# Kinetics of the cooperative binding of glucose to dimeric yeast hexokinase P-I

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Kinetic studies of the cooperative binding of glucose to yeast hexokinase P-I at pH 6.5 have been carried out using the fluorescence temperature-jump technique. Three relaxation effects were observed: a fast low-amplitude effect which could only be resolved at low glucose concentrations ($\tau_1^{-1}$ = 500–800 $s^{-1}$), an intermediate effect ($\tau_2$) which showed a linear dependence of reciprocal relaxation time on concentration, and a slow effect ($\tau_3$) which showed a curved dependence on glucose concentration, increasing from $\approx$ 28 $s^{-1}$ at low concentrations to 250 $s^{-1}$ at high levels. The findings are interpreted in terms of the concerted Monod–Wyman–Changeux mechanism, the two faster relaxations being assigned to binding to the R and T states, and the slow relaxation to isomerization between the states. Quantitative fitting of the kinetic data to the mechanism has been carried out using independent estimates of the equilibrium parameters of the model; these have been derived from equilibrium dialysis data and by determining the enhancement of the intrinsic ATPase activity of the enzyme by the non-phosphorylatable sugar lyxose, which switches the conformation of the enzyme to the active R state.

## INTRODUCTION

The two isoenzymes of yeast hexokinase, designated P-I and P-II (alternatively A and B) are dimers of subunit molecular mass 52 kDa [1–6] of known amino acid sequence [7,8]. The individual roles of these two isoenzyme forms is not yet understood [9]. Glucose repression in *Saccharomyces cerevisiae* is directly associated with sugar phosphorylation by both isoenzymes, although the P-II isoenzyme appears to have a dominant role [10]. Both enzymes are known to become phosphorylated in cells grown under conditions of derepression at low concentrations of glucose, but the function and consequences of this modification are unclear, although there do not appear to be significant effects on $V_{max.}$ or $K_m$ for the reaction [11,12]. Glycolysis in *S. cerevisiae* has been thought to be controlled mainly by phosphofructokinase and pyruvate kinase: yeast hexokinases, unlike mammalian hexokinases, are not inhibited by glucose-6-phosphate, and the early findings of apparent regulatory effects such as citrate activation, transient kinetic effects and negative cooperativity with respect to nucleotides were clearly established to be artifacts arising from aluminium contamination [13]. However, it is recognized that regulation at these points is not sufficient to control glucose utilization and that some other element of regulation must exist in the early steps of glycolysis. It has recently been found that trehalose-6-phosphate competitively inhibits both isoenzymes of yeast hexokinases and this inhibition may have physiological significance at intracellular levels of trehalose-6-phosphate (about 200 $\mu$M) [14]; hexokinase appears to be a site of regulation in yeast as in other organisms, but with trehalose-6-phosphate playing the role of feedback inhibitor more usually associated with glucose-6-phosphate.

The yeast enzyme is structurally well-characterized [15–20]. Each subunit of the homodimer comprises two domains and in the open conformation these are separated by a deep cleft containing the sugar-binding site. The binding of glucose promotes a hinge-bending movement that brings the two domains together, partially closing the cleft around the sugar molecule; this conformational change promotes the binding of metal-ATP substrate and apparently protects the activated ATP from attack by water [21]. Although this picture of induced-fit is common to both isoenzymes, we have shown that there are major differences in glucose-binding behaviour of the two forms; under conditions of physiological pH (6.5–7) binding to dimeric P-I shows strong positive cooperativity, whereas for the P-II isoenzyme the two sites are equivalent and binding is non-cooperative [22–24].

The study described here is concerned with the homotropic cooperative binding of glucose to the P-I isoenzyme. The two classical models of cooperativity, the concerted model of Monod, Wyman and Changeux (MWC) [25], and the sequential model of Koshland, Nemethy and Filmer [26], both assume the presence of two conformational states, the low affinity tense (T) state and the high affinity relaxed (R) state; for hexokinase these states can be equated with the open and closed conformations respectively. These two models of cooperativity are limiting cases of a more general mechanism involving the possible population of all of the states of a multi-subunit enzyme in which each subunit can exist in one of two conformations [27]. The binding of glucose to hexokinase causes a large quenching of tryptophan fluorescence, which can be used to monitor the strength and kinetics of binding [24,28]. We report here a fluorescence temperature-jump study of the kinetics of the cooperative binding to hexokinase PI. To provide a basis for quantitative fitting of the kinetic results, we have estimated the proportion of enzyme in the active or closed conformation in the absence of glucose. This has been done by determining the intrinsic ATPase activity of the P-I isoenzyme, considered to be a property of the active form of the enzyme [29], in the absence and presence of lyxose; there is considerable evidence, which is also borne out by our present findings, that lyxose induces similar conformational changes to those produced by glucose, at least in the presence of nucleotide [21,30–32]. Analysis of the results shows that binding follows the concerted (MWC) model.

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## MATERIALS AND METHODS

Experimental procedures for enzyme purification and characterization [22,24], equilibrium dialysis [23], fluorescence titrations and analytical ultracentrifugation [24] have been described previously. The enzyme is isolated in its non-phosphorylated form under the yeast growth conditions used.

### ATPase activity

The ATPase activity of hexokinase was determined spectrophotometrically following the procedure of DelaFuente et al. [32] from the rate of oxidation of NADH in the presence of phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase. Assays were carried out in 50 mM Tris/HCl, pH 6.5, with a known amount (0.5–1.0 mg) of hexokinase which had been dialysed overnight. One unit of activity corresponds to 1 $\mu$mol/min of ATP hydrolysis. The stimulatory effect of D-lyxose on activity was determined using sugar (from Sigma) that had been three times re-crystallized from 80% aqueous ethanol; the glucose content of this preparation was determined to be $< 1$ part in $10^5$.

### Kinetic measurements

Fluorescence temperature-jump measurements were carried out on apparatus described fully elsewhere [33,34]. Temperature-jumps were generated using either a 52.5 nF or a 20 nF capacitor; at the low ionic strength used in these experiments (50 mM Tris/HCl) the rise times for heating with the two capacitors were about 16 $\mu$s and 5 $\mu$s respectively. The averaged results of about 4–6 separate experiments were taken for each set of conditions. Results were obtained by non-linear least-squares analysis of the digitized relaxation traces; errors in relaxation times varied from 10% to 20% when the amplitude of the effect was small, or when the separation of two relaxation effects was less than a factor of 5–10. Figure 1 illustrates a representative trace showing two relaxation effects.

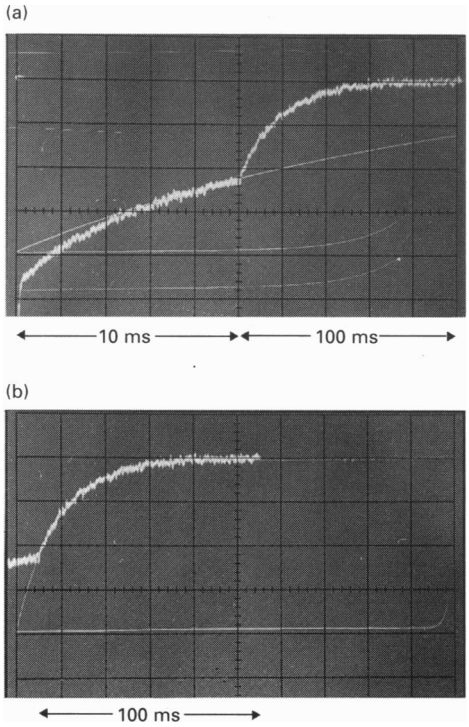



**Figure 1 Traces of the relaxations observed with hexokinase P-I (0.8 mg/ml) and glucose**

The buffer conditions were 50 mM Tris/HCl, pH 6.5, 0.6 mM glucose. Rise time filter, 10 $\mu$s; total signal, 3.0 V; sensitivity, 10 mV/cm. In (**a**) the time axis is split as shown (2 ms/cm from 0–5 cm and 20 ms/cm from 5–10 cm) to illustrate the two relaxation effects. (**b**) Time axis 20 ms/cm. The figure shows the slower relaxation. The smooth line in (**b**) is a fit to a single exponential corresponding to the slow relaxation; the smooth line in (**a**) is a scaled continuation of this line on the faster time axis, shown to illustrate the faster relaxation effect. The two reciprocal relaxation times evaluated by non-linear least squares analysis were $\tau_2^{-1} = 460\ s^{-1}$ and $\tau_3^{-1} = 57\ s^{-1}$.

## RESULTS

### Kinetic measurements

Temperature-jump experiments were carried out in 50 mM Tris/HCl buffer at a final temperature of 10 °C and a final pH of 6.5; under these conditions hexokinase P-I is completely dimeric [22]. Relaxation effects were detected only with mixtures of enzyme and glucose. Three relaxation effects were detected; one very fast ($\tau_1$) and incompletely resolved effect with a small amplitude, that was observed only at very low concentrations of glucose, and two other effects ($\tau_2$ and $\tau_3$) that were resolvable over the whole range of glucose concentration examined. Because of difficulties in resolution, the existence of three distinct effects had not previously been appreciated, and the two faster effects had appeared to be a single rapid process. This created the misleading impression of a minimum in the concentration dependence of the reciprocal relaxation time, to which we had attributed a different interpretation to that presented here [35]. The relaxation trace in Figure 1 shows the two resolved effects; the faster ($\tau_2$) had a reciprocal relaxation time of about 460 $s^{-1}$ and the slower ($\tau_3$) a reciprocal relaxation time of 57 $s^{-1}$. Figure 2 shows the dependences of these relaxation times on glucose concentration at a fixed enzyme concentration of 0.8 mg/ml, corresponding to 16 $\mu$M monomer subunits; the concentration of enzyme was almost invariably much lower than that of glucose in these experiments. The incompletely resolved fast relaxation effect ($\tau_1$) had a reciprocal relaxation time of about 500–800 $s^{-1}$ at low glucose levels, which increased out of the observable range as the glucose concentration reached 0.05–0.1 mM. For the faster of the two resolved times, $\tau_2^{-1}$ showed a linear dependence on concentration, whereas $\tau_3^{-1}$ showed a curved dependence, tending towards a plateau at saturating levels of glucose. These two relaxation times were independent of enzyme concentration at a fixed excess level of glucose (0.2 mM) over the range 0.02–1.7 mg/ml (results not shown), indicating that neither effect was due to the occurrence of association–dissociation reactions. Relaxations attributable to the monomer–dimer reaction of hexokinase have been shown to intervene only at higher pH and ionic strength, and these relaxations are slow and depend strongly on enzyme concentration [34].

### ATPase activity and lyxose activation

The intrinsic ATPase activity of hexokinase is very weak compared with its ability to phosphorylate glucose. Previous measurements of the activity, which relate to mixtures of isoenzymes, almost certainly mixed with the proteolysed S-forms, give values in the range 2–4 mU/mg enzyme [32,36]. In the present study with the purified P-I isoenzyme, the activity was 0.90 mU/mg enzyme, with a 95% confidence range of

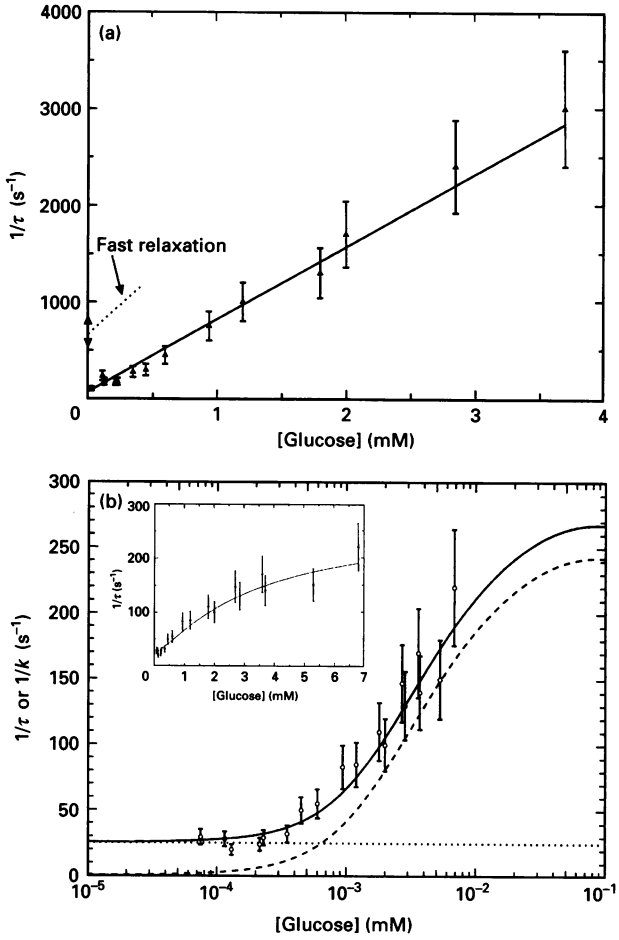



**Figure 2 Dependence of the reciprocal relaxation times on glucose concentration**

The concentration of enzyme was 0.8 mg/ml and the buffer conditions were as in Figure 1. (**a**) The approximate dependence of the fast relaxation ($\tau_1$) is shown by the dotted line and the arrow at zero glucose concentration. $\tau_2^{-1}$ (▲) depends linearly on concentration; the line shows the least squares fit discussed in the text. (**b**) The dependence of $\tau_3^{-1}$ (○) against glucose concentration is shown on a logarithmic axis. The solid line is the fit of $\tau_3^{-1}$ to the kinetic parameters shown in Table 1, as discussed in the text, and the dashed and dotted lines represent fits of the forward ($k_f$) and back ($k_b$) rate constants of the T↔R isomerization respectively. The inset shows the concentration dependence of $\tau_3^{-1}$ on a linear axis to illustrate saturation at high glucose concentrations.

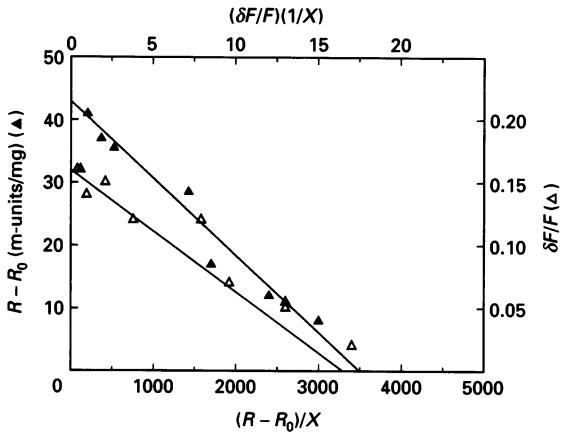



**Figure 3 Effect of lyxose concentration of the ATPase activity and fluorescence of hexokinase**

The ATPase activation (▲), expressed as $(R-R_0)$, where $R$ and $R_0$ are the activities in the presence and absence of lyxose respectively is plotted against $(R-R_0)/X$ where $X$ is the concentration of lyxose. The linearity of the dependence indicates that activation is non-cooperative. The intercept of the $y$-axis ($\approx 43$) corresponds to the activation at saturating concentrations of lyxose, and the slope to the activation constant ($\approx 8$ mM). The results of the fluorescence titration of lyxose binding (△) are plotted similarly as $\delta F/F$, where $\delta F$ and $F$ are the fluorescence quenching and initial fluorescence respectively, against $(\delta F/F)$ $(1/X)$ where $X$ is lyxose concentration. The linear dependence indicates non-cooperative binding, with a maximum quenching and binding constant of 16% and 11 mM respectively. Although the transformation used allows the ATPase and fluorescence results to be compared readily over the full range of lyxose concentration used, the data points are not equally weighted, and therefore the best values of the ATPase and fluorescence parameters were determined by unweighted non-linear least-squares analysis of the raw data.

0.65–1.30 mU/mg ($n = 18$). Commercial preparations of enzyme had activities of about 4–6 mU/mg under similar conditions, the higher activity probably being due to the presence of small quantities of contaminating pyrophosphatase [32]. This activity was strongly enhanced by D-lyxose; Figure 3 shows the dependence of $(R-R_0)$ upon $(R-R_0)/X$, where $R$ and $R_0$ are the rates in the presence and absence of lyxose respectively, and $X$ is the concentration of lyxose. The value of the fully activated rate derived from the intercept on the vertical axis was 43 mU/mg; the errors in determining the activated rate (± approx. 5%) were much smaller for the basal rate. The linearity of the plot indicates that lyxose activation is non-cooperative, and the activation constant was determined to be about 8 mM. D-Lyxose activates the ATPase activity by a factor of 49 (with a 95% confidence range of 35–70); this factor can be compared with previous factors of 20–30 for crystallized mixed isoenzymes [32,37].

The binding of lyxose to hexokinase P-I was investigated by monitoring the change in intrinsic enzyme fluorescence. In agreement with previous work [30,31], we found that lyxose produces a very small change in fluorescence even when added at high concentrations (2–3% quenching at 250 mM). Whether this is due to absence of binding, or failure to induce closure of the hexokinase cleft is not established. However, in the presence of saturating concentrations of MgATP, lyxose binds to the enzyme, producing a very similar change in fluorescence to that caused by glucose. Figure 3 also shows the result of such a fluorescence titration plotted as $(\delta F/F)$ against $(\delta F/F)(1/X)$, in which $F$ is the fluorescence in the absence of lyxose, $-\delta F$ is the quenching and $X$ is the concentration of lyxose. The maximum fluorescence quenching at saturating concentrations of lyxose was 16%; binding was non-cooperative and the binding constant was about 11 mM, similar to the activation constant observed in the ATPase reaction.

## ANALYSIS AND INTERPRETATION

### Glucose binding

Under the conditions of this study, hexokinase P-I is completely dimeric even in the presence of saturating concentrations of glucose, and binding shows strong positive cooperativity [22,23]. Figure 4 shows the results of an equilibrium dialysis study of the binding; the Hill co-efficient (15–85% saturation) was 1.6. Non-linear regression analysis of this direct-binding isotherm was carried out according to the concerted MWC equation for a

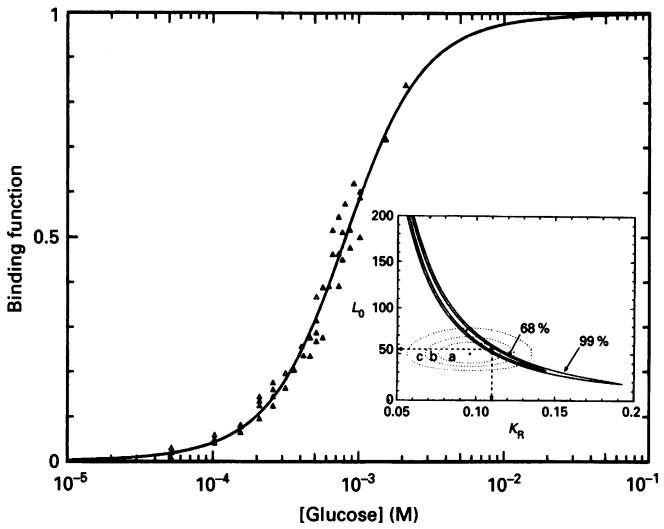
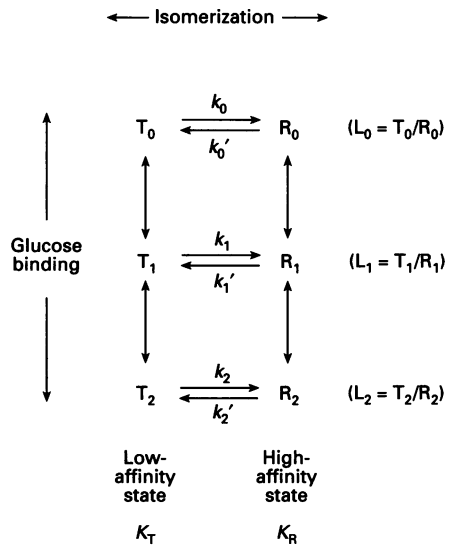



**Figure 4 Binding of glucose to hexokinase P-I determined by equilibrium dialysis**

Conditions are as in Figure 1. The inset illustrates the approximate 68 and 99% confidence contours for $L_0$ and $K_R$ from the dialysis data as solid lines. As discussed in the text, there is a strong curvilinear correlation between the $L_0$ and $K_R$ values. The dotted lines indicate approximate joint confidence contours at the 68% (a), 95% (b) and 99% (c) levels for values of $L_0$ and $K_R$ determined respectively from the ATPase activation data and the concentration dependence of $\tau_2$ (see text). The diamond and broken lines indicate the values of $L_0$ and $K_R$ which best fit all of the available data.

dimer, in which the parameters $L_0$, $K_R$ and $K_T$ have their usual meaning (Scheme 1) and [G] is concentration of glucose:




**Scheme 1**

$$y = \frac{([G]/K_R)(1+[G]/K_R)+L_0([G]/K_T)(1+[G]/K_T)}{(1+[G]/K_R)^2+L_0(1+[G]/K_T)^2} \quad (1)$$

The best fit values of $\hat{L}_0$, $\hat{K}_R$ and $\hat{K}_T$ were 73, 0.095 mM and 3 mM respectively. Analysis of the confidence limits of this fit according to the sum of squares procedure of Hoare [37] reveals several features worthy of comment. First, the fit is insensitive to the value of $K_T$ above a value of 1.5 mM, and secondly there is strong curvilinear correlation between acceptable values of $L_0$ and $K_R$. The inset of Figure 4 illustrates with solid lines the approximate 68 and 99% confidence contours of this fit. Refinement of these estimates of the MWC parameters using data from ATPase and kinetic measurements is considered later.

## Assignment of relaxation processes

The relaxation behaviour expected for a concerted MWC mechanism (Scheme 1) has been discussed extensively [27,38]. The vertical steps representing direct binding can be treated as two uncoupled second-order reactions, provided that the conformational changes are relatively slow and that the concentration of glucose is effectively buffered (which was almost invariably the case in the present experiments). Under these conditions, the reciprocal relaxation times would be expected to increase linearly with increasing concentrations of glucose. With the assumption of rapid vertical equilibration, the slow conformational changes are associated with only one relaxation time, which is the sum of the averaged microscopic rate constants for the conformational changes, weighted according to the occupation of the states (Scheme 1).

Three relaxation effects were observed, although the very fast effect (which was reproducibly present) was of low amplitude and poorly resolved, and the only conclusion that can be drawn with any confidence is that its limiting value at low concentrations of glucose was about 500–800 $s^{-1}$, and that it increased with increasing concentration of glucose. The effect $\tau_2$ shows a concentration dependence expected of a direct-binding process:

$$\tau_2^{-1} = k_D + k_A([E]+[G]) \quad (2)$$

where [E] and [G] indicate the free concentrations of enzyme sites and glucose respectively. Least-squares analysis of the data for this effect yielded the following values and standard errors: for $k_D$, $72 \pm 17\ s^{-1}$ and $k_A$, $(7.5 \pm 0.4) \times 10^5\ M^{-1} \cdot s^{-1}$. The derived value of the equilibrium dissociation constant ($k_D/k_A$) for this relaxation effect, $(9.6 \pm 2.0) \times 10^{-5}$ M, was in good agreement with the value of $K_R$ fitted from the equilibrium dialysis data (Figure 4). Therefore this relaxation effect can be attributed to binding to the high-affinity (R) site. It is possible that the unresolved very fast effect represents binding to the weak (T) site; the limiting value of $\approx$ 500–800 $s^{-1}$ at low glucose concentration would represent a $k_D$ about 7–12 times faster than that observed for the R state, compatible with the trend that differences in binding affinities are often reflected in 'off' rates rather than the 'on' rate.

The slow relaxation $\tau_3$ can be assigned to the isomerization steps between T and R states; quantitative fitting of this to the MWC mechanism is considered later.

## Conformational state and ATPase activity

In the absence of glucose, hexokinase exists in an equilibrium between open and closed conformations whose ratio according to the MWC model is given by $L_0$ ($= T_0/R_0$). The intrinsic ATPase activity of the enzyme is considered to arise from that fraction of the enzyme in the closed or R state. Binding of D-lyxose in the presence of nucleotide induces the formation of the closed state with a concomitant increase in the level of ATPase activity (Figure 3). The fact that the fluorescence change observed with saturating levels of lyxose (16% quenching) was very similar to that found with glucose, suggests that there is effectively complete transformation to the closed state of the enzyme. On the basis that the conformational transition is complete, and the reasonable assumption that lyxose is not excluding water from the active site [21], it follows that the ATPase activity at saturating levels of lyxose represents the characteristic activity of the closed conformation, and the activity in the absence of sugar represents

the fraction of enzyme in that conformation. The enhancement of ATPase activity can therefore be used to estimate a value of $L_0$ of 49, with a 95% confidence range 35–70.

### Quantitative analysis

The equilibrium dialysis results taken alone do not allow unique values of $L_0$ and $K_R$ to be specified; the two parameters can assume a wide range of values, although pairs of values are highly correlated (Figure 4). However, independent estimates of the values of $L_0$ and $K_R$, available from the ATPase and relaxation data respectively, can be used to specify these parameters more precisely. The inset to Figure 4 illustrates approximate joint confidence contours for $L_0$ and $K_R$ superimposed on those for the direct-binding data. The values of the parameters that provide the best fit to all of the data are $L_0 = 55$ and $K_R = 0.11$ mM. Although this pair of values is not unique, the combination of different experimental approaches does limit the acceptable range to reasonable bounds.

The slow relaxation effect arising from the isomerization between the T and R states is given by the following equation

$$1/\tau_3 = k_f + k_b \quad (3)$$

where $k_f$ and $k_b$ are average rate constants for the individual isomerization steps shown in Scheme 1, weighted according to the occupancy of the various states. Under the simplifying assumption, which is valid under the present conditions, that the concentration of glucose is buffered during the slow relaxation step, the dependence of these average rate constants on glucose concentration are given by the following equations:

$$k_f = \frac{[k_0 + (2k_1[G]/K_T) + (k_2[G]^2/K_T^2)]}{[1 + [G]/K_T]^2} \quad (4)$$

$$k_b = \frac{[k'_0 + (2k'_1[G]/K_R) + (k'_2[G]^2/K_R^2)]}{[1 + [G]/K_R]^2} \quad (5)$$

A further simplification of the analysis is suggested by examining the concentration dependence of $1/\tau_3$ at low levels of glucose (Figure 2b). The limiting value of $1/\tau_3$ at low concentrations of glucose was about 28 $s^{-1}$. On the basis that $L_0$ ($= T_0/R_0$) was 55, it follows that $k_0 \approx 0.5$ $s^{-1}$ and $k'_0 \approx 27.5$ $s^{-1}$. The value of $1/\tau_3$ remains constant at this level up to a glucose concentration of about 0.3–0.4 mM. In this region the $T_1$ and $T_2$ states are almost unoccupied because of the high value of $K_T$, so $k_0$, which is very small, remains the dominant term in $k_f$. However, the $R_1$ and $R_2$ states become heavily occupied; at 0.3 mM, $[G]/K_R \approx 3$ and the ratio of the populations $R_0:R_1:R_2$ are 1:6:9. Since $1/\tau_3$ is constant in this region, it follows that the back rate-constants of the three individual steps are equal within the limits of error (i.e. $k'_0 = k'_1 = k'_2 \approx 27.5$ $s^{-1}$).

On the basis that the rate constant for the back reaction ($k_b$) is independent of glucose concentration and equal to 27.5 $s^{-1}$, it follows that $1/\tau_3 - 27.5$ equals the forward rate constant $k_f$. Also, the equilibrium constants of the individual isomerization steps must decrease for thermodynamic reasons ($K_R/K_T < 1$), and since the $k_b$ values of the individual steps are constant, the rate constants $k_1$ and $k_2$ must correspondingly increase:

$$k_1 = k_0(K_T/K_R)$$

$$k_2 = k_0(K_T/K_R)^2$$

Introducing these simplifying conditions and thermodynamic relations into eqn. 4 yields the following expression for $k_f$:

$$1/\tau_3 = k_b + k_0\frac{(1 + [G]/K_R)^2}{(1 + [G]/K_T)^2} \quad (6)$$

**Table 1 Rate constants and equilibrium constants for the individual mechanistic steps of the cooperative binding**

| Step | Rate constants | Equilibrium constants |
|---|---|---|
| R-state binding | $k_A = (7.5 \pm 0.4) \times 10^5$ $M^{-1} \cdot s^{-1}$<br>$k_D = (72 \pm 17)$ $s^{-1}$ | $K_R = 0.11$ mM |
| T-state binding | $k_A \approx (2–4) \times 10^5$ $M^{-1} \cdot s^{-1}$<br>$k_D \approx 500–800$ $s^{-1}$ | $K_T \approx 2.5$ mM |
| Isomerization | $k_0 = 0.5$ $s^{-1}$<br>$k'_0 = 27.5$ $s^{-1}$ | $L_0 = 55$ |
| | $k_1 = 11$ $s^{-1}$<br>$k'_1 = 27.5$ $s^{-1}$ | $L_1 = 2.5$ |
| | $k_2 = 265$ $s^{-1}$<br>$k'_2 = 27.5$ $s^{-1}$ | $L_2 = 0.1$ |

The data for $1/\tau_3$ were fitted to this expression by non-linear regression, using fixed values of $k_b$, $k_0$ and $K_R$, to obtain the best fit value of $K_T$ of 2.5 mM; the solid line in Figure 2(b) indicates that this fit is very satisfactory. The values of the parameters used for this fit are shown in Table 1, together with the derived values of the rate constants for the individual steps.

## DISCUSSION

Hexokinase P-I shows strong positive cooperativity in the binding of glucose at pH 6.5 under conditions where the enzyme existed exclusively in the dimeric form even in the presence of high concentrations of glucose [22]. Under these conditions, three relaxation effects were observed which, as discussed above, cannot be attributed to the association–dissociation reaction of hexokinase; this reaction is known to intervene at much higher pH and ionic strength [34]. The present results are interpreted within the framework of the concerted MWC model which is conceptually more straightforward and involves fewer independent parameters than either the sequential model or the more general model of Eigen [27]. Three relaxations are expected from the MWC model under the limiting conditions of glucose buffering and slow conformational changes, two attributable to direct binding and one to isomerization. The assignment of the relaxation effects and their quantitative analysis is facilitated by the availability of independent estimates of binding and allosteric constants. Thus, assignment of $\tau_2$ to glucose binding to the R state is based on its concentration dependence and on the agreement between the kinetically derived value of $K_R$ with that obtained from equilibrium dialysis; this derived value in turn provides an independent estimate of $K_R$ which can be used to set bounds on other parameters derived from the equilibrium dialysis results. The fact that direct binding to the R state produces a detectable relaxation indicates that glucose causes a quenching of fluorescence independent of any conformational change, and it implies that fluorescence does not solely monitor the T to R state transition. The assignment of the fast effect $\tau_1$ (500–800 $s^{-1}$) to binding to the T state is admittedly tentative. Based on the experience of other systems, a fast relaxation associated with binding to the weaker T state would be expected, but further analysis of this effect is not warranted by the data; the values of $k_A$ for this process (Table 1) are derived indirectly from the $K_T$ value.

A value for the allosteric constant in the absence of glucose ($L_0$) can be estimated from the fit of the equilibrium dialysis data to the MWC binding equation, but as discussed above, there is

a strong correlation in acceptable estimates of $L_0$ and $K_R$, which limits the precision of this estimate. The availability of an independent estimate of the allosteric constant, based on determining the effect of lyxose on the intrinsic ATPase activity of the enzyme, allows the estimates of $L_0$ and $K_R$ to be refined. The data for lyxose binding and activation of ATPase activity are essentially in agrement with previous work, with some quantitative differences. We find that lyxose alone binds weakly, if it all, to hexokinase, in agreement with earlier fluorescence [31] and crystallographic [20] findings. However, binding is much stronger in the presence of nucleotide, and, based on the observed change in fluorescence, lyxose induces a similar conformational change to that induced by glucose. Unlike glucose, lyxose binds non-cooperatively, and the binding constant under these conditions (10–11 mM), is similar to the activation constant for the ATPase activity. Previous studies found binding and activation constants of 4–5 mM, the difference is likely to be due to the enzyme form used in the earlier work, which was probably mixed and proteolysed isoenzymes [32,36]. The differences in enzyme preparation probably also explain the difference in lyxose activation. With purified P-I isoenzyme we find that saturating levels of lyxose activate ATPase by a factor of about 50, in contrast to earlier estimates of 20–25 [32,36]; this higher factor is due to the lower intrinsic activity of the isolated P-I preparation.

The combination of data from equilibrium dialysis, ATPase activity and relaxation kinetics for $\tau_2$, permit reasonably secure estimates of $L_0$ and $K_R$ to be made for analysis of the slow relaxation time $\tau_3$ arising from isomerization. The most striking qualitative feature about $\tau_3$ is that it was constant up to a glucose concentration where the $R_1$ and $R_2$ states would be highly populated, suggesting that the three back rate constants (R→T) were equal. This is analogous to the situation in the classic example of $NAD^+$-binding to yeast glyceraldehyde 3-phosphate dehydrogenase, in which however it was the forward rate constants (T→R) that were the same, and the relaxation time for isomerization fell dramatically to a plateau level over the corresponding range of ligand concentrations [38]. There is no *a priori* reason why the T→R isomerization rate constants should be equal rather than the R→T, or indeed why either should be equal [39], but, as in the case of the $NAD^+$-binding, this fortuitous equality for the hexokinase relaxations does simplify subsequent analysis. Fitting the experimental data for $\tau_3$ to obtain the best fit for $K_T$ yields good agreement with the predictions of the simple concerted model, but it must be acknowledged, first that the kinetic and equilibrium parameters collected in Table 1, although consistent, do not represent a unique solution, and secondly that agreement with the MWC model does not exclude other models.

Temperature-jump relaxations with the two isoenzymes of hexokinase have previously been detected using the indicator probe Phenol Red [40]. These relaxations represent pH-dependent isomerizations which were very rapid ($\sim 2 \times 10^4\ s^{-1}$ for hexokinase P-I at pH 6.5) compared with the isomerizations observed here, and they clearly relate to different processes. The pH-dependent isomerizations are very rapid compared with the enzymic turnover number which is about 30–40 $s^{-1}$ at pH 6.5, whereas the cooperative T→R isomerizations are comparable with the turnover rate. It has been suggested that hysteresis in yeast hexokinase activity could be due to isomerization or dissociation of the molecule [41]. The slow isomerizations observed here, like the slow association–dissociation reactions observed previously [34], would be able to generate hysteretic effects.

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Received 13 June 1994/6 September 1994; accepted 9 September 1994