Fructose is a good substrate for rat liver ‘glucokinase’ (hexokinase D)

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Rat liver ‘glucokinase’ (hexokinase D) catalyses the phosphorylation of fructose with a maximal velocity about 2.5-fold higher than that for the phosphorylation of glucose. The saturation function is hyperbolic and the half-saturation concentration is about 300 mM. Fructose is a competitive inhibitor of the phosphorylation of glucose with a $K_i$ of 107 mM. Fructose protects hexokinase D against inactivation by 5,5'-dithiobis- (2-nitrobenzoic acid), and the apparent dissociation constants are about 300 mM in the presence of different concentrations of the inhibitor. The co-operativity of the enzyme in the phosphorylation of glucose can be abolished by addition of fructose to the reaction medium. Fructose appears to be no better as a substrate for the other mammalian hexokinases than it is for hexokinase D. It is proposed that the name ‘glucokinase’ ought to be reserved for enzymes that are truly specific for glucose, such as those of micro-organisms and invertebrates, and that liver glucokinase must be called hexokinase D (or hexokinase IV) within the classification EC 2.7.1.1.

Ever since the first work on yeast hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) it has been known that this enzyme catalyses the phosphorylation of the C-6 position of glucose, mannose and fructose (Colowick & Kalckar, 1941, 1943; Slim et al., 1950). Mammalian hexokinases from brain and muscle exhibit the same broad sugar specificity (Ochoa, 1941; Sols & Crane, 1954; Crane & Sols, 1955). Thus the discovery in rat and guinea-pig liver of an enzyme that seemed to be rather specific for glucose was of particular interest (DiPietro et al., 1962; Walker, 1962, 1963; Vinuela et al., 1963). This enzyme exhibited, however, a rather high half-saturation point of about 10 mM for glucose, and coexisted in the liver with a hexokinase that more closely resembled the enzymes of brain and muscle, which have a $K_m$ of about 0.1 mM. The new enzyme was named glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2), in part as recognition of an earlier observation by Slim et al. (1950), who had shown that under certain conditions liver extracts were able to phosphorylate glucose but not fructose, and had postulated the existence of a glucokinase in the liver.

Rat liver hexokinases (including glucokinase) were later resolved into four isoenzymes that were

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named A, B, C and D (González et al., 1964, 1967) or I, II, III and IV (Katzen et al., 1965). Isoenzyme D (IV) corresponded to glucokinase, because of its high $K_m$ for glucose and its low activity against 100 mM-fructose (Walker, 1963; González et al., 1964, 1967; Salas et al., 1965). With the recognition that the saturation function of glucokinase with glucose is sigmoidal (Niemeyer et al., 1975a,b; Storer & Cornish-Bowden, 1976b), we became interested in the kinetic properties of the enzyme with several sugar substrates and on their effect as competitive inhibitors on the phosphorylation of glucose (Niemeyer et al., 1978; Cárdenas et al., 1979; Olavarria et al., 1982).

In the present paper we report data for the phosphorylation of fructose catalysed by glucokinase and show that this isoenzyme is no more specific for glucose than are the other mammalian hexokinases. Thus the name 'glucokinase' appears to be a misnomer and ought to be replaced by hexokinase D, which is the name that we use in the present paper. Portions of this work have been published in abstract form (Cárdenas et al., 1981).

Experimental

Materials

ATP (disodium salt), NADP+, $\alpha$-D-glucose, $\beta$-D-fructose, D-glucosamine, glucose-6-phosphate dehydrogenase (EC 1.1.1.49, type VII, from
baker’s yeast), phosphoglucose isomerase (EC 5.3.1.9, type III, from yeast), Tris, EDTA, dithiothreitol, 2-mercaptoethanol, 5,5’-dithiobis-(2-nitrobenzoic acid) and 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide were products from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEAE-cellulose was either from Sigma Chemical Co. or from Whatman (Maidstone, Kent, U.K.) (DE-52). Sephadex G-100 and G-150 and CH-Sepharose 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Sepharose-N-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose was prepared by coupling D-glucosamine with CH-Sepharose 4B by means of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide, under conditions recommended by Pharmacia Fine Chemicals. Other chemicals were the highest grade commercially available.

Enzyme preparation

Hexokinase D was purified from the livers of well-fed rats by the procedure described in Cárdenas et al. (1978), which involves successive chromatographic separations on DEAE-cellulose, Sephadex G-100 or G-150 and Sepharose-N-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose. Most of the experiments were done with enzyme at the Sephadex step (specific activity 3–6 units/mg of protein), but the same results were obtained with enzyme of specific activity of 70 units/mg of protein.

Enzyme assay

Hexokinase D activity with fructose as substrate was assayed by measuring the production of fructose 6-phosphate through the reduction of NADP+ in the presence of an excess of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase. The increase in absorbance at 340 nm was monitored in a Gilford 2400 spectrophotometer, with circulating water at 30°C. The standard reaction mixture contained (final concentrations), in a total volume of 0.5 ml: 80 mM-Tris/HCl buffer, pH 8.0; 100 mM-KCl; 6 mM-MgCl2 or 1 mM-MgCl2, over the ATP concentration as indicated; 0.1 mM-EDTA; 2.5 mM-dithiothreitol; 5 mM-ATP (or other concentrations as indicated); 0.5 mM-NADP+; 0.7–0.8 unit of glucose-6-phosphate dehydrogenase/ml and 2 units of phosphoglucose isomerase/ml. When glucose was the substrate the isomerase was omitted. The concentrations of Mg2+ used ensured that the MgATP2- concentration would be a high and nearly constant proportion (86%) of the total ATP concentration (Storer & Cornish-Bowden, 1976a). The reaction was started by the addition of the sugar substrate unless otherwise indicated. When fructose was the substrate the omission of the isomerase from the assay medium gave no detectable increase in absorbance, at least during the first 10 min of incubation, and even at the highest fructose concentration, indicating no contamination with glucose. When fructose was used as an inhibitor of glucose phosphorylation blanks without ATP were performed at each glucose concentration and corresponded to glucose dehydrogenase activity of glucose-6-phosphate dehydrogenase. The absorbance at 100 mM-glucose was generally below 5% of the hexokinase activity, and at below 10 mM-glucose was negligible. With fructose as the substrate similar blanks gave undetectable values, even at the highest concentration. One unit of enzyme activity is defined as the amount of enzyme required to catalyse the conversion of 1 µmol of glucose/min at 30°C in standard conditions.

Enzyme inactivation by 5,5’-dithiobis-(2-nitrobenzoic acid)

Samples of the enzyme preparation were freed from dithiothreitol or 2-mercaptoethanol by using a small DEAE-cellulose column. The enzyme was eluted with 20 mM-glycylglycine/NaOH buffer, pH 8.0, containing 300 mM-KCl, 5% (v/v) glycerol and 1 mM-EDTA. The procedure also concentrated the preparation. About 50 munits of the enzyme were treated with variable concentrations of 5,5’-dithiobis-(2-nitrobenzoic acid) at 30°C in 0.5 ml of the same glycylglycine buffer in the presence of various fructose concentrations. Samples were removed for hexokinase assay at various time intervals, with 100 mM-glucose as substrate. This sugar concentration quenched any possible effect of 5,5’-dithiobis-(2-nitrobenzoic acid) during the assay. 5,5’-Dithiobis-(2-nitrobenzoic acid) was prepared fresh for each experiment in 100 mM-glycylglycine buffer, pH 8.0, containing 1 mM-EDTA.

Protein determination

Protein concentration was calculated from the absorbance at 260 and 280 nm by the procedure of Warburg & Christian (Dawson et al., 1972).

Results

Saturation function

In contrast with the behaviour with glucose, the saturation function of hexokinase D with fructose was hyperbolic [H (Hill coefficient) = 1.0], as is shown in Fig. 1. The apparent K_m values, at pH 8.0, were between 276 and 380 mM (median = 325 mM), measured with several different enzyme preparations with specific activities in the range 2–70 units/mg of protein. The K_m for the reactive tautomer of fructose is only about one-quarter of these values if it is considered that in
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Fig. 1. Hyperbolic kinetics of hexokinase D with fructose Experimental conditions were as described in the Experimental section. The straight line seen in the Eadie–Hofstee plot shown in the inset indicates that the enzyme obeys Michaelis–Menten kinetics \( (h = 1.0) \). \( K_m = 276 \text{mM} \).

aqueous solution, at 25–30°C, only about 25% of the fructose is in the furanose form (Gottschalk, 1943a,b; Que & Gray, 1974), which is the only one able to act as substrate of hexokinases (Slein et al., 1950). As with glucose (Cárdenas et al., 1979), decreasing the pH from 8.0 to 7.5 did not modify the Hill coefficient with fructose, but increased the half-saturation concentration by about 30% (420 mM at pH 7.5). Most previous studies of the saturation function of hexokinase D with fructose gave very high values of \( K_m \) (>800 mM), which were in some cases even higher than the concentration range used in the experiments (Salas et al., 1965; Parry & Walker, 1966; Grossman et al., 1974). An exception was the report by González et al. (1967), which gave a low \( K_m \) value, similar to the one with glucose. In general, all these results introduced doubts on the real capacity of the enzyme for phosphorylating fructose. We do not have a satisfactory explanation for the disagreement between the previous studies and the present results.

Relative maximal velocities with glucose and fructose

In spite of the higher value of half-saturation concentration with fructose than with glucose, the apparent maximal velocities with fructose (obtained by double-reciprocal plots, Hanes–Woelf plots or Eadie–Hofstee plots) were always 2.1–2.8-fold higher than the ones with glucose under the same conditions. The apparent maximal velocities with glucose were obtained by utilizing a computer program with \( h \), \( K_{0.5} \) and \( V_{max} \) varied to yield the best statistical fit of the equation

\[
v = V_{max}[S]/(K_{0.5} + [S])
\]

to the data or by graphical extrapolation. A variation of no more than 5% was obtained by the two methods. With the purpose of establishing a more rigorous comparison between the maximal velocities for glucose and fructose, initial velocities were measured in parallel experiments with the same enzyme solution with variation of the concentration of the hexose and ATP at a constant ratio, as suggested by Gulbinsky & Cleland (1968). The ratio \([glucose]/[ATP]\) was 10:1, and the ratio \([fructose]/[ATP]\) was 200:1. With both hexoses a parabolic graph was obtained when \( 1/v \) was plotted against \( 1/[ATP] \) (Fig. 2), as expected for a ternary-complex (sequential) kinetic mechanism (Gulbinsky & Cleland, 1968). (In the case of glucose the non-Michaelian kinetics do not allow this conclusion to be drawn.) Extrapolation gave different
intercepts on the ordinate for glucose and fructose, which indicated a maximal velocity 2.6-fold higher with fructose than with glucose, in excellent agreement with previous results, as commented above. This determination contrasts with experiments of the same design performed with 2-deoxyglucose, which showed that the maximal velocity with this substrate was only 73% of the value with glucose (Monasterio, 1980).

**Kinetic mechanism of hexokinase D with fructose**

Data from initial-velocity studies could be described by a ternary-complex mechanism, as shown by the Hanes–Woolf plots of Fig. 3. Secondary plots were linear (insets in Fig. 3), thus confirming the hyperbolic nature of the saturation function of hexokinase D with fructose and with MgATP at different concentrations of the fixed

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**Fig. 3. Initial-velocity studies of hexokinase D with fructose as substrate**

ATP total concentrations were 0.3, 0.6, 0.9 and 1.2 mM, with total Mg\(^{2+}\) concentration exceeding the total ATP concentration by 1.0 mM. The corresponding concentrations of MgATP were calculated (Storer & Cornish-Bowden, 1976a) to be 0.26, 0.52, 0.77 and 1.03 mM respectively. The fructose concentrations were 200, 400, 750 and 900 mM. Inset: secondary plots of slopes (○) and intercepts (●) of the Hanes–Woolf representations. (a) Fixed ATP concentrations and varied fructose concentrations. (b) Fixed fructose concentrations and varied ATP concentrations. In both cases the total ATP concentration has been plotted.
substrate. The equation for this type of behaviour is as follows (Cleland, 1963):

\[ v = V \cdot [A][B]/(K_{a}K_{b} + [A]K_{b} + [B]K_{a} + [A][B]) \]

where \([A]\) is the concentration of fructose and \([B]\) the concentration of MgATP. The constants have the following values as determined from the data of Fig. 3: \(K_{a} = 174 \text{mM}, \ K_{ia} = 1025 \text{mM} \) and \(K_{b} = 0.35 \text{mM}, \) and \(K_{ia}K_{b}/K_{a} = 2.0 \text{mM} \). The linearity of the plots and the clear intersection of the primary plots in the third quadrant agree with the behaviour observed with 2-deoxyglucose as substrate (Monasterio, 1980).

**Protection against inactivation by 5,5'-dithiobis-(2-nitrobenzoic acid)**

5,5'-Dithiobis-(2-nitrobenzoic acid) inactivates hexokinase D, the inactivation being reversed by dithiothreitol and blocked by glucose (Monasterio, 1980; Monasterio et al., 1981; Niemeyer et al., 1981). A reversible enzyme–5,5'-dithiobis-(2-nitrobenzoic acid) complex is formed previous to the chemical modification (Monasterio et al., 1981). When fructose was used as a protector of the inactivation of hexokinase D by 5,5'-dithiobis-(2-nitrobenzoic acid) it was as effective as glucose, blocking enzyme inactivation almost completely when added at zero time at concentrations above \(K_{m}\). The apparent dissociation constant estimated by plotting the reciprocal of the observed rate constant of inactivation versus ligand concentrations was about 300 \text{mM}, which agrees with the apparent \(K_{m}\) value (Fig. 4). The effect of fructose on hexokinase D inactivation by 5,5'-dithiobis-(2-nitrobenzoic acid) resembles the mixed pattern of enzyme inhibition, as the dissociation constant of the enzyme–fructose complex is virtually independent of the concentration of 5,5'-dithiobis-(2-nitrobenzoic acid) (results not shown). Similar behaviour has been observed with glucose as a protector (Monasterio et al., 1981).

**Effect of fructose on glucose phosphorylation**

Fructose had been described as a competitive inhibitor of ‘glucokinase’ with respect to glucose (Salas et al., 1965; Parry & Walker, 1966; González et al., 1967). We re-examined its behaviour in the light of the kinetic co-operativity of the enzyme with glucose. Fig. 5 shows a Dixon plot drawn with data obtained at relatively high concentrations of glucose (4–30 \text{mM}) in order to give straight lines (M. L. Cárdenas, E. Rabajille & H. Niemeyer, unpublished work). The competitive character of fructose with respect to glucose is shown by the set of parallel lines of the Cornish-Bowden graph of the inset (Cornish-Bowden, 1974). The inhibition constant was estimated as 107 \text{mM}, a value that is not in close agreement with the \(K_{i}\) value of 300 \text{mM} obtained from the protection studies. This discrepancy may be related to the complexity of the kinetics of hexokinase D; thus we have found that the inhibitory capacity of competitive inhibitors such as mannose and \(N\)-acetylglucosamine at low glucose concentrations decreases as the glucose concentration decreases (M. L. Cárdenas, E. Rabajille & H. Niemeyer, unpublished work). Our \(K_{i}\) value is lower than the one reported by Parry & Walker (1966) (350 \text{mM}), but this discrepancy is not very surprising, as they also obtained a very high value of \(K_{m}\) (about 2 \text{M}), as already mentioned above.

The presence of fructose in the assay mixture abolished the co-operativity of the saturation function for glucose. Thus with 300 \text{mM}-fructose a straight line is obtained in a double-reciprocal plot (Fig. 6), with a corresponding Hill coefficient of 1.0. The effect of different fructose concentration on the Hill coefficient and the half-saturation concentration of glucose as substrate is shown in Fig. 7. A similar effect on the saturation function with glucose is observed when mannose, 2-deoxyglucose or \(N\)-acetylglucosamine is used as inhibitor (Cárdenas et al., 1979, and unpublished work). In the present work the Hill plot and the Hill coefficient have been used only to ascertain the

![Fig. 4. Protection by fructose of hexokinase D inactivation by 5,5'-dithiobis-(2-nitrobenzoic acid)](image)
Fig. 5. **Dixon plots of the inhibition by fructose of glucose phosphorylation by hexokinase D**

The phosphorylation of glucose was measured at several fixed concentrations of this substrate, namely 4 (○), 8 (●), 15 (●) and 30 mM (●), and various fructose concentrations (40–900 mM) as indicated. The reaction was initiated by the addition of ATP (5 mM, final concentration). $K_i = 107$ mM-fructose. Inset: Parallel Cornish-Bowden plots indicate that any uncompetitive component of the inhibition was negligible.

Fig. 6. **Double-reciprocal plot of the phosphorylation of glucose in the absence and presence of fructose**

The phosphorylation of variable concentrations of glucose (1–200 mM) by hexokinase D (3–6 munits) was measured in the absence (●) or in the presence (○) of 300 mM-fructose. The reaction was initiated by the addition of ATP (5 mM, final concentration).

○, $K_{0.5} = 3.6$ mM-glucose, $h = 1.6$; ○, $K_{0.5} = 13.6$ mM-glucose, $h = 1.0$. The kinetic parameters were determined as indicated in Fig. 7 legend.

Fig. 7. **Effect of fructose on the kinetic parameters $K_{0.5}$ and $h$ of the saturation function of hexokinase D with glucose**

The phosphorylation of glucose (range 1–200 mM) by 4 munits of enzyme was measured at different fructose concentrations, as indicated in the Figure. The reaction was initiated by the addition of ATP (5 mM, final concentration). The kinetic parameters were obtained by using a computer program with $h$, $K_{0.5}$ and $V_{max}$ varied to yield the best statistical fit of the equation

$$v = \frac{V_{max} [S]}{(K_{0.5}^h + [S]^h)}$$

to the data. Similar values of $h$ and $K_{0.5}$ (variation of less than 5%) are obtained from a Hill plot in which a $V_{max}$ value calculated as described above or estimated graphically was used.
Table 1. Kinetic parameters for hexokinase isoenzymes with glucose and fructose as substrates at pH 7.5

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>$K_{Glc}^{0.5}$ (mM)</th>
<th>$K_{Fr}^{0.5}$ (mM)</th>
<th>$K_{Glc}^{0.5}/K_{Fr}^{0.5}$</th>
<th>$V_{Glc}/V_{Fr}$</th>
<th>$V_{Fr}K_{Glc}^{0.5}$</th>
<th>$V_{Glc}K_{Fr}^{0.5}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase A*</td>
<td>0.044</td>
<td>3.1</td>
<td>70.5</td>
<td>1.1</td>
<td>0.016</td>
<td>0.052</td>
</tr>
<tr>
<td>Hexokinase B*</td>
<td>0.130</td>
<td>3.0</td>
<td>23.1</td>
<td>1.2</td>
<td>0.022</td>
<td>0.043</td>
</tr>
<tr>
<td>Hexokinase C*</td>
<td>0.020</td>
<td>1.2</td>
<td>60.0</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexokinase D</td>
<td>7.5†</td>
<td>420‡</td>
<td>56.0</td>
<td>2.4‡</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* From Niemeyer et al. (1975b).
† From Niemeyer et al. (1975a).
‡ Present work.

degree of co-operativity without implication of mechanism.

Discussion

Since the first reports there has been general agreement in considering that hexokinase D (glucokinase) shows a very low activity with fructose by comparison with the other animal hexokinases (Viñuela et al., 1963; González et al., 1964; Parry & Walker, 1966; Niemeyer et al., 1975b; Weinhouse, 1976). This idea was mainly a consequence of the relative velocities of phosphorylation of fructose and glucose at a sugar concentration of 100 mM. At this concentration hexokinases A, B and C catalyse the phosphorylation of fructose better than glucose (1.1–1.3-fold) (Niemeyer et al., 1975b), but for hexokinase D the ratio of phosphorylation of fructose and glucose is only about 0.2:1. However, if instead of comparing the relative velocities at one substrate concentration we examine the quotient $V_{Fr}/K_{Glc}^{0.5}/V_{Glc}K_{Fr}^{0.5}$ (Table 1), we can see that the four hexokinases show similar specificity for fructose, and in all of them glucose is a more specific substrate than fructose. For an enzyme obeying Michaelis–Menten kinetics the ratio $k_{cat}/K_m = k_{cat}/K_{0.5}$ is the best measure of specificity (Fersht, 1977); although it is not strictly correct for a co-operative enzyme such as hexokinase D, it is clearly preferable to other parameters. Table 1 shows that, not only does glucokinase have a much higher $K_{0.5}$ for fructose than for glucose, but this is also true for hexokinases A, B and C. On the other hand, hexokinase D has a higher maximal velocity with fructose than with glucose.

Although in the past the relative capacity for phosphorylating fructose at a concentration of 100 mM might have been a good argument for using the name glucokinase and a separate EC number, this name is no longer valid. In fact, even without our present knowledge of the specificity of the liver isoenzyme, some authors have questioned whether glucokinase is the proper name for it (Walker, 1966; Niemeyer et al., 1975b; Ureta et al., 1979; Pollard-Knight & Cornish-Bowden, 1982). Especially, Ureta et al. (1979) have made an extensive analysis of the problem.

The name glucokinase and the classification as EC 2.7.1.2 strictly implies an enzyme that is specific for glucose, which is inappropriate for the liver enzyme, since it can also catalyse the phosphorylation of mannose, fructose and 2-deoxyglucose, sugars that are also competitive inhibitors of the phosphorylation of glucose. In these properties the liver enzyme differs from the glucokinases from Aerobacter aerogenes (Kamel et al., 1966), Brevibacterium fuscom (Saito, 1965), Bacillus stearotherophilus (Hengartner & Zuber, 1973), Streptococcus mutans OMZ 70 (Porter et al., 1982), Dictyostelium discoideum (Baumann, 1969), Schistosoma mansoni (Bueding & Mackinnon, 1955) and Echinococcus granulosus (Aposin & Aravena, 1959), which are truly specific for glucose, but resembles multiple-substrate hexokinases of yeast and mammals. In addition, the analysis of Table 1 indicates that there is really nothing special about the liver hexokinase D in relation to fructose specificity. Furthermore, it seems that neither hexokinase D nor the other hexokinases are responsible for the phosphorylation of fructose in vivo, since the phosphorylation of fructose in the liver occurs in position C-1 by the action of fructokinase (Hers, 1955). Thus, instead of glucokinase, the name hexokinase D (or hexokinase IV), within the classification number of EC 2.7.1.1, would be more appropriate and is the one used throughout the present paper. The name 'glucokinase' should be reserved more properly for those enzymes known to be specific for glucose, such as those isolated from several invertebrates and micro-organisms, as mentioned above.

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