Suppression of kinetic cooperativity of hexokinase D (glucokinase) by competitive inhibitors

A slow transition model

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Hexokinase D (‘glucokinase’) displays positive cooperativity with mannose with the same $h$ values (1.5 – 1.6) as with glucose but with higher $K_{0.5}$ values (8 mM at pH 8.0 and 12 mM at pH 7.5). In contrast, fructose and 2-deoxyglucose exhibit Michaelian kinetics [Cárdenas, M. L., Rabajille, E., and Niemeyer, H. (1979) Arch. Biol. Med. Exp. 12, 571–580; Cárdenas, M. L., Rabajille, E., and Niemeyer, H. (1984) Biochem. J. 222, 363–370]. Mannose, fructose, 2-deoxyglucose and $N$-acetylglucosamine acted as competitive inhibitors of glucose phosphorylation and decreased the cooperativity with glucose. Their relative efficiency for reducing the value of $h$ to 1.0 was: fructose > mannose > 2-deoxyglucose > $N$-acetylglucosamine. Galactose, which is not a substrate nor an inhibitor, was unable to change the cooperativity. The competitive inhibition of glucose phosphorylation by $N$-acetylglucosamine or mannose was cooperative at very low glucose concentrations (< 0.5 $K_{0.5}$), suggesting the interaction of the inhibitors with more than one enzyme form. These and previously reported results are discussed on the basis of a slow transition model, which assumes that hexokinase D exists mainly in one conformational transition to $E_h$. This new conformation would have a higher affinity for the sugar substrates and a higher catalytic activity than $E_k$. Cooperativity would emerge from shifts of the steady-state distribution between the two enzyme forms as the sugar concentration increase. The inhibitors would suppress cooperativity with glucose by inducing or trapping the $E_k$ conformation. In addition, the model postulates that the different kinetic behaviour of hexokinase D with the different sugar substrates, cooperative with glucose and mannose and Michaelian with 2-deoxyglucose and fructose, is the consequence of differences in the velocities of the conformational transitions induced by the sugar substrates.

‘Glucokinase’ (ATP: D-glucose 6-phosphotransferase) is one of four hexokinase isoenzymes in rat liver and it is also named hexokinase D [1, 2] or hexokinase IV [3]. However, in view of its substrate specificity in relation to the other animal hexokinases either of these latter names, within the classification EC 2.7.1.1, would be more appropriate [4]. For this reason we shall use the name of hexokinase D in this report.

The enzyme displays a sigmoidal saturation function for glucose with a Hill coefficient of 1.5 – 1.6 and a half-saturation concentration of about 7.5 mM at pH 7.5 [5 – 7]. This cooperative behaviour accords well with the presumed function of the enzyme [8] and has also been observed in isolated hepatocytes [9]. The molecular interpretation of hexokinase D cooperativity poses some problems, as it is a monomeric protein with only one active site [10 – 13] to which classical equilibrium models of cooperativity [14, 15] cannot be applied. As the cooperativity must be purely kinetic in origin steady-state models have been proposed [16 – 19]. In an attempt to have a better understanding of the molecular mechanisms responsible for the kinetic cooperativity of hexokinase D, we studied the kinetic behaviour with different sugar substrates and the effect of competitive inhibitors on the cooperativity with glucose. The first results appeared very promising as the kinetics with 2-deoxyglucose was Michaelian and $N$-acylglucosamine decreased the cooperativity with glucose [17]. In this report we present a detailed study on the phosphorylation of mannose and on the effects of mannose, 2-deoxyglucose and $N$-acylglucosamine, as competitive inhibitors of the reaction with glucose. We discuss our results with a slow transition model that considers an ordered binding of substrates and which apparently gives account of the known experimental facts.

MATERIALS AND METHODS

Materials

Substrates, inhibitors, coenzymes, auxiliary enzymes and DEAE-cellulose were products from Sigma Chemical Co. (St. Louis, MO, USA). Other materials were as in Cárdenas et al. [11].

Enzyme preparation

Hexokinase D was purified from the liver of well fed rats by a procedure previously described [11], which involves successive use of DEAE-cellulose, Sephadex G-100 or G-150 and Sepharose-N-(6-amino hexanoyl)-2-amino-2-deoxy-D-glucopyranose (affinity column). Most of the experiments...
were done with enzyme at the Sephadex step (spec. act. 3–6 units/mg protein), which is free of any other hexokinase activity. For practical considerations a partially purified enzyme was preferred instead of the homogeneous enzyme, as comparison between samples of enzymes with different degree of purification gave the same kinetic parameters and only differed in specific activity.

**Enzyme assay**

The reaction kinetics was monitored by spectrophotometry at 340 nm [11]. The reaction mixture contained (final concentrations) in a total volume of 0.5 ml: 80 mM Tris/HCl pH 8.0 or 7.5; 100 mM KCl; 6 mM MgCl2; 0.1 mM EDTA; 2.5 mM dithiothreitol; 5 mM ATP; 0.5 mM NADP and variable amounts of hexokinase D. When glucose was the substrate, 0.6–1.0 unit/ml glucose-6-phosphate dehydrogenase was added. In the assay with mannose as substrate, 8 units/ml phosphomannose isomerase, 4.6 units/ml phosphoglucone isomerase and 1.4 units/ml glucose-6-phosphate dehydrogenase were present. The concentrations of Mg2+ used ensured that the MgATP2- concentration would be a high and nearly constant proportion (86%) of the total ATP concentration [21]. The hexokinase D concentration was not always the same for a given set of rate measurements but was varied to ensure accurate velocity determinations. The reaction velocity was always proportional to the enzyme concentration thus it was easy to normalize the rate measurements. The system was preincubated at 30°C for 5 min prior to starting the reaction by the addition of the sugar substrate unless otherwise is indicated. Sugar concentrations, when used as substrates or inhibitors, were as indicated in legends to figures. Blanks without ATP corresponded to glucose dehydrogenase activity of glucose-6-phosphate dehydrogenase and were performed at each glucose concentration. The absorbance at 100 mM glucose was generally below 5% hexokinase D activity, and was negligible below 10 mM glucose. When mannose was the substrate the value of the blank without ATP was undetectable even at the highest concentrations used. When mannose was used as an inhibitor of glucose phosphorylation, the blanks with ATP but without glucose at different mannose concentrations also gave undetectable values and for periods as long as 30 min. When 2-deoxyglucose was used as an inhibitor of glucose phosphorylation the blanks with ATP but without glucose were undetectable during the first minutes of incubation. However, after a lag period, variable in length depending on the concentration of 2-deoxyglucose and of hexokinase D, a progressive increase of absorbance was observed due to a low but significant catalytic activity of glucose-6-phosphate dehydrogenase with 2-deoxyglucose 6-phosphate.

This acceleration was practically undetectable in the experiments with relatively high glucose concentrations (Fig. 10, Table 1), but it constitutes a problem for estimating the velocity at low glucose concentrations, where higher amounts than usual of hexokinase D (10–100 mU/assay) are required and consequently a higher amount of 2-deoxyglucose 6-phosphate is formed. A similar acceleration was detected when fructose, but not mannose, was used as an inhibitor. These observations agree with the report that 2-deoxyglucose 6-phosphate and fructose 6-phosphate, but not mannose 6-phosphate, are secondary substrates of glucose-6-phosphate dehydrogenase [22].

One unit of enzyme activity is defined as the amount of enzyme required to catalyze the conversion of 1 μmol glucose in 1 min at 30°C in the assay conditions.

In all cases recorder traces were straight during the periods of measurement and initial rates were found with a ruler and pencil. The kinetic parameters were obtained 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. Similar values of $h$ and $K_{0.5}$ (variation of less than 5%) were obtained from a Hill plot, in which a $V_{max}$ value, calculated as described above, or estimated graphically was used.

**Protein determination**

Protein determination was calculated from the absorbance at 260 nm and 280 nm by the procedure of Warburg and Christian [23].

**RESULTS**

**Saturation function of hexokinase D with mannose**

Mannose is a good substrate for hexokinase D and exhibits a cooperative effect similar to that of glucose, as is shown in Fig. 1. Thus, with both substrates a non-linear Eadie-Hofstee function is obtained (concave downwards curve in inset of Fig. 1). The Hill coefficient ($h$) was about 1.5, i.e. the same value as obtained with glucose [5, 6]. In contrast, the saturation functions of hexokinase D with 2-deoxyglucose [17, 24] and with fructose [4] were hyperbolic ($h = 1.0$). In this report the Hill coefficient and the Hill plot have been used only to measure the degree of cooperativity with no implication of mechanism. As in the case of glucose, the $h$ value for mannose did not vary when preparations of different degree of purification (spec. act. 0.2–70 unit/mg protein) were used nor when a different assay procedure was used; nor did it vary after changing the pH of the assay medium from 7.5 to 8.0. The $K_{0.5}$ value (concentration for half-maximal velocity) was slightly higher with mannose than with glucose, being about 8 mM at pH 8.0 and about 12 mM at pH 7.5. The maximal velocity with mannose is slightly lower than with glucose (about 90%). The sigmoidal dependence of velocity on mannose concentration with a Hill coefficient of about 1.5 is shown not only by rat hexokinase D but also by the enzyme of other animal species as hamster (Mesocricetus auratus), Chilean frog (Calyptocephalella caudiverbera) and Chilean turtle (Geochelone chilensis); this agrees with results for glucose as substrate [5].

**Effects of alternative substrates and N-acetylglucosamine on glucose phosphorylation**

Several alternative sugar substrates and the analogue N-acetylglucosamine have been described as competitive inhibitors of hexokinase D with respect to glucose [2, 25–27]. We explored again their behaviour as inhibitors in view of the kinetic cooperativity of the enzyme with some of the substrates and the hyperbolic kinetics with others, as mentioned above.

**N-acetylglucosamine.** We found that this sugar behaves as a competitive inhibitor of hexokinase D in agreement with previous reports. Dixon plots permitted the estimation of $K_i$ values, which fall between 0.11 mM and 0.15 mM (median = 0.14 mM) in several experiments performed with different hexokinase D preparations at either pH 7.5 or 8.0. These values were lower than those reported previously (0.38–0.5 mM) [2, 25–27]. Only glucose concentrations over 4 mM were used for our calculations since they gave linear graphs
Fig. 1. Kinetic cooperativity of hexokinase D with glucose or mannose as substrates. Activities were measured at pH 8.0, as described in Materials and Methods. All the experimental points were used to draw the Eadie-Hofstee graphs of the insets, but the concentrations higher than four times $K_{0.5}$ were omitted for the direct representations. The kinetic parameters were: (A) glucose, $V_{\text{max}} = 9$ mU, $K_{0.5} = 4$ mM, $h = 1.6$; (B) mannose, $V_{\text{max}} = 7.9$ mU, $K_{0.5} = 8.0$ mM, $h = 1.5$

Fig. 2. Dixon plot of the inhibition by N-acetylglucosamine of hexokinase D at different glucose concentrations. The enzyme was assayed at pH 8.0 in the presence of different concentrations of GlcNAc as indicated. (A) The assays were performed with 6.5—13 mU hexokinase D (the results were normalized to an activity of 6.5 mU) in the presence of 2 (○), 6 (●), 10 (□), 20 (■) mM glucose. Inset: (----) The experiment performed at 2 mM glucose; the arrow indicates the highest concentration represented in the main graph. (---) Another experiment at 2 mM glucose, performed with 4.3 mU hexokinase D (another preparation). (B) 25—100 mU hexokinase D (normalized to 100 mU) were assayed at 0.2 (○), 0.6 (●), 2 (□) mM glucose. (C) 130 mU hexokinase D were assayed at 0.1 (○) or 0.2 (●) mM glucose

Fig. 3. Effect of glucose concentration upon inhibition of hexokinase D by N-acetylglucosamine. The enzyme was assayed at variable concentrations of glucose in the absence ($v_a$) and in the presence of diverse fixed GlcNAc concentrations ($v_i$), at pH 8.0. (A) 48 mU hexokinase in the presence of 0.38 mM GlcNAc. (B) 10—100 mU enzyme were used depending on the inhibitor concentrations. 0.048 (○), 0.08 (●), 0.16 (□), 0.36 (■), 0.60 (△), 1.0 (▲) mM GlcNAc

(Fig. 2A). With very low glucose concentrations (≤ 2 mM), Dixon plots gave curves with downward curvature, mimicking partial competitive inhibition as illustrated in the inset of Fig. 2A. Similar curves were obtained with 0.1, 0.2 or 0.6 mM glucose in the assay medium (Fig. 2B, 2C). Tangents drawn at zero concentrations of the inhibitor tend not to intersect in the upper left quadrant, suggesting the presence of an apparent uncompetitive component, as if N-acetylglucosamine were interacting with different affinities with more than one enzyme form, and that the relative proportions of these forms are dependent on glucose concentrations. The mutual effects of glucose and N-acetylglucosamine on their interactions with hexokinase D at low glucose concentrations are illustrated in Fig. 3, in which the relative activities $v_i/v_a$, i.e. the ratio of velocities in the presence and in the absence of the inhibitor was studied as a function of glucose concentrations. As glucose concentrations decrease below about 1—2 mM the relative activities increase, which is not expected for a competitive inhibition. By increasing glucose beyond these values, the relative activities increase, as normally happens in that type of inhibition. Notice that the N-acetylglucosamine concentra-
Fig. 4. Cooperativity of N-acetylglucosamine inhibition of hexokinase D at low glucose concentration. 30 mU enzyme were assayed at pH 7.5 at 0.2 (●) or 1.0 (○) mM glucose in the presence of variable concentrations of GlcNAc as indicated.

Fig. 5. Hill plots of glucose phosphorylation in the presence of increasing N-acetylglucosamine concentrations. The phosphorylation of glucose (range 1–700 mM) by hexokinase D (4–8 mU) was measured at pH 8.0 in the absence (○) and in the presence of 0.36 (●), 0.96 (■), 2.8 (▲), 5.6 (△) 10.0 (◆) mM GlcNAc. The kinetic parameters, $K_a$ and $h$, at each GlcNAc concentration were the following: (○) 5.2 mM glucose, 1.52; (●) 14.5 mM glucose, 1.35; (■) 23 mM glucose, 1.33; (▲) 58 mM glucose, 1.21; (△) 115 mM glucose, 1.13; (◆) 268 mM glucose, 1.03.

Mannose. The competitive character of mannose with respect to glucose is shown in Fig. 6. The double-reciprocal plot of the saturation function of hexokinase D with glucose in the presence of a relatively low concentration of mannose (6 mM) was still curved upwards and could be linearized by using glucose concentrations to the power $h$ (inset of Fig. 6). However, at 20 mM mannose a straight line was obtained ($h = 0.96$). It was found, as in the case of N-acetylglucosamine, that the progressive increase in mannose concentration induces a progressive decrease of $h$ (progressive decrease of the slope of the Hill plots) and an increase of $K_0$ (Table 1). Also, as in the case of N-acetylglucosamine, there was a cooperative effect of mannose at very low glucose concentrations (Fig. 7A), which decreased as the concentration of glucose increases. This effect may also be seen when the relative enzyme activities ($v/v_o$) are studied as a function of glucose concentrations, in which case the inhibition was progressively less in the presence of glucose concentrations lower than 2 mM (Fig. 7B). To calculate the inhibition constant $K_i$ a Dixon plot was applied to data from experiments performed.
Table 1. Decreasing cooperativity of glucose phosphorylation in the presence of increasing concentrations of mannose and deoxyglucose

Experimental details are given in the text. Glucose-phosphorylating activity was measured at pH 8.0 in the presence of variable concentrations of glucose and of the inhibitor. The reaction was initiated by the addition of 5 mM ATP. In the experiment with mannose, hexokinase D was at the Sephadex step of purification (spec. act. 4.1 U/mg protein), and 4–8 mU were used. In the experiment with 2-deoxyglucose an enzyme (8 μM) after affinity chromatography was used (spec. act. 31 U/mg protein).

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with glucose at relatively high concentrations ($\geq K_{0.5}$), in which case reasonable straight lines were obtained (Fig. 8A). A $K_i$ of 3.1 mM was estimated, which is lower than the value of 16 mM for the rabbit enzyme [26] and the value of 14 mM for rat hexokinase D [27] previously reported. When low glucose concentrations ($< K_{0.5}$) were employed, the Dixon plot [28] and the Cornish-Bowden plot [29] revealed an apparent uncompetitive component (Fig. 8B).

2-Deoxyglucose. Fig. 9 shows that this compound is also a competitive inhibitor with respect to glucose in the hexokinase reaction, and that the cooperativity with glucose decreases in the presence of 2-deoxyglucose. Table 1 indicates that at a concentration of 200 mM 2-deoxyglucose (about 10 $K_m$) the saturation function for glucose became hyperbolic ($h = 1.0$). Experiments with 2-deoxyglucose at very low glucose concentrations, similar to the ones performed with mannose or N-acetylglucosamine, are not reported because of difficulties in the estimation of velocity with the required degree of security (see Materials and Methods).

Specificity

The relative efficiency of mannose, 2-deoxyglucose and N-acetylglucosamine in their effects on the cooperative behaviour of glucose phosphorylation appears in Table 2, where
the concentrations of the inhibitors are expressed as a function of their respective half-saturation concentration values, inhibition constant values or both. Data with fructose [4] are also included. It is interesting to note that fructose, a relatively poor inhibitor, appears as the most efficient, as the relative concentration necessary to bring the Hill coefficient to 1.0 was about three times lower than the relative concentration required by mannose and ten times lower than that required by 2-deoxyglucose. N-Acetylglucosamine was the least efficient as its concentration had to be about 100 times its \( K_i \) in order to give a Hill coefficient approaching 1.0.

The action of the inhibitors thus appears to be very specific since in each case a different concentration is required to suppress the cooperativity with glucose. Nevertheless in order to rule out an unspecified effect due to an increase in the concentration of alcohol groups and in viscosity originated by the presence of the high sugar concentrations we tested the effect of galactose, which had been described to be neither a substrate nor an inhibitor of hexokinase D [26, 27, 30]. We confirmed that galactose is not phosphorylated by hexokinase D and that it does not act as an inhibitor. Its presence in the assay medium, even at concentrations as high as 320 mM, does not alter the cooperativity with glucose. This fact constitutes strong evidence in favour of the specificity of the effect of fructose, mannose, 2-deoxyglucose and N-acetylglucosamine.

Long-chain acyl-coenzymes A have been described as competitive inhibitors of hexokinase D with both glucose and ATP, but do not alter the positive cooperativity with glucose [31]. In this case an allosteric site has been postulated for the binding of these compounds [32].

**DISCUSSION**

The molecular mechanism responsible for the kinetic cooperativity of hexokinase D with respect to two of its sugar substrates, glucose and mannose, is of particular interest because hexokinase D is a monomeric protein under native and denaturing conditions [10, 13] and also under the normal assay conditions [11]. Thus the cooperativity cannot be due to interactions between subunits [14, 15] or to an association/dissociation phenomenon induced by the substrates accompanied by changes in kinetic properties according to the degree of oligomerization [33]. Affinity labelling experiments have shown only one active site [12]. Thus the cooperativity of hexokinase D must be explained in purely kinetic terms, being necessary to postulate a steady-state model with at least two kinetically distinct pathways for the generation of products. We have postulated models [17, 19] with isomerization of the enzyme along the lines of the slow transition model proposed by Ainslie et al. [34]. Storer and Cornish-Bowden [16] have postulated a 'mnemonical mechanism' as the one developed by Ricard et al. [35] from the idea of enzyme memory [36]. Any postulated model for the cooperativity of hexokinase D ought to be able to explain at least the following observations: (a) sigmoidal kinetics with glucose and mannose and hyperbolic saturation function with 2-deoxyglucose and fructose [4–6, 17, 24] (and this paper); (b) suppression of sigmoidicity and increase of affinity for glucose at low ATP concentrations, and hyperbolic behaviour with respect to this substrate [6, 17, 20]; (c) suppression of cooperativity with respect to glucose in the presence of competitive inhibitors [17] (this paper); (d) cooperative inhibition of some of these inhibitors at low glucose concentrations (this paper).

In our models [17, 19] a random order of addition of substrates was postulated based on indirect evidence obtained from studies on the kinetics of hexokinase D with 2-deoxyglucose as substrate [24], and on chemical modification of the enzyme and the protection displayed by substrates [37]. A random mechanism in the steady state appeared attractive at first as it permits the generation of cooperative behaviour in a monomeric enzyme [38]. However, in order to explain at a molecular level that the two pathways were not kinetically equivalent we postulated slow conformational changes of the enzyme [17]. In Olavarria et al. [19] an extension of this model was simulated on an analog computer, using a stochastic method developed by Olavarria [39, 40]. It was shown that the simulated model was capable of accounting for some of the observations mentioned above. The existence of a random pathway is supported by isotope exchange studies [18], which have indicated that under conditions of chemical equilibrium a pathway exists in which MgATP binds to the free enzyme to give a binary complex capable of reacting with glucose. However, exchange experiments in the steady state showed that the main catalytic pathway corresponds to an ordered
The reaction would occur mainly through the ternary complex (glucose-affinity form) and in the first catalytic cycles the glucose (El form, and would predominate in the absence of ligands but with an ordered mechanism in which glucose is the first substrate and glucose 6-phosphate as the last product [18]. This last result suggests that the random order of substrates probably does not contribute significantly to the mechanism of cooperativity.

In view of these considerations we have attempted to discuss the results presented above and to explain the kinetic behaviour of glucokinase on the basis of a slow transition model, such as the one discussed in Olavarria et al. [19], but with an ordered mechanism in which glucose is the first substrate.

As in the previous models hexokinase D would exist basically in two conformations: El (circle) and EII (square) (Fig. 10). El would be the thermodynamically more stable form, and would predominate in the absence of ligands (k-1 > k-2). As glucose would increase, the rate of the transition from El to EII would depend on the MgATP concentration. Thus as the MgATP concentration decreases that fraction of the binary complex El would experience a kinetic lag can be predicted, but although we have looked for it we have not been able to see this hysteretic behaviour in the conditions of our assay.

The fraction of the binary complex El · Glc experiencing the transition would depend on the MgATP concentration. Thus as the MgATP concentration decreases that fraction of the binary complex El · Glc would decrease, allowing the operation of the catalysis mainly through the ternary complex EII · Glc · MgATP. This would explain the observation that the half-saturation glucose concentration decreases as the MgATP concentrations decrease [17]. Under these conditions there would still be the possibility of two routes [(EI → EI · Glc → EI · Glc · MgATP) and EII → EII · Glc · MgATP] to form the ternary complex EII · Glc · MgATP, depending on glucose concentration. As the experimental results have shown that at very low MgATP concentrations hyperbolic behaviour exists, these two routes must become equivalent.

The first indications of the possibility of the existence of two conformations of hexokinase D came out from studies of inhibition of glucose phosphorylation by the alternative substrate mannose and the analogue N-acetylglucosamine [17] (this paper). These observations suggested two simultaneous and opposite actions of the inhibitors; on one side, the competitive inhibition and, on the other, the activation due to the generation of a more active conformation. In fact, the experiments performed with very low glucose concentrations (< K0.5) suggested the generation of an enzyme form EII in the presence of N-acetylglucosamine or mannose in addition to the form El, which would be highly predominant in the absence of inhibitors. In contrast EII would be the prevailing form in the presence of high glucose concentrations (K0.5) and would have a high affinity for N-acetylglucosamine, i.e. a very low Ki, as experimentally found (0.11–0.14 mM).

In this case only the competitive inhibition of El by N-acetylglucosamine and mannose was apparent. As glucose concentrations decrease below K0.5, the proportion of the El form would increase, and if this conformer has lower affinity than the EII form for the inhibitors, inhibition constants Ki* of higher values than Ki would be expressed, giving the kinetic features of a mixed inhibition.

Cooperativity of glucose phosphorylation can be suppressed in the presence of high concentrations of the alternative substrates and of N-acetylglucosamine. This can be explained assuming that under these conditions only one conformation exists. Owing to the higher affinity of the inhibitors for El with respect to EII, they would be able to trap the El form generated by glucose, thus stabilizing this enzyme conformation. On the other hand, as the inhibitors have different efficiency in suppressing cooperativity with glucose, a difference that is not correlated to their affinities (Table 2), we postulate that the transition rates of the various binary enzyme-inhibitor complexes are different. Direct simulation studies of the kinetic cooperativity of hexokinase D have shown that changes in the velocities of the conformational transitions alter the cooperativity of the system and hyperbolic or cooperative behaviour can be obtained [19].

In the case of 2-deoxyglucose, which is the least efficient substrate in suppressing cooperativity with glucose, it can be
postulated that although this sugar derivative is able to induce the transition from $E_1$ to $E_0$, the transition rate would be slow enough in relation to the catalytic constant to permit, when used as substrate, the basic operation of the catalytic cycle through the ternary complex $E_1 \cdot dGlc \cdot MgATP$ to explain the hyperbolic kinetics of hexokinase D with 2-deoxyglucose. The fact that the maximal velocity with 2-deoxyglucose is about 0.7 $V_{max}$ with glucose is compatible with the postulation that the catalytic cycle through the $E_1 \cdot dGlc \cdot MgATP$ complex is slower than through $E_0 \cdot dGlc \cdot MgATP$.

Fructose also has hyperbolic kinetics and abolishes the cooperativity of glucose phosphorylation [4]. As in the case of 2-deoxyglucose, the operation of one catalytic cycle may be assumed. However, in this case we postulate that the cycle operates through $E_0 \cdot Fru \cdot MgATP$, because: (a) the maximal velocity with fructose is more than twice that with glucose, and (b) fructose is more efficient than 2-deoxyglucose in suppressing the cooperativity of glucose phosphorylation. These two facts suggest that fructose not only acts by trapping the $E_0$ form generated by glucose, but also by contributing to the generation of the $E_0$ form through $E_1 \cdot Fru \cdot MgATP$. Fructose before binding ATP and only one kinetic pathway would exist.

Cooperativity of glucokinase with mannose can be explained in the same way as in the case of glucose, that is, accepting that the two cycles would contribute to the formation of products. As mannose can both generate and trap the $E_0$ conformation, it would be very efficient in suppressing cooperativity of glucose phosphorylation. Using the same conceptual scheme, the low efficiency of N-acetylglucosamine in suppressing cooperativity of glucose phosphorylation can be explained assuming that the analogue is able to trap the $E_0$ form generated by glucose because of its high affinity for this form, but is unable by itself readily to generate the $E_0$ conformation.

Thus we postulate that the different kinetic behaviour with the different sugars substrates would be the consequence of differences in the velocities of the conformational transitions induced by the sugar substrates.

The main difference between the model that we have discussed here and the mnemonological model [16, 18] is that in the latter both enzyme conformations lead to the same enzyme - glucose complex. Although our data can also be explained by a mnemonical model we consider that at present there is no evidence that would allow one to establish whether only one or more than one hexokinase - glucose complex exists. Thus the model presented above may be considered less restrictive because it allows the possibility that the two enzyme - glucose forms, corresponding to the two enzyme conformations, may be different. While this manuscript was under consideration Pollard-Knight and Cornish-Bowden [41] reported a change in the kinetic properties of hexokinase D by $^2H_2O$, which we have interpreted by an extended version of the mnemonical model.

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