase II in diabetes can be explained in this way (4–6). An explanation derived from the present studies might be the following: Starvation and diabetes may lower the level of glucose in insulin-sensitive tissues. This will lead to an increased rate of unfolding of hexokinase II to inactive forms which might be rapidly activated when glucose is again elevated in the tissues. On the other hand, reactivation may not be a functional pathway for recovery if the greater sensitivity of these forms to proteolysis results in their destruction. Rat muscle hexokinase type II was shown to undergo the same glucose-sensitive inactivation

and reactivation phenomena observed with the mouse tumor enzyme. Activatable hexokinase was, therefore, sought in extracts of hearts of rats made streptozotocin diabetic following the procedure of Katzen *et al.* (5).

Thirteen days after drug treatment the heart cytosol hexokinase had fallen about 60% and the hexokinase II component as determined by DE52 chromatography represented only 43% compared with 67% for normal, in agreement with the earlier report (5). No increase in the activity of the whole cytosol fraction was observed after the glucose (0.1 M)–mercaptoethanol (0.1 M) incubation (Table III). Subsequent studies using 3-day-treated rats gave similar results. Since it could be shown that inactive tumor hexokinase II was recovered in good yield from normal rat heart cytosol fractions in the region of elution of rat heart hexokinase II (Table III), the distribution and activation of the isoenzymes from 3-day streptozotocin rat hearts was

TABLE III
LACK OF REACTIVATION OF HEXOKINASE FROM
STREPTOZOTOCIN-DIABETIC RAT HEART^a

Expt		Hexokinase activity (units)		Isoenzyme Ratio II/I
		Before activation	After activation	
1	Normal (seven hearts = 3.5 g)	4.8	—	2
	13-Day streptozotocin (seven hearts = 2.5 g)	2.17	2.09	0.8
2	Normal (2.58 units + tumor (1.3 units inactivated))	I 0.64	0.64	2.1
		II 1.38	2.03	
3	3-Day streptozotocin	I 0.66	0.65	1.2
		II 0.82	0.82	

^a In expt 1 the cytosol fraction of 13-day streptozotocin rats were assayed before and after glucose–mercaptoethanol activation. Hexokinase I and II were determined by DE52 column separation. Experiment 2 shows that inactivated tumor hexokinase II when added to normal heart homogenate is recovered with the rat heart hexokinase II peak. The failure to observe activatable hexokinase in this peak from 3-day streptozotocin hearts is shown in expt 3.

determined after ion-exchange separation. As noted, a significant fall in hexokinase II was seen in 3 days but no activation was detected.

It has been implied that disturbances in the normal tertiary structure of proteins facilitates their degradation in normal cells (12, 13) and that a correlation exists between protein half-lives *in vivo* and their sensitivity *in vitro* to known proteases (14, 15). The observed sensitivity of hexokinase II to inactivation in the presence of low glucose and its modulation by effectors may be of importance in determining the proteolytic degradation rate in starvation and diabetes in insulin-sensitive tissues. On the other hand, the activation phenomenon requiring glucose and mercaptoethanol and modulated by reaction effectors may be important, not only in the reversal of transient inactivation but also post-translationally in completing protein synthesis at the folding stage. Such a role for NAD⁺ has been suggested by Deal and Constantinides (16) for folding of glyceraldehyde-*P* dehydrogenase.

The influence of insulin on the hexokinase composition of tissues would then be indirect on both degradation and synthesis of hexokinase II, acting through its regulation of glucose permeability. Fat pads from alloxan-diabetic rats have elevated hexokinases after 20-hr incubation with high glucose without insulin (6). Insulin without glucose leads to lesser increase (6) or no increase with fat pads from 48-hr fasted rats (17). Significant effects with insulin plus pyruvate have been interpreted as a requirement for an energy source (17) or for intermediates (18). The superiority of glucose and the facilitation by intermediates would be consistent with a role in post-translational control at the stage of folding the hexokinase II precursor. On the other hand, glucose plus insulin-like serum factors failed to inhibit hexokinase breakdown

in kidney fibroblasts treated with cycloheximide (19). Further research to explain the loss and reappearance of hexokinase II *in vivo* will depend on being able to follow the turnover of the enzyme. To this end a pure enzyme and suitable antibody will be required.

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