

Solvent isotope effects on the glucokinase reaction Negative co-operativity and a large inverse isotope effect in $^2\text{H}_2\text{O}$

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The solvent isotope effects on the reaction catalysed by rat-liver glucokinase have been studied. At low concentrations of glucose and high concentrations of MgATP^{2-} there is an inverse solvent isotope effect of 3.5. At high glucose concentrations there is a normal solvent isotope effect of 1.3. In $^1\text{H}_2\text{O}$ there is positive co-operativity with respect to glucose [Storer, A. C. and Cornish-Bowden, A. (1976) *Biochem. J.* 159, 7–14], but this is changed to negative co-operativity in $^2\text{H}_2\text{O}$. The half-saturation points for both glucose and MgATP^{2-} are decreased in $^2\text{H}_2\text{O}$ compared with those in $^1\text{H}_2\text{O}$. Explanations of these effects in terms of the mnemonical model proposed by Storer and Cornish-Bowden [*Biochem. J.* 65, 61–69 (1977)] were considered in computer simulation. Two interpretations could account for the results, either a decrease in the rate of interconversion of the two forms of free enzyme postulated in the model, or an increase in the affinity for glucose of the enzyme form with the lower affinity in $^1\text{H}_2\text{O}$. The results of a proton-inventory analysis were consistent with either of these interpretations. The solvent isotope effects thus provide additional evidence for the mnemonical model as an explanation of glucokinase co-operativity.

Hexokinase type IV (or type D) is the isoenzyme principally responsible for the phosphorylation of glucose in the livers of mammals and other vertebrates. The name 'glucokinase' is commonly applied to this isoenzyme and for convenience we shall do so in this paper. It shows positive co-operativity with respect to glucose in the physiological range but Michaelis-Menten kinetics with respect to ATP under all conditions [1, 2]. Such properties are consistent with the presumed function of glucose phosphorylation in the liver as a means of controlling the blood-glucose concentration.

Glucokinase is monomeric under all conditions of the assay [3–5] and has only a single active site [6]. Hence quasi-equilibrium models involving subunit interactions [7, 8] or reversible association of the enzyme [9, 10] cannot explain glucokinase co-operativity.

To account for the co-operativity Storer and Cornish-Bowden [11] proposed a kinetic model based on the 'mnemonical' model of Ricard et al. [12], applied originally to wheat-germ hexokinase L_1 [13]. The essential feature of the model, which has since received support from isotope-exchange studies [14], is that free glucokinase exists in two forms E and E' that bind glucose with different affinities (Fig. 1). We have reviewed the properties of glucokinase more fully elsewhere [15].

In view of the postulated dependence of the co-operativity on the conformational states of glucokinase, and because of the drastic effects that $^2\text{H}_2\text{O}$ can sometimes have on protein structural equilibria [16–18] we have studied the kinetics of

the glucokinase reaction in $^2\text{H}_2\text{O}$. A preliminary report of those results has appeared [19].

MATERIALS AND METHODS

Materials

Glucokinase was purified to a specific activity of 2.5 kat/kg from the livers of well fed Wistar rats, essentially by the method of Holroyde et al. [3]. After purification the enzyme was stored in 0.3-M KCl, 1-mM dithiothreitol, 50-mM glucose, 30% (v/v) glycerol, 20-mM triethanolamine HCl buffer, pH 7.0 at -20°C . In experiments where very low concentrations of glucose were required the excess present in the storage buffer was removed by passing the enzyme down a Sephadex G-25 column pre-equilibrated with 20-mM triethanolamine/HCl buffer at pH 7.0 containing 0.1-M KCl, 1-mM dithiothreitol, 10% (v/v) glycerol and 1-mM EDTA. Residual glucose, if any, was determined by the ferricyanide assay for reducing sugars [20]. Glucose added with the enzyme was always taken into

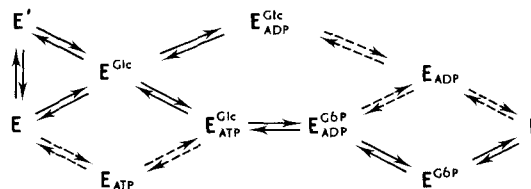


Fig. 1. Proposed mechanism for glucokinase. If the steps indicated by broken lines are omitted this mechanism constitutes the mnemonical mechanism, but with these steps included it represents the mechanism proposed by Cárdenas et al. [5]. Abbreviations: Glc, glucose; ATP, MgATP^{2-} ; ADP, MgADP^- ; G6P, glucose 6-phosphate

Enzyme. Hexokinase, ATP:D-hexose 6-phosphotransferase (EC 2.7.1.1), type IV; this isoenzyme is commonly known as glucokinase.

Symbol. The symbol L represents both ^1H and ^2H . Thus pL is a generalized pH that applies to mixtures of $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$.

account when calculating the glucose concentration in reaction mixtures.

Other materials

ATP (disodium salt), glycylglycine, 4-morpholinepropane-sulphonic acid, dithiothreitol, NADP⁺, glucose-6-phosphate dehydrogenase (type VII from baker's yeast) and 99.8 atom % ²H₂O were obtained from Sigma Chemical Co. Ltd. MgCl₂ was the anhydrous salt from Aldrich Chemical Company.

Standard solutions

The ATP concentration was determined spectrophotometrically [21], and concentrations of MgATP²⁻ in reaction mixtures were calculated from the total concentrations of MgCl₂ and ATP as described previously [22]. In all reaction mixtures the total Mg²⁺ concentration exceeded the total ATP concentration by 1.0 mM, an excess sufficient to maintain the concentration of MgATP²⁻ at a high and almost constant proportion (86 ± 2%) of the total ATP concentration and ATP⁴⁻ and free Mg²⁺ at low concentrations [22].

The pL of solutions in ²H₂O was taken as 0.4 greater than the reading of the pH-meter in all cases [23]. ¹H₂O solutions were titrated to the correct pL using KO¹H or ¹HCl, and ²H₂O solutions were similarly titrated with KO²H and ²HCl.

Enzyme assays

Glucokinase was assayed by coupling the production of glucose 6-phosphate to the reduction of NADP⁺ by means of glucose-6-phosphate dehydrogenase as coupling enzyme, under the conditions given previously [24], which were based on the assay of DiPietro and Weinhouse [25].

Recorder traces were essentially straight during the first 3 min of each assay and initial rates were obtained with a ruler and pencil. This linearity indicated that there was no exchange of hydrogen atoms important in the reaction during the time course of the experiment [26].

The solvent isotope effect is defined as the rate in ¹H₂O divided by the rate in ²H₂O. A normal isotope effect is greater than unity, i.e. the reaction is faster in ¹H₂O; whereas there is an inverse isotope effect if the reaction is faster in ²H₂O.

Analysis of data

Computer simulation was used to investigate the properties of different models. For each of these the rate equation was derived automatically by means of the program described previously [27] after it had been modified to be capable of carrying out numerical simulation with specified values of rate constants.

Non-linear regression was done by the method of Cornish-Bowden and Koshland [28] modified as described by Wharton et al. [29].

Computing was done on an International Computers Ltd, 1906A computer.

THEORY

General theory of the proton inventory

A proton-inventory analysis [26, 30, 31] is an experimental way of determining the number of exchanging protons

involved in a reaction and the contribution of each proton to the total isotope effect. It is necessary to determine the rate of a reaction as a function of the mole fraction of ²H₂O. Solvent isotope effects in ¹H₂O:²H₂O mixtures can be described by the general form of the Gross-Butler equation, expressed here for a rate constant *k*:

$$k_n = \frac{k_0 \Pi (1 - n + n\phi_i)}{\Pi (1 - n + n\phi_j)}$$

In this equation *n* is the atom fraction of ²H in the mixed solvent system; *k*₀ is the rate constant in pure ¹H₂O; *k*_{*n*} is the rate constant in solvent in which the atom fraction of ²H is *n*; ϕ_i and ϕ_j are the isotope fractionation factors for the *i*th transition-state proton and *j*th reactant proton respectively. The Π in the numerator indicates that we require the product of the expressions in parentheses evaluated for all *i* (all transition-state protons), and the Π in the denominator expresses the same requirement for all reactant protons. A fractionation factor is the equilibrium constant for exchange of ²H with solvent for a particular site and is thus a measure of the preference of the site for ²H over ¹H relative to the corresponding preference of a single site in the solvent molecule [32].

One can apply essentially the same equation to solvent isotope effects on equilibria, except that now the rate constants *k*₀ and *k*_{*n*} are replaced by equilibrium constants *K*₀ and *K*_{*n*} respectively, and the product state of the reaction replaces the transition state.

Although the general form of the Gross-Butler equation may seem hopelessly complicated, with an impossibly large number of protons to be considered, it is greatly simplified in practice by the fact that for protons unaffected by the reaction the transition-state and reactant fractionation factors are equal and thus the corresponding terms cancel. In general one can assess the number of protons involved in a reaction by the form of a plot of *k*_{*n*} against *n*. In the simplest case, a straight line indicates that the Gross-Butler equation is linear in *n*, so that only one fractionation factor different from unity remains in the numerator after cancellation and none remain in the denominator: this would indicate a 'one-proton' transition state. Plots curving upwards would indicate transition states with two or more protons. A downward curvature may be produced if a larger inverse isotope effect is partially offset by smaller normal isotope effects, etc., as discussed by Schowen [26].

In the present work observed plots of *k*_{*n*} against *n* were fitted by non-linear regression to various special cases of the Gross-Butler equation. The special cases were obtained from plausible models that had been shown by computer simulation to be capable of describing the kinetics of glucokinase.

Specific theory for the proton inventory of the glucokinase reaction

Let us consider the expression for the isotope effect on the rate of binding of glucose to an equilibrium mixture of two forms E and E' with an equilibrium constant *K* for the ratio [E]/[E']. If *k* and *k*' are the rate constants for binding of glucose to E and E' respectively, the observed rate constant *k*_{obs} will be the mean of *k* and *k*' weighted according to the proportions of E and E' in the mixture:

$$k_{\text{obs}} = (k' + kK)/(1 + K).$$

If replacement of $^1\text{H}_2\text{O}$ by $^2\text{H}_2\text{O}$ has no effect on k and k' , but has an effect on K because of m protons that have fractionation factors ϕ and ϕ' in E and E' respectively, the value of the equilibrium constant becomes

$$K_n = K_0(1 - n + n\phi')^m / (1 - n + n\phi)^m$$

where K_0 and K_n are the values of K in pure $^1\text{H}_2\text{O}$ and in a mixture with mole fraction n of $^2\text{H}_2\text{O}$ respectively. This must now be substituted for K in the expression for k_{obs} :

$$k_{\text{obs}} = \frac{k' + \frac{kK_0(1 - n + n\phi')^m}{(1 - n + n\phi)^m}}{1 + \frac{K_0(1 - n + n\phi')^m}{(1 - n + n\phi)^m}}$$

In a similar way one can derive an equation to describe the effect on the observed kinetics of a solvent isotope effect on any step in the reaction.

RESULTS

pL profile

At saturating concentrations of glucose and MgATP the pL curves are similar, but the pL optimum is about 0.5 higher in $^2\text{H}_2\text{O}$ than in $^1\text{H}_2\text{O}$ and there is a normal solvent isotope effect of 1.3 (Fig. 2). The shift in pL optimum is of the magnitude expected if the ionization of a weak acid is important in catalysis under the conditions used [33]. It also indicates that the deuterated solvent does not drastically effect the conformations of the enzyme complexes. The lack of effect on the shape of the pL profile may be contrasted with observations recently reported for a mixture of isoenzymes of hexokinase from yeast [34]: for this preparation the pL profile in $^2\text{H}_2\text{O}$ showed an anomalously steep dependence of the rate on pL on the low-pL side of the maximum, a feature that was absent from the pL profile in $^1\text{H}_2\text{O}$.

In the remainder of the present study rates were measured both at the pL optima and at equal pL values in both solvents to eliminate effects of any changes in pL optima resulting from changes in substrate concentrations. There were, however, only slight differences between the two sets of results, as expected from the broadness of the pL profiles. Only the results obtained at equal pL values will be shown.

Dependence of solvent isotope effects on the glucose concentration

The rate of the glucokinase-catalysed phosphorylation of glucose was measured at 4.3-mM MgATP (near-saturating) and at glucose concentrations in the range 0.1–20 mM in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ (Fig. 3). In $^1\text{H}_2\text{O}$ there is positive co-operativity with respect to glucose and the half-saturation point for glucose is 5 mM, as in previous work [2]. In $^2\text{H}_2\text{O}$, however, there is negative co-operativity and the enzyme is half-saturated at 3.5-mM glucose. These differences indicate a solvent isotope effect of 0.29 at 0.1-mM glucose (i.e. an inverse solvent isotope effect of 3.5). As the glucose concentration increases the inverse solvent isotope effect declines, so that there is no isotope effect at 1.8-mM glucose and a normal isotope effect of 1.3 at saturating glucose, i.e. the solvent isotope effect on the limiting rate V is 1.3. The results were

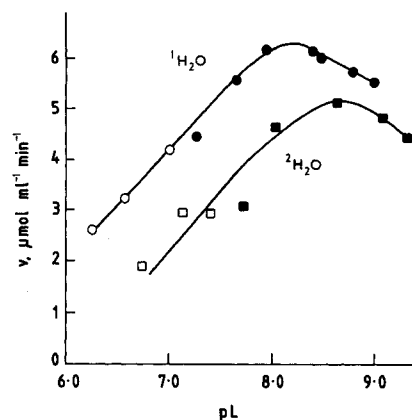


Fig. 2. pL profiles. Plots of rate v against pL (generalized pH defined in $^2\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}/^1\text{H}_2\text{O}$ mixtures as well as in $^1\text{H}_2\text{O}$) are shown for the glucokinase-catalysed reaction in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ as indicated. Points shown with open symbols refer to experiments in 4-morpholinepropanesulphonic acid buffer, those with filled symbols to ones in glycylglycine buffer

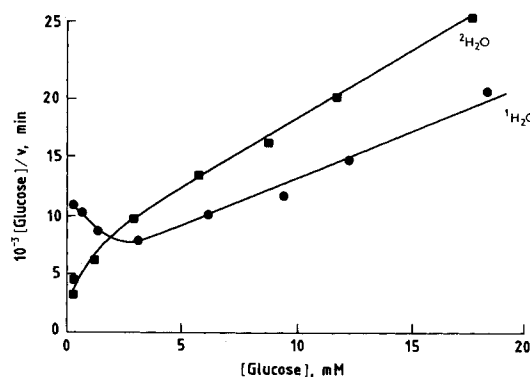


Fig. 3. Positive co-operativity in $^1\text{H}_2\text{O}$; negative co-operativity in $^2\text{H}_2\text{O}$. Rates were measured in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ as indicated in 4.3-mM MgATP. The upward curvature of the plot in $^1\text{H}_2\text{O}$ confirms the positive co-operativity reported previously [1,2], but the downward curvature of the other plot shows that this is converted into negative co-operativity in $^2\text{H}_2\text{O}$

unaffected by whether the enzyme had been stored and equilibrated before the experiment in $^1\text{H}_2\text{O}$ or $^2\text{H}_2\text{O}$.

The behaviour was similar at low concentrations of MgATP, but the inverse solvent isotope effect with 0.1-mM glucose was increased to 4.5 and the concentration at which there was no isotope effect was shifted to 2.5-mM glucose.

A fuller investigation of the kinetics in $^2\text{H}_2\text{O}$ with respect to glucose is shown in Fig. 4. The enzyme was half-saturated at 3.5-mM glucose at all MgATP concentrations, as compared with a value of 5-mM glucose in $^2\text{H}_2\text{O}$.

MgATP kinetics

As in $^1\text{H}_2\text{O}$ [2], glucokinase displayed Michaelis-Menten kinetics with respect to MgATP at all glucose concentrations (Fig. 5), the apparent K_m for MgATP being lowered from 0.55 mM in $^1\text{H}_2\text{O}$ to 0.28 mM in $^2\text{H}_2\text{O}$. Both the nature and the magnitude of the solvent isotope effect were virtually unaffected by the MgATP concentration.

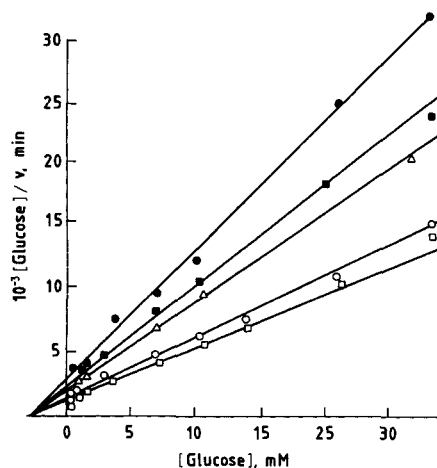


Fig. 4. Dependence of the rate on the glucose concentration in $^2\text{H}_2\text{O}$. MgATP concentrations were 0.48 mM (●), 0.87 mM (■), 1.5 mM (△), 2.1 mM (○) and 3.3 mM (□)

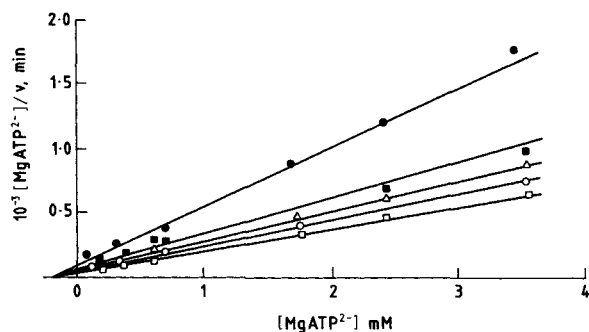


Fig. 5. Michaelis-Menten kinetics with respect to MgATP. Glucose concentrations were 1 mM (●), 10 mM (■), 25 mM (△), 50 mM (○) and 100 mM (□). The lack of curvature at all glucose concentrations indicates that there is no co-operativity with respect to MgATP

At 1-mM glucose the inverse isotope effect was increased by lowering the MgATP concentration; even at 100-mM glucose the normal isotope effect seen at saturating MgATP can be converted to an inverse isotope effect at very low MgATP concentrations. These results indicate that the solvent isotope effect is due to an equilibrium rather than a rate effect, because lowering the MgATP concentration must cause the binding of glucose to come to equilibrium: if the isotope effect were a rate effect it would be abolished under these conditions.

Simulation studies

Simulation of the mnemonical model (Fig. 1) for glucokinase revealed two possible explanations of the solvent isotope effects.

The existence of ^2H isotope effects on equilibria between different states of a protein is well documented [16–18], and so the first possibility we considered is that the equilibrium constant between the two forms of free enzyme is altered in $^2\text{H}_2\text{O}$. The results with respect both to glucose and to MgATP can be accounted for if E, the form of free enzyme released at the end of the catalytic cycle (see Fig. 1), is stabilized in $^2\text{H}_2\text{O}$ relative to the other form E'. As little as a 10-fold change in

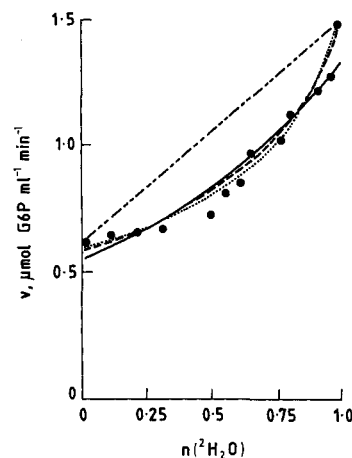


Fig. 6. Proton inventory for the glucokinase-catalysed reaction. Experiments were done in mixtures of $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ at various mole fractions n of $^2\text{H}_2\text{O}$ as indicated, with 0.25-mM glucose and 4.3-mM MgATP. In addition to the experimental points (●) the figure shows lines for the models compared in Table 1: linear (----); medium effect (—); opposing effects from reactant-state and product-state protons (····); and the two models considered in computer simulation (·-·-·), which are not distinguishable on this scale

equilibrium constant (a 20-fold decrease in the rate constant for conversion of E into E' with a twofold decrease in the reverse rate constant) appears sufficient to effectively abolish the positive co-operativity. However, negative co-operativity cannot be generated by changing these rate constants alone, but it becomes possible if it is assumed that E is capable of binding MgATP before glucose at very low glucose and high MgATP concentrations. Isotope-exchange studies have provided evidence that this alternative pathway exists [14], supplementing evidence from studies of the kinetics with glucose analogues [5].

Simulation also showed that the results could be accounted for if $^2\text{H}_2\text{O}$ were to increase the rate constant for binding of glucose to E', the form of free enzyme that binds glucose more weakly in $^1\text{H}_2\text{O}$. In this case an increase in rate constant by a factor of about 100 would be sufficient. With this model no participation of an alternative pathway for binding substrates is needed in order to generate negative co-operativity, but the affinity of E' for glucose would have to become greater than that of E. The mechanism of glucokinase catalysis in $^2\text{H}_2\text{O}$ would then correspond to that reported for wheat-germ hexokinase L₁ (in $^1\text{H}_2\text{O}$) [13].

The proton inventory

Although the results can be explained on a theoretical basis in terms of the mnemonical model, a proton-inventory analysis [26, 30–32] provides a more experimental approach to determining the origin of solvent isotope effects. The plot of observed rate against mole fraction of $^2\text{H}_2\text{O}$ obtained for the glucokinase-catalysed reaction in mixture of $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ at 0.25-mM glucose and 4.3-mM MgATP showed an upward curvature, with a net inverse isotope effect of 2.5, as expected from previous results at this glucose concentration in $^2\text{H}_2\text{O}$. However, model discrimination was poor as several different models gave a reasonably good fit to the data. The experiment was therefore repeated but with observations concentrated at

Table 1. *Non-linear regression results for the proton inventory*

| Model for isotope effects | Number of parameters | Residual sum of squares | <i>F</i> value for lack of fit |
|--|----------------------|-------------------------|--------------------------------|
| Linear (one-proton transition state) | 2 | 0.34959 | 10.91 ^b |
| Log-linear (medium effect) | 2 | 0.19251 | 5.54 ^b |
| Opposing effects of one transition-state proton and one reactant-state protons | 3 | 0.08110 | 2.04 ^a |
| Stabilization of E in ² H ₂ O | 5 | 0.08344 | 2.56 ^a |
| Increased rate of glucose binding to E' in ² H ₂ O | 5 | 0.08345 | 2.57 ^a |

^a Lack of fit not significant at 5% level of confidence.

^b Lack of fit significant at 1% level of confidence.

Table 2. *Analysis of variance calculation for stabilization of E*

The table illustrates the method [35] of analysing the residual sum of squares into pure-error and lack-of-fit components, using the fourth of the models listed in Table 1 as an example. The residual sum of squares is obtained from fitting 30 observations to the five-parameter model by non-linear regression. The pure-error estimate is found from the reproducibility of the replicate observations and the lack-of-fit estimate by subtraction. The *F* value of 2.56 is less than the value 2.6 tabulated for 10 and 15 degrees of freedom at the 95% level of confidence [35], and indicates that lack of fit is not significant, i.e. that the model cannot be rejected on the basis of the replicate observations

| Source of variation | Degree of freedom | Sum of squares | Mean square | <i>F</i> value |
|---------------------|-------------------|----------------|-------------|----------------|
| Residual | 25 | 0.08344 | | |
| Lack of fit | 10 | 0.05265 | 0.005265 | 2.564 |
| Pure error | 15 | 0.03079 | 0.002053 | |

the mole fractions where the models considered differed most from one another, i.e. in the ranges 0.4–0.7 and 0.9–1.0. This gave the results shown in Fig. 6, together with curves calculated from equations commonly invoked to account for proton-inventory plots of the general appearance observed [26] as well as equations derived from the two explanations of the solvent isotope effects considered in the simulation.

The results of fitting the data to five models are shown in Table 1. The first three of these are included primarily because models of this type are normally considered in proton-inventory studies and not because they appear particularly appropriate for the glucokinase reaction. It may be seen that two of these models fit badly enough to be rejected, but the third, in which the isotope effect is considered to arise from opposing effects of one proton in the transition state and one proton in the reactant state, actually fits somewhat better than either of the two models considered in the simulation, though both of these fit within experimental error. In all cases the significance of lack of fit was assessed by standard methods [35], the details of the calculation being shown for one of the models in Table 2.

DISCUSSION

Simulation studies revealed two possible explanations of the solvent isotope effects on the glucokinase reaction. The first of these involves stabilization of E, the form of free enzyme that in ¹H₂O has the higher affinity for glucose. In this interpretation, at low glucose concentrations in ¹H₂O E would relax to E', the form of free enzyme with the lower affinity in ¹H₂O for glucose, and the observed rates would mainly reflect binding of glucose to E'. In ²H₂O, however, stabilization of E would decrease the likelihood of relaxation to E, with the result that the reaction would be faster in ²H₂O. This ceases to be an important consideration at higher glucose concentrations, because then E is sequestered as an enzyme-glucose complex before it has time to relax to E': thus under these conditions there is little difference between the rates in ¹H₂O and in ²H₂O. At very high glucose concentrations we can account for the slower reaction in ²H₂O by supposing that we are now observing the isotope effects on a different step in the mechanism. This may be, for example, the release of glucose 6-phosphate from the enzyme, which is probably the rate-limiting step when the enzyme is saturated with substrates [14].

The second explanation of the results involves an increase in the affinity of E' for glucose in ²H₂O. This would again result in a faster reaction in ²H₂O than in ¹H₂O at low glucose concentrations, i.e. an inverse solvent isotope effect. As with the other explanation, increasing the concentration of glucose to saturation would bring about an isotope effect on a different step of the mechanism, one that proceeds more slowly in ²H₂O.

Both hypotheses account for the effects of MgATP on the kinetics observed in ²H₂O by supposing that lowering the concentration of MgATP causes the two forms of free enzyme to equilibrate with one another and with the enzyme-glucose complex. The fact that the inverse isotope effect is still evident under these conditions means that it must be an equilibrium rather than a rate effect.

The proton-inventory analysis provides further evidence for these interpretations, though it does not, unfortunately, discriminate between them. A plot with a downward curvature, such as the one observed in Fig. 6, is usually interpreted as implying one or more transition-state protons with ground-state fractionation factors of unity. The greatest curvature possible with this approach occurs when the number of such protons is infinite, a so-called medium effect. A greater curvature than this can only be due to a contribution from the ground state and so in our experiments the medium effect can be ruled out. Similar considerations apply to equilibrium isotope effects with the product state substituted for the transition state.

The analysis shown in Table 1 supports the impression given by Fig. 6, that the medium effect fits the observations badly but that opposing effects of transition-state and ground-state protons fit well. However, neither of these models can easily be related to the glucokinase reaction, and so it is fortunate that almost as good a fit is given by the models that we have found from computer simulation to give a good description of glucokinase co-operativity.

Each of the three models that fit the data well contains two fractionation factors, with values of 0.77–0.79 and 0.30 that are essentially the same for each model. The latter value of 0.30 is extremely low by comparison with values that have been measured in other systems. However, most of those have been for chemical systems: although they are reported not to vary

with the molecular environment [36] it would be rash to assume that this remains the case in the extremely complicated environment of the active site of an enzyme. One biological system that has been studied, formate-tetrahydrofolate ligase, shows a fractionation factor of 0.36 [16], another very low value.

A possible interpretation of the fractionation factors in the glucokinase-catalysed reaction may be derived from the chemical studies of Kreevoy and Liang [37]. They calculated that certain kinds of homoconjugate complexes A_2H^- can give isotopic fractionation factors in the range 0.25–0.35 if there is a strong hydrogen bond within the complex. The fractionation factor in such a case rises to a value around 0.7–0.8 as the hydrogen bond is weakened by increasing the distance between the two residues. The data for glucokinase suggest therefore that there is a particularly strong hydrogen bond between two residues in the free enzyme form E' with a fractionation factor of 0.30. If binding of glucose brings about a conformational change that increases the separation between the two hydrogen-bonded atoms there could be an increase in the fractionation factor to the value of 0.77–0.79 estimated for the enzyme-glucose complex.

A similar interpretation is possible if the isotope effect is due to stabilization in 2H_2O of E . One could envisage that after release of the product glucose 6-phosphate the glucose binding site has a more 'open' structure in E than in E' . This more 'open' structure may relax to E' , the form of free enzyme with the lower affinity for glucose, so as to make the binding site less accessible to glucose. A weak hydrogen bond in E with a fractionation factor of 0.77–0.79 could be strengthened in the conformational change accompanying the relaxation so that the fractionation factor became 0.30 in the relaxed form E' . This is clearly rather speculative, but it may account for the differences in the affinities of the two forms for glucose.

We prefer the latter interpretation, in terms of stabilization of E in 2H_2O , as there has to be an increase in 2H_2O of the affinity of E' for glucose over that of E if we are to account for the negative co-operativity observed in 2H_2O . To support this view there is a wealth of data giving evidence that 2H_2O stabilizes proteins. Thus, pyruvate carboxylase [38], glutamate dehydrogenase [39, 40], lactate dehydrogenase [40, 42] and fatty acid synthase [41] are some of the enzymes that have been reported to be stabilized against dissociation or denaturation by 2H_2O . The actual mechanism of this stabilization is a matter of some debate, but the effects of 2H_2O on protein structural equilibria have generally been interpreted as resulting from the stabilization of hydrophobic interactions [42] or hydrogen bonds [43] in 2H_2O . One of the differences between the two postulated forms of free glucokinase may therefore involve such interactions, which could constitute a medium effect: although the analysis in Table 1 shows that the curvature of Fig. 6 is too great to be explained solely as a medium effect it is certainly possible that a medium effect contributes to the results. The results for glucokinase do, however, resemble those for formate-tetrahydrofolate ligase [16], in which there is a single ground-state proton with a fractionation factor of 0.36, and also the conformation-associated ionization in ribonuclease [18].

We believe, therefore, that the solvent isotope effects that we have reported provide support for the mnemonic model as an explanation of the co-operativity that glucokinase shows with respect to glucose, as proposed previously [11, 14].

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