PRESENCE OF FRUCTOSE-6-PHOSPHATE,2-KINASE IN PANCREATIC ISLETS

Willy J. MALAISSE, Francine MALAISSE-LAGAE and Abdullah SENER
Laboratory of Experimental Medicine, Brussels Free University, Brussels 1000, Belgium

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1. Introduction

In July–August 1981, three reports appeared on the presence in rat liver of a novel enzyme catalyzing the formation of fructose-2,6-bisphosphate (fructose-2,6-P_2) from fructose-6-P and ATP-Mg [1–3]. Fructose-2,6-P_2 was identified as a potent activator of phosphofructokinase [4–6]. Exposure of intact pancreatic islets to a high concentration of glucose (20 mM) results in the activation of phosphofructokinase, a phenomenon tentatively attributed to the generation of fructose-2,6-P_2, which indeed activates phosphofructokinase in an islet homogenate [7]. In support of this interpretation, it is shown here that pancreatic islets indeed display fructose-6-P,2-kinase activity.

2. Materials and methods

Groups of 950–1150 islets isolated from the pancreas of fed albino rats were placed in 0.18 ml of a Tris–HCl buffer (200 mM, pH 7.4) containing 5 mM $MgCl_2$ and 0.5 mM EGTA. After exposing the tubes containing the islets to liquid nitrogen, the islets were homogenized by mechanical vibration [8]. After centrifugation for 3 min at 5000 × *g*, an aliquot (160 μl) of the supernatant solution was brought to a final volume of 180 μl containing, as final concentration and except if otherwise mentioned, Tris–HCl (200 mM, pH 7.4), $MgCl_2$ (5 mM), EGTA (0.5 mM), ATP (5 mM) and fructose-6-P (5 mM). After incubation for 5–20 min at 30°C, the reaction was halted by adding 20 μl NaOH (1.0 M) and heating for 20 min at 80°C. The tubes were again centrifuged for 3 min at 5000 × *g* and aliquots (20 μl) of the supernatant solution examined for their content in fructose-2,6-P_2. For such a purpose, we used a reaction mixture (0.98 ml) containing (final concentrations), except if otherwise mentioned, Hepes–NaOH (50 mM, pH 7.4), EDTA (0.2 mM), $MgCl_2$ (10 mM), NH_4Cl (1 mM), dithiothreitol (2.5 mM), fructose-6-P (0.25 mM), ATP (10 mM), NADH (0.16 mM), desalted aldolase (0.4 units), triose-P-isomerase (2.4 units), α-glycero-P dehydrogenase (0.4 units). The reaction was initiated by addition of purified muscle phosphofructokinase and the basal activity recorded spectrophotometrically over 10–15 min incubation at 30°C. The sample (20 μl) derived from the first reaction was then added to the assay cuvette and the increase in reaction velocity measured over the ensuing 10–15 min. Thereafter, fructose-2,6-P_2 (kindly given by Drs E. van Schaftingen and H.-G. Hers; see [9]) was added in a small volume (10 μl) to yield 250 nM final conc. and the reaction velocity again monitored over 10–15 min. The reaction velocity was judged from the decrease in NADH absorbance at 340 nm. In some experiments, the sample (20 μl) derived from the first reaction was first mixed with 5 μl HCl (0.5 M) and allowed to stand for 20 min at room temperature prior to addition to the assay cuvette. The pH of the acid-treated sample (≤3.0) was not sufficiently low to affect either the final pH of the reaction mixture or the responsiveness of phosphofructokinase to exogenous fructose-2,6-P_2. All results are expressed as the mean (±SEM) together with the number of individual determinations (*n*).

3. Results

3.1. *Assay of fructose-2,6-P_2*

In a prior study of fructose-6-P,2-kinase, aliquots of the alkali-treated reaction mixtures were assayed for their content in fructose-2,6-P_2, i.e., the activator of phosphofructokinase, in the presence of 15 munits/ml purified muscle phosphofructokinase, 1 mM fructose-6-P and 1.25 mM ATP [1]. Using the same concentration of phosphofructokinase, we found that the

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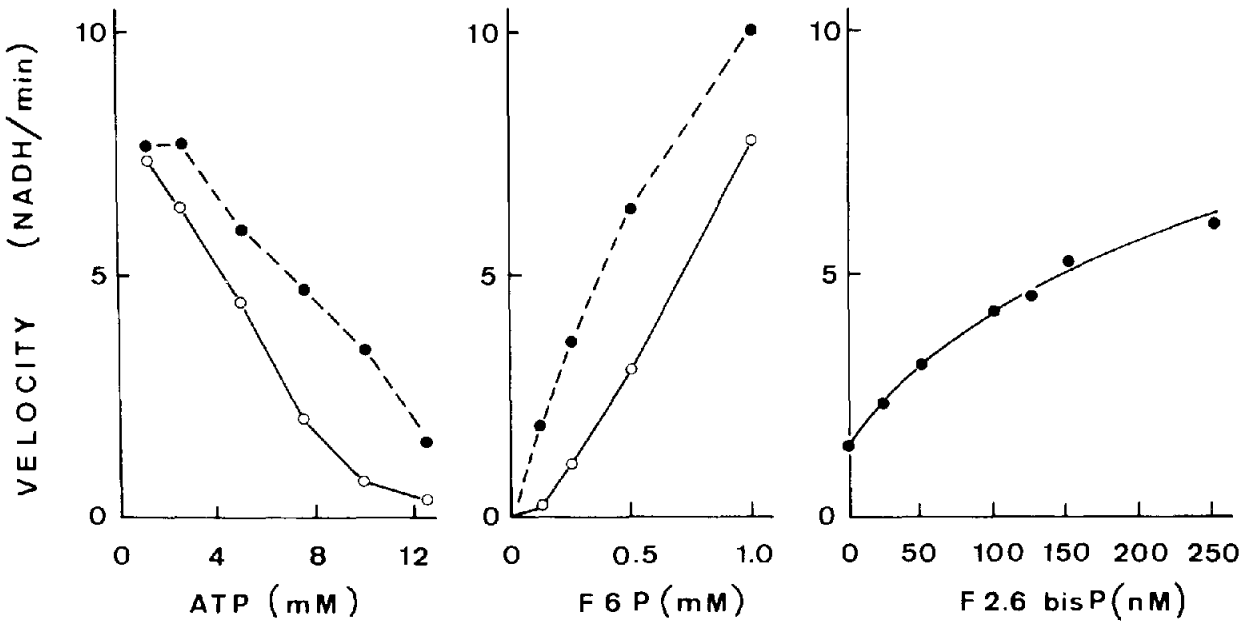

Fig.1. Velocity of the reaction catalyzed by purified phosphofructokinase (expressed as nmol NADH consumed/min) as a function of [ATP] (left; the initial concentration of fructose-6-P amounted to 0.25 mM), fructose-6-P (middle; initial ATP was 10 mM) and fructose-2,6-P_2 (right; the initial concentrations of fructose-6-P and ATP amounted to 0.25 mM and 10 mM, respectively). (Left, middle), data collected in the absence of activator (○——○) are compared to those collected in the presence of 250 nM fructose-2,6-P_2 (●– – –●). Mean values are derived from 2 or more individual determinations.

concentration of ATP had to be increased to a much higher value (8–10 mM) in order to obtain a close-to-optimal difference in reaction velocity in the absence and presence of the activor, respectively (fig.1, left) and this despite the relatively low concentration of fructose-6-P (0.25 mM) used in this series of experiments. The latter concentration of fructose-6-P was selected because, in the presence of 10 mM ATP, it also yielded a close-to-optimal difference in reaction velocity in the absence and presence of the activator, respectively (fig.1, middle). Thus, in further experiments, the assay of the activator was always performed in the presence of 15 munits/ml purified phosphofructokinase, 0.25 mM fructose-6-P and 10 mM ATP. Under the latter experimental conditions, the reaction velocity in the absence of fructose-2,6-P_2 averaged 1.10 ± 0.08 nmol NADH consumed/min (n = 63), which represented 26.0 ± 1.2% (n = 9) of the velocity recorded, in the same assay cuvette, in the presence of 250 nM fructose-2,6-P_2. The increase in reaction velocity attributable to the activator was dose-related in the 25–250 nM range of fructose-2,6-P_2 (fig.1, right). To minimize the variability of results from one experiment to another, we found it suitable to express the enhancing effect of unknown or standards amounts of the activator (i.e., the difference in reaction velocity prior to and after addition of the activator) relative to the increase in reaction velocity attributable to a high concentration of fructose-2,6-P_2 (250 nM). Thus, the basal velocity (no activator), the partially stimulated velocity (unknown or standards amounts of activator) and the close-to-maximal velocity (250 nM activator) were always measured in succession in the same assay cuvette. The concentration of activator in the samples was then calculated by reference to the abacus established at increasing concentrations of fructose-2,6-P_2 (standards).

The results illustrated fig.1, middle indicate that, under the present experimental conditions, a change in the concentration of fructose-6-P (over 0.25–0.50 mM) affected much more markedly the basal reaction velocity (i.e., that measured in the absence of activator) than the absolute value for the increment in reaction velocity attributable to fructose-2,6-P_2. This is important since the reaction mixture used for the characterization of fructose-6-P,2-kinase contains fructose-6-P, which could be transferred to the cuvette used for the assay of fructose-2,6-P_2. Under these experimental

conditions, if none of the fructose-6-P present in the first reaction mixture were either converted into another hexose-phosphate or destroyed by heating of the alkali-treated samples, the initial concentration of fructose-6-P in the second reaction mixture (i.e., that used for the assay of the activator) would be increased from 0.25–0.34 mM. In the absence of activator, such a rise in fructose-6-P concentration increased the velocity of the reaction catalyzed by phosphofructokinase to 145.9 ± 12.8% (n = 10) of the paired control values found in the presence of 0.25 mM fructose-6-P. As a result, the ratio in reaction velocity in the absence and presence of the activator (250 nM), respectively, increased from 26.0 ± 1.2% (n = 9) to 36.7 ± 2.6% (n = 6) as the fructose-6-P level was raised from 0.25–0.34 mM. However, the absolute value for the increment in reaction velocity attributable to the activator was not significantly different at the two concentrations of fructose-6-P, averaging 3.95 ± 0.37 and 4.22 ± 0.28 nmol NADH consumed/min at 0.25 and 0.34 mM fructose-6-P, respectively.

3.2. *Characterization of fructose-6-P,2-kinase*

The alkali-treated reaction mixture obtained after incubation of the islet homogenate in the presence of fructose-6-phosphate (5 mM) and ATP (5 mM), when added to the assay cuvette containing purified phosphofructokinase, markedly increased the reaction velocity. In a series of 12 expt, the samples obtained after 20 min incubation in the presence of the islet homogenate increased the reaction velocity from a basal value of 1.18 ± 0.17 to 3.00 ± 0.45 nmol NADH consumed/min, the velocity measured in the presence of 250 nM fructose-2,6-P_2 in the same cuvette averaging 6.91 ± 0.45 nmol NADH consumed/min (n = 12 in each case). In these experiments, the 20 μl samples

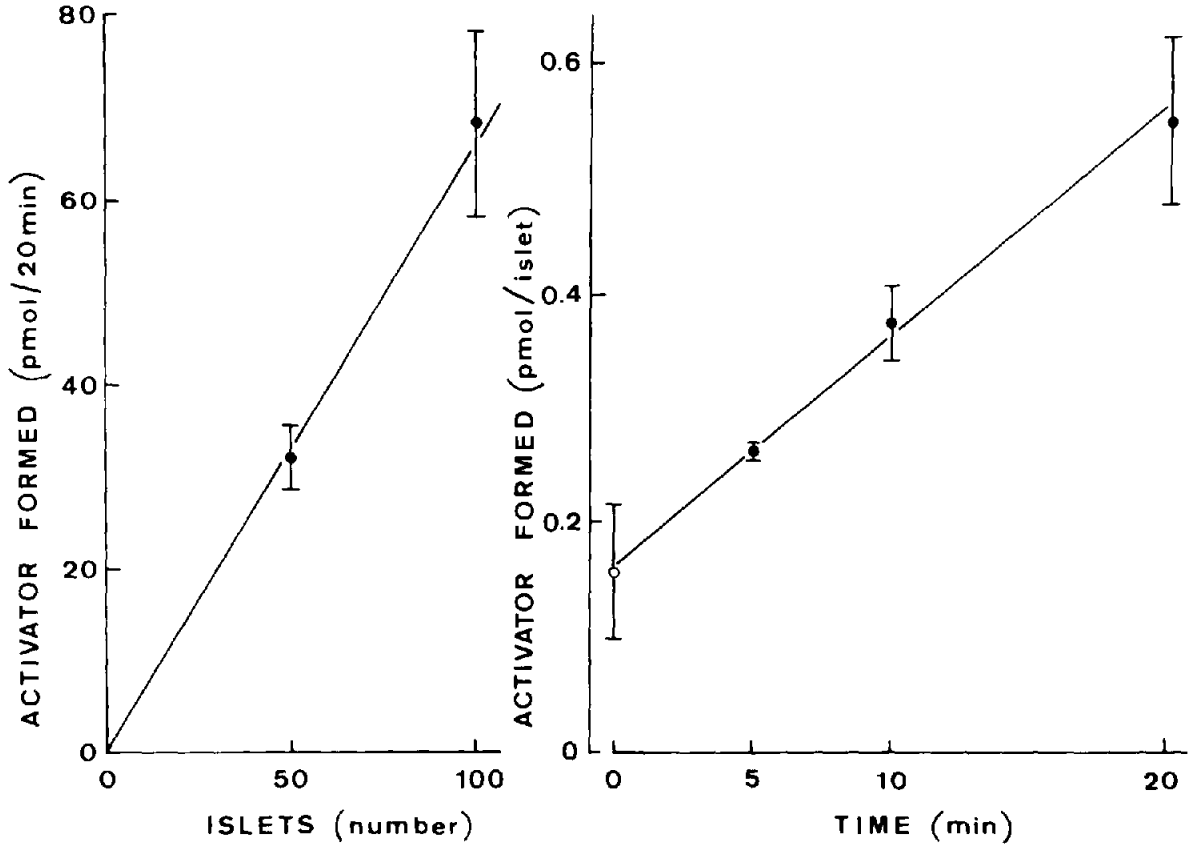

Fig.2. Left: relationship between the amount of activator formed and the number of homogenized islets present in the sample. Right: time course for the formation of the activator. The zero time value corresponds to the readings obtained with acid-treated samples. Mean values (±SEM) refer to 3–10 individual determinations.

derived from the first reaction mixture and added to the assay cuvette corresponded to 80 ± 2 islet-equivalents. From these data, the activity of fructose-6-P,2-kinase was estimated at 27.4 ± 4.3 fmol . min^{-1} . $islet^{-1}$ or, taking into account a mean protein content of 0.8 μg/islet [10], 0.034 ± 0.005 munits/mg islet protein.

The amount of activator generated during the first reaction was proportional to the volume of the sample assayed and length of incubation (fig.2). The reaction velocity in the presence of 0.5 mM fructose-6-P averaged 52.3 ± 7.3% (n = 4) of the paired value found in the presence of 5.0 mM fructose-6-P.

Several observations indicated that the increase in the velocity of the reaction catalyzed by phosphofructokinase was due, mainly if not exclusively, to the generation of an activator during the first reaction, namely that aiming at the characterization of fructose-6-P,2-kinase:

(i) When the first reaction was carried out in the absence of homogenate (and, hence, when fructose-6-phosphate could not be converted to any other hexose-phosphate), the increase in velocity of the reaction catalyzed by phosphofructokinase averaged ⩽20.8 ± 6.2% (n = 5) of that attributable, in the same experiment, to the sample obtained after 20 min incubation in the presence of the islet homogenate.

(ii) When the first reaction was carried out for 20 min in the presence of the islet homogenate but absence of fructose-6-P, the apparent concentration of activator represented ⩽3.2 ± 1.2% of the paired value found in the presence of fructose-6-P (5.0 mM).

(iii) When the samples derived from the first reaction mixture were acidified and then incubated for 20 min at room temperature in order to destroy most of the fructose-2,6-P_2 [11], their apparent concentration in activator averaged 28.8 ± 10.7% (n = 7) of the paired value found in a non-acidified sample.

4. Discussion

These data demonstrate that the islets contain an enzyme able to catalyze the formation from fructose-6-P and ATP-Mg of an acid-labile activator of phosphofructokinase. This situation is superimposable to that in liver, where sufficiently large amounts of tissue were available to partially purify the enzyme and to identify by chromatography the reaction product as fructose-2,6-P_2 [1–3]. It is remarkable that the activity of fructose-6-P,2-kinase in liver (i.e., 0.044 mU/mg protein) was almost the same as that here found in pancreatic islets (i.e., 0.034 mU/mg protein).

The presence of fructose-6-P,2-kinase in pancreatic islets adds further support to the view that fructose-2,6-P_2 is responsible for the glucose-induced activation of phosphofructokinase in intact islets. Moreover, preliminary measurements indicate that glucose indeed increases the tissue content of fructose-2,6-P_2 in intact islets (unpublished). It now remains to explore whether glucose stimulates the synthesis of the activator merely by a mass action phenomenon or via enzyme activation [12].

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