High glucose concentrations partially release hexokinase from inhibition by glucose 6-phosphate

(diabetes/kinetics)

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ABSTRACT The phosphorylation of glucose by human erythrocyte hexokinase follows classical Michaelis-Menten kinetics; hexokinase manifests maximum activity at 5 mM glucose, and no further increase in activity can be measured at higher glucose concentrations. However, the erythrocytes of diabetics and normal erythrocytes incubated with high concentrations of glucose contain increased concentrations of glucose 6-phosphate. To elucidate the mechanism of accumulation of glucose 6-phosphate when erythrocytes are exposed to high glucose concentrations, hexokinase activity was examined in the presence of naturally occurring inhibitors, such as glucose 1,6-bisphosphate, 2,3-diphosphoglycerate, ADP, and glucose 6-phosphate at physiological concentrations. Without inhibitors or in the presence of glucose 1,6-bisphosphate, 2,3-diphosphoglycerate, and ADP, maximum hexokinase activity was observed at 5 mM glucose concentration. On the contrary, in the presence of glucose 6-phosphate, hexokinase activity increased at glucose concentrations >5 mM; inhibition by glucose 6-phosphate was partially competitive with glucose. The relief by glucose of glucose 6-phosphate inhibition of hexokinase is a possible explanation of the increased glucose 6-phosphate level in diabetic erythrocytes.

The erythrocytes of diabetic patients contain increased levels of the glycohemoglobin hemoglobin A1c. The concentration of glucose 6-phosphate has been found to be increased in the erythrocytes of diabetic patients (1–3), and it has been suggested that glucose 6-phosphate may be the precursor of the carbohydrate variety of this glycohemoglobin. The mechanism by which erythrocyte glucose 6-phosphate levels become elevated in diabetics is by no means obvious, however. The human erythrocyte is highly permeable to glucose. Human erythrocyte hexokinase has a very low Km for glucose (50–80 × 10⁻⁶ M), and it is saturated with glucose even at normal plasma glucose levels (5 mM). Accordingly, the increase in glucose 6-phosphate concentration in diabetic erythrocytes is obviously not merely the direct result of increased availability of glucose to hexokinase.

It has been proposed that acidemia due to ketoacidosis sometimes seen in severe or untreated diabetics might cause elevation of glucose 6-phosphate levels in the erythrocytes of diabetics, because phosphofructokinase activity is inhibited by the lower pH (4). However, most diabetics are not acidotic. Moreover, Stevens et al. (1) and Tegos and Beutler (3) observed no crossover in the levels of erythrocyte glycolytic intermediates in diabetics. This suggested that high glucose concentrations affected the hexokinase reaction itself.

Hexokinase normally operates in a state of partial inhibition by some glycolytic intermediates (5–8). Relief of inhibition of hexokinase by high concentrations of glucose would result in the apparent activation of the enzyme by high glucose concentrations, and although previous reports showed no competition of these inhibitors with glucose, no details were presented. In the present studies, we extensively examined the velocity of the hexokinase reaction in the presence of inhibitors and high concentrations of glucose in order to shed light on the cause of the elevation of glucose 6-phosphate levels in the erythrocytes of diabetics.

EXPERIMENTAL PROCEDURES

Partial Purification of Hexokinase from Human Erythrocytes. Hexokinase was partially purified by a modification of the method of Rijken and Staal (9). Three hundred milliliters of blood was freed of leukocytes and platelets by passing through a cellulose column (10), and the erythrocytes were washed three times with isotonic saline. The packed erythrocytes were lysed with 1 vol of 0.4% saponin in H₂O for 1 hr. The hemolysate was mixed with 0.5 vol of 50% suspension of DEAE-cellulose (DE52) equilibrated with 10 mM potassium phosphate buffer (pH 7.3) containing 3 mM 2-mercaptoethanol, and 3 mM NaF. After washing with the same buffer, the enzyme was eluted with 500 mM potassium phosphate buffer (pH 7.3) containing 3 mM 2-mercaptoethanol, and 3 mM NaF, and dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-cellulose column (0.8 × 23 cm) equilibrated with the same buffer. The column was washed with the same buffer and then with the same buffer containing 10 mM KCl. Elution of the enzyme was performed with a 500-mL linear gradient of 0–500 mM KCl in the same buffer containing 10 mM KCl. Fractions of 3.5 ml were collected and assayed for hexokinase, glucose-6-phosphate dehydrogenase, glucose-phosphate isomerase, and adenylate kinase (11). The fractions containing hexokinase activity and free of glucose-6-phosphate dehydrogenase, glucose phosphate isomerase, and adenylate kinase were pooled and concentrated by ultrafiltration. The enzyme was stored at −20°C in 10 mM potassium phosphate buffer (pH 7.0) containing 3 mM 2-mercaptoethanol, 3 mM NaF, 2 mM glucose, and 10% (vol/vol) glycerol. Before use, the stored enzyme was dialyzed against 10 mM Tris·HCl (pH 8.0).

Hexokinase Assay. The standard assay mixture (11) contained 50 mM Tris·HCl, pH 8.0/0.2 mM NADP/1 mM ATP/5 mM MgCl₂/0.1 international unit (IU) of glucose-6-phosphate dehydrogenase and glucose at the concentrations indicated in the text. Absorbance was measured at 340 nm at 37°C for 10–20 min.

When glucose 6-phosphate was studied as an inhibitor, it

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Abbreviation: IU, international unit(s).

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was necessary to measure hexokinase activity in another system in which ADP production was determined in the pyruvate kinase reaction. This assay system contained 50 mM Tris·HCl, pH 8.0/5 mM MgCl₂/75 mM KCl/1 mM ATP/2 mM phosphoenolpyruvate/0.2 mM NADH/3 IU of pyruvate kinase/3 IU of lactate dehydrogenase. The reaction was followed by measuring optical density at 340 nm at 37°C for 3 min. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of glucose 6-phosphate or 1 μmol of ADP per min. In both systems, the reaction was started by adding 0.004 unit of hexokinase per ml of reaction mixture. A cuvette without hexokinase served as the blank.

RESULTS

Glucose 6-Phosphate Level After Incubation of Human Erythrocytes with Glucose. Fasting blood was drawn into heparin from normal volunteers. After centrifugation at 2000 × g for 10 min, packed erythrocytes were suspended in 1 vol of 0.1 M Hepes buffer (pH 7.4) containing 135 mM NaCl in the presence of 5 mM and 30 mM glucose. Incubation was continued for 1 hr at 37°C in a shaking bath. The pH of both suspensions was the same at the end of the incubation. The reaction was terminated by adding 4 vol of ice-cold 4% perchloric acid. The concentration of glucose 6-phosphate was measured as described (11). In eight separate experiments, the glucose 6-phosphate concentration in the presence of 5 mM glucose was found to be 30.19 ± 0.88 μmol per liter of packed erythrocytes (mean ± SEM); in the presence of 30 mM glucose, the glucose 6-phosphate concentration was 35.8 ± 1.58 μM. The paired differences were 5.61 ± 1.79 μM (mean ± SEM), this difference being statistically significant at P < 0.05.

Studies with Purified Erythrocyte Hexokinase. Fig. 1 shows the effect of high concentrations of glucose on the inhibition of hexokinase by glucose 6-phosphate using the PK-LDH assay method. Without glucose 6-phosphate, hexokinase activity was maximal at 5 mM glucose and 87.6% ± 1.5% (mean ± SEM) of the Vₘₐₓ at 0.5 mM glucose; glucose concentrations >5 mM resulted in no increase of activity. Since the Kₘₐₓ for glucose is 30–80 × 10⁻⁶ M, these findings were in perfect agreement with the predicted value from the Michaelis–Menten equation, indicating the accuracy of the method used here. It is clear that hexokinase is completely saturated at 5 mM glucose. In the presence of glucose 6-phosphate, on the contrary, the activity is slightly, but significantly increased at glucose concentrations >5 mM. These results indicate that hexokinase inhibition by glucose 6-phosphate is partially relieved by high concentrations of glucose. The data have been replotted in the inserted figure to provide insight into the relationship between glucose concentration and inhibition by glucose 6-phosphate. The curves obtained suggest the presence of a mixed type of inhibition (12).

Using the standard assay, hexokinase activity in the absence and presence of glucose 1,6-bisphosphate, 2,3-diphosphoglycerate, and ADP at various glucose concentrations was examined (data not given). In the absence of inhibitors, hexokinase activity reached its maximum activity at 5 mM glucose; higher glucose concentration resulted in no increase of activity. In the presence of physiological concentrations of glucose 1,6-bisphosphate (200 μM), 2,3-diphosphoglycerate (5 mM), and ADP (1 mM), hexokinase activity also plateaued at 5 mM and higher glucose concentrations, indicating that a high concentration of glucose is not competitive with the action of these inhibitors. These findings are consistent with previous reports (7, 8). At higher concentrations of inhibitors, the same results were obtained.

![Fig. 1](image-url)  
**Fig. 1.** Effect of high glucose concentration on hexokinase inhibition by glucose 6-phosphate. Hexokinase activity is expressed as percent of the activity at 5 mM glucose at each concentration of glucose 6-phosphate. The mean values (± SEM) were obtained from several independent experiments and each experiment was performed in duplicate. (Inset) Eadie plot of the data. Initial velocity (v) is expressed as ΔA₅₅₀/min and glucose concentration, S, is in mM. G6P, glucose 6-phosphate.
**DISCUSSION**

The elevated glucose 6-phosphate level that has been found in the erythrocytes of diabetics can also be documented in normal erythrocytes that are incubated in vitro with high concentrations of glucose under conditions of carefully controlled pH. Thus, it cannot be due to any of the variables that are incidental to the diabetic state. If the accumulation of glucose 6-phosphate were due to inhibition of glucose phosphate isomerase, phosphofructokinase, or other glycolytic enzymes, a decrease in metabolic intermediates downstream from the inhibited step would be expected, but no such crossover has been detected in vivo (1, 3). Thus, it seemed most likely that enhanced phosphorylation of glucose was responsible for the increased level of glucose 6-phosphate that occurred at high glucose concentrations. Since hexokinase was fully saturated with glucose even at a concentration of 5 mM, and since the enzyme normally functions under circumstances when it is under marked inhibition, relief of inhibition by glucose seemed a likely answer to the problem.

To our knowledge, however, no effect of high glucose concentration on the inhibitory kinetics of the enzyme had been reported previously. Using the standard assay system, we found the inhibitory effect of glucose 1,6-bisphosphate, 2,3-diphosphoglycerate, and ADP unaffected by glucose. The effect of glucose on inhibition by glucose 6-phosphate was more difficult to study, because it is this product of the reaction that is measured in the standard assay system. An assay was used in which the formation of ADP was estimated. In using this approach, the accuracy of measurement of initial reaction velocity was of critical importance because during the reaction, glucose 6-phosphate accumulates rapidly, making it difficult to obtain a linear reaction rate. Only 0.004 unit of hexokinase per ml was used and the reaction was recorded for only 3 min. These modifications and using mean values obtained from several experiments made it possible to obtain reliable results.

The partial relief of hexokinase inhibition by glucose 6-phosphate shown in our present work, although small, is probably sufficient to account for the modest accumulation of glucose 6-phosphate in the erythrocytes of diabetic patients. The exact steady-state concentration of glucose 6-phosphate in erythrocytes cannot readily be computed, because it is a function not only of hexokinase activity, but also of the activities of phosphofructokinase and probably, to a limited extent, of glucose-6-phosphate dehydrogenase. These activities, in turn, are controlled not only by their substrates, but also by effectors including 2,3-diphosphoglycerate, ATP, ADP, NADP, and NADPH. The glucose-6-phosphate dehydrogenase reaction depends primarily on the availability of NADP and, hence, is not expected to be greatly affected by an increase in the glucose 6-phosphate level of the erythrocytes. However, at physiological substrate concentrations, phosphofructokinase activity is strongly dependent on the level of fructose 6-phosphate, which, through glucose phosphate isomerase, exists in a 1:3 equilibrium mixture with glucose 6-phosphate in erythrocytes. Thus, the rate of removal of glucose 6-phosphate would be dependent on its rate of formation and a new steady state would be expected at glucose 6-phosphate levels that are roughly dependent on the rate of glucose 6-phosphate formation by hexokinase. The increase in hexokinase activity when the glucose concentration was increased from 5 to 40 mM at a 30 μM concentration of glucose 6-phosphate was 5.5%. By comparison, the increase in the glucose 6-phosphate level found in intact erythrocyte incubated at 30 mM glucose when compared with 5 mM glucose was 18.6%. Thus, the increases in hexokinase activity and of glucose 6-phosphate levels were found to be of a similar order of magnitude. These considerations indicate that our findings are consistent with the mechanism that we propose to account for the increased level of glucose 6-phosphate in erythrocytes exposed to high glucose concentrations.

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