Anomeric specificity of D-glucose phosphorylation by rat liver glucose-6-phosphatase

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The anomeric specificity of D-glucose phosphorylation by hepatic glucose-6-phosphatase was examined in rat liver microsomes incubated in the presence of carbamoyl phosphate. At 10 °C, the K_m for the equilibrated hexose and phosphate donor was close to 56 mM and 11 mM, respectively. The enzymic activity, which was increased in diabetic rats, was about 40 % lower in untreated than in sonicated microsomes. No anomeric difference in affinity was found in sonicated microsomes. In untreated microsomes, however, the K_m for β -D-glucose was slightly lower than that for α -D-glucose. The maximal velocity was higher with β - than α -D-glucose in both untreated and sonicated microsomes. These data indicate that the phosphotransferase activity of glucose-6-phosphatase cannot account for the higher rate of glycolysis and glycogen synthesis found in hepatocytes exposed to α - rather than β -D-glucose.

INTRODUCTION

It was recently reported that the rates of glycolysis and glycogen synthesis are both higher in hepatocytes exposed to α - rather than β -D-glucose [1]. This situation is unlikely to be attributable to the anomeric behaviour of liver hexokinase isoenzymes, since both the low- K_m hexokinase and high- K_m glucokinase display a higher maximal velocity with β - than α -D-glucose [2]. Moreover, the modest preference, in terms of affinity, of these hexokinases for α -D-glucose does not appear sufficient to compensate for the difference in maximal velocity, at least at physiological hexose concentration [2]. However, a third enzyme, namely glucose-6-phosphatase, might participate in the phosphorylation of D-glucose by intact hepatocytes [3]. We have therefore examined, in the present study, the anomeric behaviour of liver glucose-6-phosphatase under experimental conditions designed to assess its catalytic activity as a phosphotransferase. To our knowledge, previous attempts to characterize the anomeric specificity of glucose-6-phosphatase were restricted to the study of its hydrolytic activity [4].

MATERIALS AND METHODS

Most experiments were performed in normal female albino rats (180–200 g body wt.) fed *ad libitum*. In one series of experiments, the rats were injected intravenously with streptozotocin (60 mg/kg body wt.) and examined 5–7 days later, when they had lost 9.4 ± 1.2 g and were severely hyperglycaemic (plasma glucose concentration 26.1 ± 1.6 mM versus a control value of 8.4 ± 0.6 mM).

Samples of liver (1.0 g each) were homogenized in 2.0 ml of a Hepes/KOH buffer (2 mM, pH 7.4) containing mannitol (220 mM) and sucrose (70 mM), using a mechanical homogenizer (Braun, Melsungen, Germany) with six passes at 1250 rev./min. The homogenate was then brought to a total volume of 10 ml with the same buffer and centrifuged for 10 min at 600 g and 4 °C to obtain the postnuclear supernatant. In most experiments, the

microsomes were then prepared through two successive centrifugations, the first for 10 min at 12000 g to deposit mitochondria and the second for 60 min at 100000 g to isolate the microsomal pellet. In some experiments, however, the postnuclear supernatant was immediately centrifuged for 60 min at 100000 g. Table 1 documents the distribution of protein, lactate dehydrogenase, glutamate dehydrogenase and glucose-6-phosphatase (hydrolytic activity) in the latter experiments. In all cases, the pellet obtained after 60 min centrifugation at 100000 g was resuspended in the same buffer so that 1.0 ml of the suspension now contained the material derived from 0.20–0.25 g wet wt. of liver. Whenever required, the microsomes were sonicated for 3×10 s at a 10 μ m amplitude (Soniprep 150; MSE Scientific Instruments, Crawley, Sussex, U.K.). All these manipulations were conducted at 4 °C.

The assay of glucose-6-phosphatase (phosphotransferase activity) was performed over 30 min incubation at 10 °C in 25 μ l aliquots of the microsomal suspension or homogenate mixed with 75 μ l of reaction mixture to yield the following final concentrations: mannitol, 55 mM; sucrose, 17.5 mM; Hepes/NaOH, 50 mM (pH 7.0); KCl, 150 mM; EDTA, 1.0 mM, $MgCl_2$, 7.0 mM. The reaction was halted by heating for 10 min at 80 °C. When the assay medium contained D-[U- ^{14}C]-glucose, the labelled hexose phosphates were separated by anion-exchange chromatography [5]. Otherwise, the hexose phosphates formed during incubation were measured enzymically through the reaction catalysed by glucose-6-phosphate dehydrogenase [6]. In the radioisotopic procedure, the blank value was measured in the presence of homogenate but absence of carbamoyl phosphate. It represented no more than 0.46 ± 0.02 % of the total radioactive content of the assay mixture ($n = 18$). In the non-isotopic technique, the results were corrected for both the blank value found in microsomes incubated with carbamoyl phosphate in the absence of D-glucose and the readings attributable to D-glucose itself in the absence of homogenate. The reaction velocity was then calculated by reference to a standard curve

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Table 1. Absolute values for the protein and enzymic content of liver postnuclear supernatants and corresponding fractional recoveries in the postmicrosomal supernatants

Results are expressed as the mean ($\pm$ S.E.M.) together with the number of individual determinations (n).

	Postnuclear supernatant (absolute values)	Postmicrosomal supernatant (% of total)
Protein	$80.9 \pm 6.7 \mu\text{g}/\text{mg}$ liver wet wt.	65.0 ± 2.2 (4)
Lactate dehydrogenase	$1048.3 \pm 62.2 \text{ pmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$	≥ 100.0 (4)
Glutamate dehydrogenase	$32.6 \pm 5.9 \text{ pmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$	1.8 ± 0.3 (6)
Glucose-6-phosphatase	$23.7 \pm 0.4 \text{ pmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$	1.0 ± 0.1 (3)

Fig. 1. Double-reciprocal plot for the phosphorylation of equilibrated D-[U-¹⁴C]glucose by sonicated microsomes incubated in the presence of 10 mM-carbamoyl phosphate (left) and 100 mM-D-glucose (right)

Mean values ($\pm$ S.E.M.) are derived from three (left) and five (right) individual experiments, and are expressed relative to the mean value found within each experiment in the presence of 100 mM-D-glucose and 10 mM-carbamoyl phosphate. Such a reference value averaged $5.09 \pm 0.25 \text{ pmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$.

established with known amounts (2.5–25.0 nmol) of D-glucose 6-phosphate.

The protein content was measured by the method of Lowry *et al.* [7] using bovine albumin as standard. The methods used to measure the activity of lactate dehydrogenase [8] in the presence of pyruvate (4.0 mM) and NADH (0.4 mM) placed in a Hepes/NaOH buffer (80 mM, pH 7.0), the activity of glutamate dehydrogenase [9] in the presence of 2-oxoglutarate (7.0 mM), ammonium acetate (50.0 mM), ADP (0.5 mM) and NADPH (0.4 mM) placed in the same Hepes buffer, and the hydrolytic activity of glucose-6-phosphatase [10] in the presence of D-[U-¹⁴C]-glucose 6-phosphate (8.0 mM) placed in a Mes/NaOH buffer (50 mM, pH 6.5) were similar to those described in the cited references. All these measurements were performed at 20 °C.

The results are always expressed as the mean ($\pm$ S.E.M.) together with the number of individual measurements

(n). The statistical significance of differences was assessed by use of Student's *t*-test. The K_m for α - and β -D-glucose was calculated as described elsewhere [6].

RESULTS

Characterization of glucose-6-phosphatase

The generation of D-[U-¹⁴C]glucose 6-phosphate from D-[U-¹⁴C]glucose and carbamoyl phosphate was proportional to the amount of protein and length of incubation with a variation of less than 3%. Both glucose 6-phosphate and carbamoyl phosphate, but not ATP, acted as phosphate donors. In sonicated microsomes incubated in the presence of 10 mM-carbamoyl phosphate, the K_m for D-[U-¹⁴C]glucose, at anomeric equilibrium, was close to 55.6 mM (Fig. 1, left panel). In the presence of 100 mM-equilibrated D-[U-¹⁴C]glucose, the K_m for carbamoyl phosphate averaged, in five

Fig. 2. Double-reciprocal plot for the phosphorylation of equilibrated D-[U-¹⁴C]glucose by untreated and sonicated microsomes incubated in the presence of 10 mM-carbamoyl phosphate

Mean values are shown together with the S.E.M. whenever it exceeded the size of the mean point.

experiments, 11.0 ± 0.9 mM (Fig. 1, right panel). The latter K_m was not affected by a decrease in hexose concentration to 50 mM (results not shown).

When the supernatant obtained after sedimentation of nuclei and cell debris, and which contained 79.4 ± 4.4 μ g of protein per mg liver wet wt., was immediately centrifuged for 60 min at 100 000 g and 4 °C, the pellet contained 30.1 ± 1.5 μ g of protein per mg liver wet wt. and displayed, in the presence of 5 mM-D-glucose and 10 mM-carbamoyl phosphate, a mean specific activity not exceeding 272 ± 11 fmol·min⁻¹· μ g⁻¹ ($n = 6$ in all cases). However, when the postnuclear supernatant was first centrifuged for 10 min at 12 000 g to deposit mitochondria and the resulting supernatant then further centrifuged for 60 min at 100 000 g to obtain the microsomal pellet, the protein content of such a pellet was decreased to 14.4 ± 0.5 μ g per mg liver wet wt. ($n = 22$) and its specific activity (also measured in the presence of 5 mM-D-glucose and 10 mM-carbamoyl phosphate) increased ($P < 0.001$) to 477 ± 34 fmol·min⁻¹· μ g⁻¹ ($n = 7$).

All these data refer to sonicated microsomes. The K_m for D-[U-¹⁴C]glucose was virtually identical in sonicated and untreated microsomes examined within the same experiments with, in this case, a K_m close to 55 mM and a maximal velocity in sonicated microsomes of 5.98 ± 0.10 pmol·min⁻¹· μ g⁻¹ (Fig. 2). The reaction velocity in untreated microsomes was lower, averaging 59.4 ± 1.2 % of the paired value found in sonicated microsomes. In considering the latter difference in reaction velocity, it should be realized that, even in sonicated

Table 2. Distribution of glucose-6-phosphatase (phosphotransferase activity) and proteins before and after centrifugation (60 min at 100 000 g) of either untreated or sonicated microsomes

Reaction velocities were measured in the presence of 10 mM-D-[U-¹⁴C]glucose and 10 mM-carbamoyl phosphate. Mean values ($\pm$ S.E.M.) are derived from quadruplicate measurements in two separate experiments and refer to 5 mg liver wet wt.

Microsomal fraction	Reaction velocity (pmol·min ⁻¹)	Protein (μ g)
Untreated microsomes		
Before centrifugation	67.0 ± 2.1	84.2 ± 0.4
After centrifugation		
Supernatant	2.7 ± 1.0	7.2 ± 0.1
Sonicated pellet	98.7 ± 2.0	76.7 ± 0.7
Sonicated microsomes		
Before centrifugation	97.7 ± 1.8	83.7 ± 1.6
After centrifugation		
Supernatant	11.1 ± 0.9	11.7 ± 0.2
Sonicated pellet	104.0 ± 1.6	72.1 ± 1.4

microsomes, only a minor fraction of the enzyme was solubilized. This is documented in Table 2, which refers to the distribution of enzymic activity in the supernatant fraction and pellet prepared by centrifugation of either untreated or sonicated microsomes. Prior to centrifugation, the enzymic activity in untreated microsomes averaged, in this series of experiments, 68.5 ± 2.1 % ($P < 0.001$) of that in sonicated microsomes. In untreated microsomes, only 2.7 ± 1.0 % of the total activity was recovered in the supernatant after centrifugation for 60 min at 100 000 g . Although sonication of the microsomes increased 4-fold ($P < 0.001$) the enzymic content of the supernatant, the bulk of the activity was still recovered in the pellet after centrifugation for 60 min at 100 000 g .

In four diabetic rats, the activity of glucose-6-phosphatase (pmol·min⁻¹· μ g⁻¹), as measured by the non-radioisotopic method in sonicated microsomes incubated in the presence of 100 mM-D-glucose and 10 mM-carbamoyl phosphate, averaged 13.10 ± 0.99 as distinct ($P < 0.001$) from 6.16 ± 0.57 in four control rats examined within the same experiments.

Anomeric specificity in affinity

In order to establish the affinity of glucose-6-phosphatase for α - and β -D-glucose, respectively, the phosphorylation of D-[U-¹⁴C]glucose (5 mM) in anomeric equilibrium was measured in the sole presence of carbamoyl phosphate (10 mM) or in the simultaneous presence of the phosphate donor and unlabelled α - or β -D-glucose (45 and 95 mM). In this series of six experiments, the reference value found in the absence of the unlabelled anomers averaged 0.333 ± 0.006 pmol·min⁻¹· μ g⁻¹ in untreated microsomes. The sonicated/untreated ratio in reaction velocity amounted to 1.602 ± 0.152 ($P < 0.02$ as compared to unity). As illustrated in Fig. 3, unlabelled α - and β -D-glucose inhibited to the same extent the phosphorylation of D-[U-¹⁴C]glucose in sonicated microsomes. Based on the mean data illustrated in Fig. 3, the K_m for α - and β -D-glucose [6] in sonicated microsomes averaged 56.8 ± 1.4

and 58.4 ± 3.5 mM, respectively. Virtually identical results (57.5 ± 3.5 mM for α -D-glucose and 59.1 ± 4.3 mM for β -D-glucose) were derived from the separate analysis of each set of data within each experiment.

A somewhat different situation was found in untreated microsomes. The phosphorylation rate of equilibrated D-[U- 14 C]glucose was indeed slightly lower in the presence of unlabelled β - rather than α -D-glucose. When the unlabelled anomers were tested at a concentration of 45 mM, the phosphorylation rate of the radioactive hexose averaged, in the presence of unlabelled β -D-glucose, $92.4 \pm 2.3\%$ ($P < 0.025$ as compared with unity) of the paired value found in the presence of unlabelled α -D-glucose. When the unlabelled anomers were tested at a concentration of 95 mM, the same ratio yielded a mean value of $95.2 \pm 1.5\%$ ($P < 0.05$). From these measure-

ments (Fig. 3), the K_m for α -D-glucose was found to average 57.7 ± 0.4 mM, as distinct from 50.8 ± 2.8 mM for β -D-glucose.

Anomeric specificity in maximal velocity

Since no anomeric difference in affinity was found in sonicated microsomes, the latter were incubated in the presence of 100 mM α - or β -D-glucose and 10 mM-carbamoyl phosphate to assess a possible anomeric difference in maximal velocity, the formation of D-glucose 6-phosphate being measured by the non-isotopic procedure. In a series of six experiments, including quadruplicate measurements for each anomer, the reaction velocity was higher with β - than α -D-glucose (Table 3, expt. 1).

The same experiments were then repeated in both untreated and sonicated microsomes. In the sonicated microsomes, the reaction velocity was again higher with β - than α -D-glucose (Table 3, expt. 2). There was no significant difference ($P > 0.2$) between the first and second series of experiments in terms of either the absolute reaction velocity or its anomeric specificity. However, the anomeric difference in reaction velocity was somewhat more marked ($P < 0.005$) in untreated than sonicated microsomes (Table 3, expt. 2). This difference in anomeric behaviour between untreated and sonicated microsomes, respectively, was borne out by the finding that the sonicated/untreated ratio in reaction velocity was, in all of the seven experiments in this series, higher in the case of α - than β -D-glucose. Thus, the sonicated/untreated ratio for α -D-glucose averaged $109.6 \pm 3.0\%$ ($n = 7$; $P < 0.02$ as compared with unity) of the paired sonicated/untreated ratio for β -D-glucose. However, when allowance was made for the anomeric difference in affinity found in untreated microsomes, the β/α ratio in true maximal velocities averaged $113.8 \pm 2.1\%$ and, hence, was no more different ($P > 0.1$) from that found, within the same experiments, in sonicated microsomes ($109.1 \pm 1.6\%$). In other terms, the anomeric difference in maximal velocity displayed the same relative magnitude in both untreated and sonicated microsomes. Incidentally, in these as in previous experiments, the absolute values for reaction velocity were higher in sonicated than untreated microsomes with a mean ratio of 1.514 ± 0.041 (pooled data obtained with both α - and β -D-glucose).

Fig. 3. Effect of unlabelled α -D-glucose ($\square$) and β -D-glucose ($\boxtimes$) upon the phosphorylation of equilibrated D-[U- 14 C]glucose (5.0 mM) by sonicated or untreated microsomes incubated in the presence of 10 mM-carbamoyl phosphate

Mean values ($\pm$ S.E.M.) are derived from six experiments, expressed relative to the mean value found within each experiment in untreated microsomes, and shown as a function of the total concentration of the hexose.

Table 3. Phosphorylation of unlabelled D-glucose anomers (100 mM) in the presence of 10 mM-carbamoyl phosphate

Mean values ($\pm$ S.E.M.) for the rate of hexose phosphorylation are expressed either in absolute terms or relative to the mean reading recorded, within the same experiment, in the presence of α -D-glucose. Also shown are the number of experiments (in parentheses) and statistical significance (P) of the anomeric difference.

Expt. no.	Microsomes	Rate of phosphorylation of:			
		α -D-Glucose		β -D-Glucose	P
		(pmol $\cdot$ min $^{-1}$ $\cdot$ μ g $^{-1}$)	(%)	(%)	
1	Sonicated	6.23 ± 0.40 (6)	100.0 ± 1.3	112.3 ± 1.4	< 0.005
2	Sonicated	7.16 ± 0.67 (7)	100.0 ± 1.3	109.1 ± 1.0	< 0.005
2	Untreated	4.56 ± 0.43 (7)	100.0 ± 1.2	119.0 ± 1.7	< 0.001

DISCUSSION

The present results reveal that, when liver glucose-6-phosphatase acts as a phosphotransferase in catalysing the conversion of D-glucose into D-glucose 6-phosphate, the enzyme yields a higher maximal velocity with β - than α -D-glucose. In this respect, glucose-6-phosphatase resembles liver hexokinase and glucokinase, which both display a higher maximal velocity with β - than α -D-glucose [2]. The anomeric difference in maximal velocity is less pronounced, however, in the case of glucose-6-phosphatase than the hexokinase isoenzymes. In the present study, the subcellular distribution of glucose-6-phosphatase and its long-term adaptation to insulin deficiency were similar to those previously found when measuring the hydrolytic rather than phosphotransferase activity of the enzyme [11]. Incidentally, in a limited series of four experiments conducted with sonicated microsomes of diabetic rats, the reaction velocity was also higher with β - than α -D-glucose (paired β/α ratio: $107.1 \pm 1.7\%$; $P < 0.025$).

In untreated, but not sonicated, microsomes, the affinity of glucose-6-phosphatase was slightly higher for β - than α -D-glucose. The difference in anomeric behaviour between untreated and sonicated microsomes, respectively, is unlikely to reflect a difference in the rate of α - and β -D-glucose transport into the microsomes. Indeed, the fact that a comparable discrepancy between untreated and sonicated microsomes was not observed in those experiments designed to assess the anomeric difference in maximal velocity and the apparent lack of stereospecificity (D- versus L-glucose) for hexose penetration into the microsomal lumen [11] both argue against the latter hypothesis. It cannot be ruled out, however, that the difference in anomeric behaviour between untreated and sonicated microsomes is somehow linked to the spatial organization of the enzyme in its microenvironment, even if the bulk of the enzyme apparently remained membrane-associated in sonicated microsomes. Anyhow, the present results indicate that the intrinsic properties of glucose-6-phosphatase, considered together with those factors which may govern the translocation of both the hexose and its 6-phosphate

ester across the microsomal membrane [11], favour modestly but significantly the phosphorylation of β - rather than α -D-glucose. This finding is not necessarily incompatible with the claim that the hydrolytic activity of glucose-6-phosphatase fails to display any anomeric preference, at least in detergent-treated microsomes [4]. Our results also imply that the phosphotransferase activity of glucose-6-phosphatase cannot be held responsible for the higher rate of glycolysis and glycogen synthesis observed in intact hepatocytes exposed to α - rather than β -D-glucose [1].

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