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Kinetics of the monomer–dimer reaction of yeast hexokinase PI

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Kinetic studies of the glucose-dependent monomer–dimer reaction of yeast hexokinase PI at pH 8.0 in the presence of 0.1 M-KCl have been carried out using the fluorescence temperature-jump technique. A slow-relaxation effect was observed which was attributed from its dependence on enzyme concentration to the monomer–dimer reaction; the reciprocal relaxation times τ^{-1} varied from 3 s^{-1} at low concentrations of glucose to 42 s^{-1} at saturating concentrations. Rate constants for association ($k_{ass.}$) and dissociation ($k_{diss.}$) were determined as a function of glucose concentration using values of the equilibrium association constant of the monomer–dimer reaction derived from sedimentation ultracentrifugation studies under similar conditions, and also from the dependence of τ^{-2} on enzyme concentration. $k_{ass.}$ was almost independent of glucose concentration and its value (2×10^5 $M^{-1} \cdot s^{-1}$) was close to that expected for a diffusion-controlled process. The influence of glucose on the monomer–dimer reaction is entirely due to effects on $k_{diss.}$, which increases from 0.21 s^{-1} in the absence of glucose to 25 s^{-1} at saturating concentrations. The monomer and dimer forms of hexokinase have different affinities and K_m values for glucose, and the results reported here imply that there may be a significant lag in the response of the monomer–dimer reaction to changes in glucose concentrations *in vivo* with consequent hysteretic effects on the hexokinase activity.

INTRODUCTION

The native PI and PII (alternatively A and B) isoenzymes of yeast hexokinase are dimers of subunit molecular mass 52 kDa [1–6], the amino acid sequences of which show 78% identity [7–9]. Binding of glucose induces a large conformational change in which the two lobes of the subunit rotate relative to each other by 12° [10], resulting in an enhancement of nucleotide binding [2,11–13]. Both isoenzymes also exist in a monomer–dimer association–dissociation equilibrium which is influenced by pH, ionic strength and substrate binding [1–4,6,14,15]. The concentration of hexokinase isoenzymes in the cell is relatively high (about 10 mg/ml [16]), and it has been suggested that, since the monomer and dimer forms of the enzyme have different affinities for glucose, the state of association of the enzyme may influence its activity under physiological conditions [15].

In previous papers we have reported studies of the binding of glucose to hexokinase PI and the proteolytically modified monomeric SI form [17–19]. At low pH (6–7) and low ionic strength, glucose binding to dimeric PI shows strong positive co-operativity, in marked contrast with the behaviour of the PII isoenzyme for which both sites in the dimer are equivalent in solution and binding is non-co-operative [20]. At pH 8.0, glucose binding to dimeric PI is non-co-operative, and there appears to be a structural transition from a co-operative to a non-co-operative dimer over the pH range 7.0–7.5. The monomer–dimer association–dissociation reaction becomes more significant at pH 8.0, particularly at higher ionic strength; glucose binds about seven times more tightly to the monomeric form of hexokinase PI than to the dimer, and as a consequence glucose promotes dissociation of the enzyme. The effects of glucose concentration, pH and ionic strength on the monomer–dimer reaction have been investigated by analytical ultracentrifugation and related quantitatively to the glucose-binding parameters of the monomeric and dimeric forms of the enzyme [17].

Although considerable information exists on the strengths of the monomer–dimer interactions of the two isoenzymes of yeast hexokinase and the factors that influence them [1–4,6,14,15, 17–20], there is no information on the kinetics of these reactions. The physiological significance of substrate-induced association–dissociation reactions in widely recognized [21–23], and clearly the rate of response of these reactions to changes in substrate concentrations is an important feature. We report here a study using relaxation kinetics of the effect of glucose on the rate of the monomer–dimer reaction of the hexokinase PI isoenzyme under conditions where glucose binding and its effect on the monomer–dimer equilibrium are well characterized.

MATERIALS AND METHODS

Experimental procedures for enzyme purification and characterization, glucose binding and fluorescence titrations and analytical ultracentrifugation have been described previously [6,17–20].

Kinetic measurements

Fluorescence temperature-jump measurements were carried out on the apparatus described fully elsewhere [24,25]. A semimicro cell (1.2 cm^3) was used, and solutions were degassed

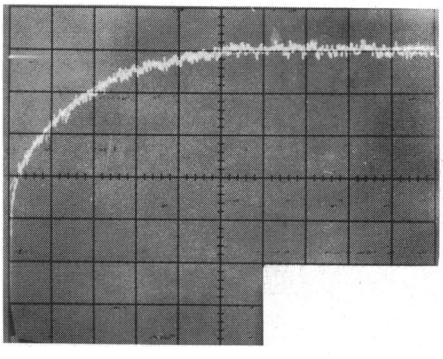

Fig. 1. Trace of the relaxation observed with hexokinase PI (1.75 mg/ml) and glucose

The buffer conditions were 50 mM-Tris/HCl, pH 8.0, 0.1 M-KCl, 0.21 mM-glucose. Time axis 100 ms/cm, rise time filter 0.1 ms; total signal 4.0 V, sensitivity 10 mV/cm. The smooth trace gives the fitted time (τ^{-1} = 6.3 s^{-1}) from an analogue computer.

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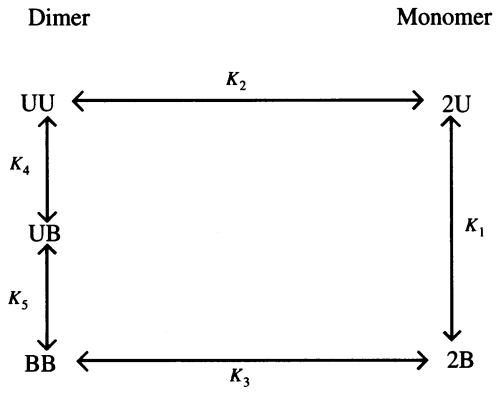


Scheme 1

before being introduced into the cell. Temperature jumps of 5–7 °C to a final temperature of 20 °C were applied, and the rise time for heating at the ionic strength used (0.12 M) was about 5 μs. The wavelengths for fluorescence excitation and emission were 285 nm and 340 nm respectively. Results were analysed by comparing the observed relaxation with a trace simulated using an analogue computer as described previously [25]. A representative relaxation trace is shown in Fig. 1. The averaged results of four to five separate experiments were taken for each set of conditions; errors in the relaxation times varied from about 10% when the amplitude of the effect was large towards 20% when it was small at very low and very high concentrations of glucose.

Linkage scheme for glucose binding

Glucose binding to the monomeric and dimeric forms of the enzyme can conveniently be represented by Scheme 1. The dissociation constants of glucose from the first and second sites on the dimer, K_4 and K_5 respectively, are related to that for the monomer, K_1, and to the association constants for the monomer–dimer reaction for the unbound (U) and bound (B) forms K_2 and K_3 respectively, by the following expression:

$$\frac{K_2}{K_3} = \frac{K_4 K_5}{(K_1)^2} \quad (1)$$

The dependence of the apparent monomer–dimer association constant $K_{ass.}$ on the concentration of glucose is given by the expression

$$K_{ass.} = K_2\left[\frac{1+([G]/K_4)+([G]^2/K_4K_5)}{1+([G]/K_1)+([G]^2/K_1^2)}\right] \quad (2)$$

RESULTS

Temperature-jump experiments were carried out in 50 mM-Tris/HCl buffer in the presence of 0.1 M-KCl at a final temperature of 20 °C and a final pH of 8.0. Relaxation effects were detected only when mixtures of enzyme and glucose were examined. Under these conditions, hexokinase PI exists as a mixture of monomer and dimer whose relative proportion depends on enzyme and glucose concentrations [17]. Relatively fast relaxation effects ($\tau < 200$ μs) were observed which can be attributed to glucose binding to the monomer and dimer species. The relaxation times of these effects were independent of enzyme concentration (under conditions where the concentration of glucose was much higher than that of the enzyme), and they were similar in magnitude to the relaxation times observed for the individual monomer [25] and dimer [19] species at high ionic strength and low ionic strength respectively. In addition to these rapid relaxations, a much slower relaxation effect (in the range 20–400 ms) was observed, which was well resolved kinetically from the fast effects. Fig. 2 shows the dependence of relaxation times of this process on the concentration of glucose at a fixed concentration of enzyme of 1.75 mg/ml, corresponding to 34 μM-monomer subunits. This rate showed the dependence on concentration of enzyme expected for a monomer–dimer association–dissociation process. Fig. 3 illustrates the dependence of τ^{-2} on the total concentration of monomer subunits at a fixed glucose concentration of 1 mM [see eqn. (6) below].

In terms of the following simplified representation of the association–dissociation reaction, in which D and M denote the dimeric species (UU, UB and BB) and monomeric species (U and B) respectively, and $k_{ass.}$ and $k_{diss.}$ are the glucose-dependent weighted-average rate constants for dissociation and association respectively

$$D \underset{k_{ass.}}{\overset{k_{diss.}}{\rightleftharpoons}} 2M \quad (3)$$

the reciprocal relaxation time is given by the following expressions in which $K_{ass.}$ is the monomer–dimer association constant ($k_{ass.}/k_{diss.}$) and [M] is the concentration of free monomers:

$$\tau^{-1} = k_{diss.} + 4[M]k_{ass.} \quad (4)$$

$$= k_{diss.}(1+4[M]K_{ass.}) \quad (5)$$

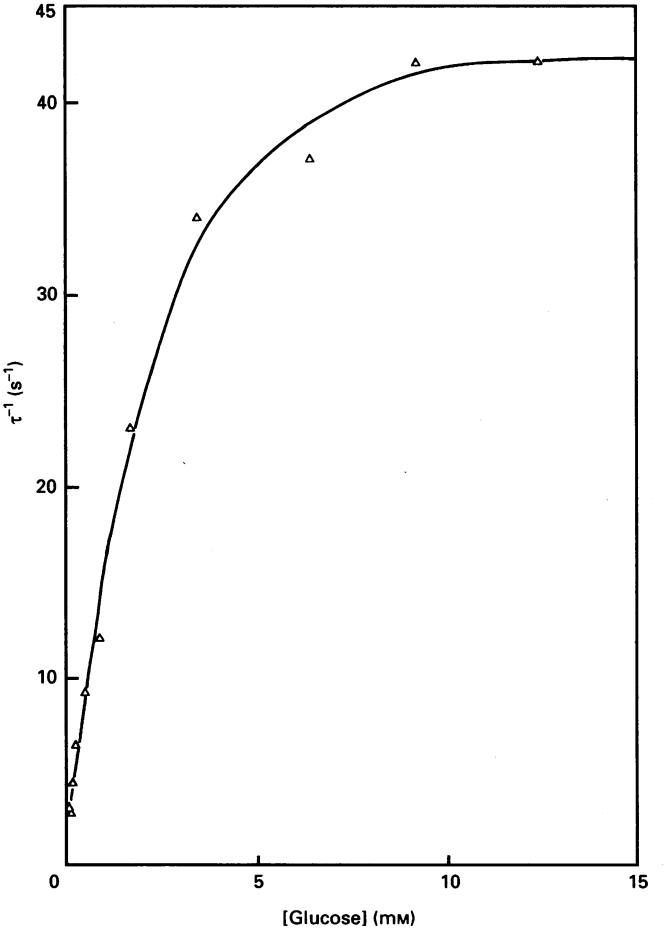


Fig. 2. Dependence of the reciprocal relaxation time with hexokinase PI on glucose concentration

The total concentration of enzyme was 1.75 mg/ml and the buffer conditions were as in Fig. 1.

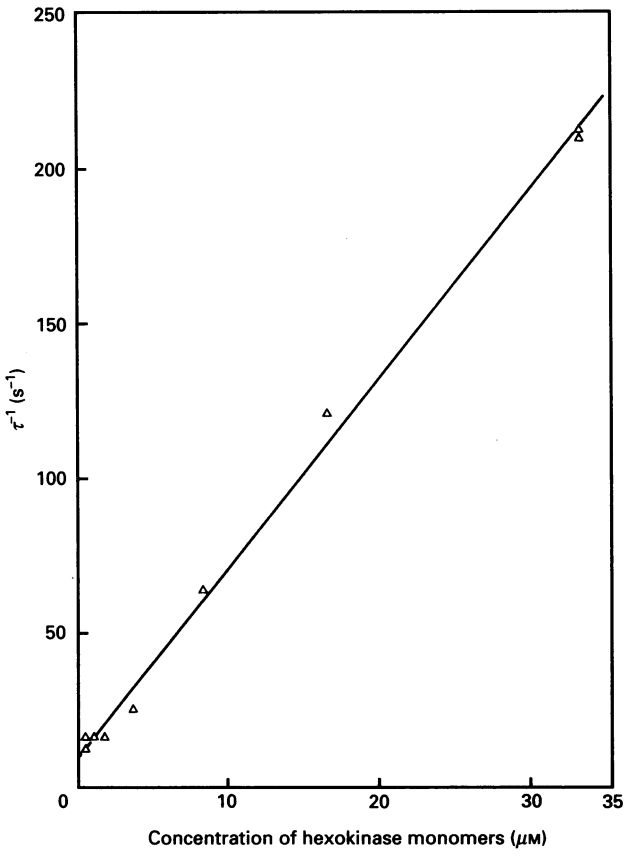


Fig. 3. Dependence of τ^{-2} on the concentration of hexokinase monomers

The concentration of glucose was 1 mM and the buffer conditions were as in Fig. 1.

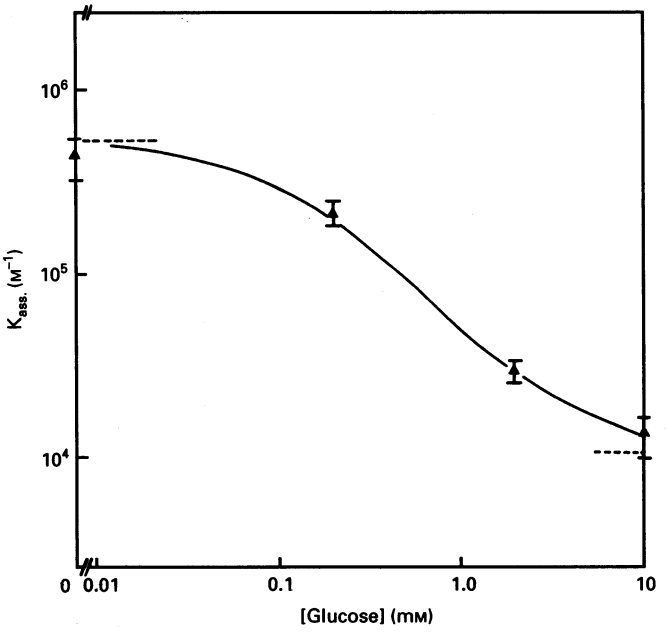


Fig. 4. Dependence of the apparent monomer–dimer association constant $K_{ass.}$ for hexokinase PI on glucose concentration

The experimental points were determined by sedimentation ultracentrifugation, and the solid curve is the best-fit dependence of these data obtained using the linkage relation discussed in the text. The dotted lines correspond to the best-fit values of $K_{ass.}$ at zero and saturating concentrations of glucose. (Note the break in the logarithmic scale to incorporate zero glucose concentration).

Analysis of these relaxation times to obtain the rate constants $k_{ass.}$ and $k_{diss.}$ from knowledge of the association constant of the monomer–dimer reaction derived from ultracentrifugation data is considered later. These rate constants may also be obtained from relaxation data without recourse to equilibrium constants by analysis of the dependence of relaxation times on enzyme concentration at a fixed concentration of glucose. Squaring eqn. (4) and noting that the total concentration of enzyme expressed in terms of monomer subunits $[M]_T$ is given by the following equations:

$$\begin{aligned}[M]_T &= [M]+2[D] \\ &= [M]+2K_{ass.}[M]^2 \\ &= [M]+2(k_{ass.}/k_{diss.})[M]^2\end{aligned}$$

yields the following expression

$$\tau^{-2} = k_{diss.}^2 + 8k_{diss.}k_{ass.}[M]_{stoic.} \qquad (6)$$

A least-squares analysis of the data in Fig. 3 yielded the following values and standard errors for the slope [6.21 ($\pm$0.22) $\times 10^6$ $M^{-1}\cdot s^{-2}$] and intercept [9.5 ($\pm$3.1) s^{-2}]. The value of $k_{diss.}$ derived from the intercept was 3.1 ($\pm$0.6) s^{-1}, and the standard error ($\pm$20%) is realistic given the uncertainty in the value of the intercept. The value of $k_{ass.}$ derived from the slope and intercept was 2.5 ($\pm$0.5) $\times 10^5$ $M^{-1}\cdot s^{-1}$; as an approximation a similar standard error ($\pm$20%) is attributed to this value, although it is recognized that estimates of the rate constants are correlated, and the slope of the least-squares plot, and hence the product $k_{diss.} \times k_{ass.}$ is defined more precisely ($\pm$3–4%) than either $k_{diss.}$ or $k_{ass.}$ The equilibrium association constant $k_{ass.}$ for the monomer–dimer reaction derived from the slope and intercept (slope/(8 $\times$ intercept) = $k_{ass.}/k_{diss.}$) was 8.1 ($\pm$2.5) $\times 10^4$ M^{-1}. This independent estimate of the monomer–dimer association constant is compared with the results from ultracentrifugation experiments later.

Linkage analysis of the monomer–dimer association constant

In order to determine the rate constants of the monomer–dimer reaction from the relaxation rates shown in Fig. 2, it is necessary to know how the apparent association constant for this reaction depends on glucose concentration. This information is available from our previous binding and ultracentrifugation studies on hexokinase PI under the same conditions which are discussed here within the framework of the linkage scheme mentioned above [17].

Apparent association constants for the monomer–dimer reaction over the concentration range 0–10 mM-glucose determined from the weight-average sedimentation coefficients as described previously are shown in Fig. 4 [17]. The dependence of the apparent association constants on glucose concentration is given by eqn. (2) above, and a standard Marquardt non-linear least-squares procedure was used to estimate K_2 by fitting the experimental values of these constants to this expression with the known values of the glucose-binding constants to the monomer and dimer [17–20]; K_1 for monomer binding was 0.23 mM, and the two sites in the dimer were equivalent to the same intrinsic binding constant (1.6 mM), which leads to values for K_4 and K_5 of 0.8 mM and 3.3 mM when the necessary statistical factors are taken into account. The agreement between the fitted curve and the experimental data shown in Fig. 4 is very satisfactory; the best-fit value of the association constant K_2 (in the absence of glucose) was 5.5 ($\pm$0.8) $\times 10^5$ M^{-1} and the corresponding value at saturating concentrations of glucose was 1.12 ($\pm$0.16) $\times 10^4$ M^{-1}.

Table 1. Glucose-dependence of relaxation times and rate constants for the association–dissociation constants

The association constants $K_{ass.}$ for the monomer–dimer reaction were taken from the curve fitted to the ultracentrifuge data in Fig. 4. $\tau^{-1}_{exp.}$ are the experimentally determined reciprocal relaxation times from which the rate constants $k_{diss.}$ and $k_{ass.}$ were calculated as described in the Results section. $\tau^{-1}_{calc.}$ are values of the reciprocal relaxation times calculated from the rate constants of the individual steps shown in Table 2.

[Glucose] (mM)	$10^{-5} \times K_{ass.}$ (M^{-1})	$k_{diss.}$ (s^{-1})	$10^{-5} \times k_{ass.}$ ($M^{-1} \cdot s^{-1}$)	$\tau^{-1}_{exp.}$ (s^{-1})	$\tau^{-1}_{calc.}$ (s^{-1})
0.027	4.7	0.28	1.3	3.0	3.1
0.053	3.75	0.29	1.1	2.8	3.6
0.11	2.85	0.52	1.5	4.4	4.5
0.21	1.9	0.93	1.8	6.5	6.3
0.42	1.1	1.82	2.0	9.2	10.3
0.84	0.60	3.1	1.8	12.0	16.0
1.65	0.34	7.5	2.5	23.0	23.5
3.43	0.22	13.4	2.9	34.0	32.2
6.4	0.17	16.2	2.7	37.0	38.4
9.2	0.145	19.5	2.8	42.0	41.6
12.4	0.135	19.5	2.6	42.0	43.6

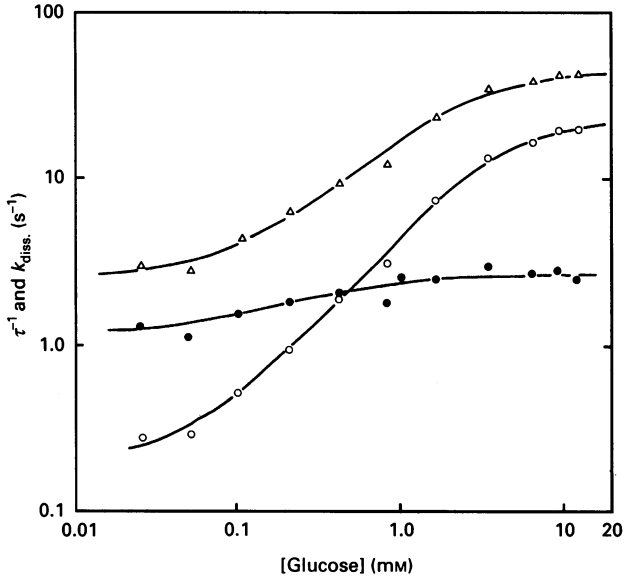


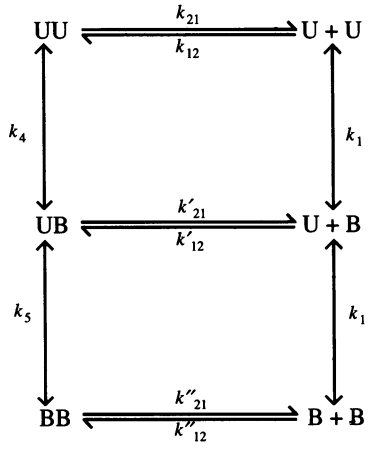
Fig. 5. Dependence of reciprocal relaxation times and rate constants on glucose concentration

Reciprocal relaxation times (△) are taken from Fig. 2 and relate to an enzyme concentration of 1.75 mg/ml. Values of $k_{diss.}$ (○) and $k_{ass.}$ (●) were evaluated from these relaxation times using the corresponding values of $K_{ass.}$ as described in the text. The solid curves are the dependences on glucose concentration expected from the values of the rate constants of the individual monomer–dimer steps shown in Table 2, and the known equilibrium constants for glucose binding to the monomeric and dimeric forms of the enzyme.

Values of the apparent association constant used for evaluation of the kinetic data were taken from this best-fit dependence.

Analysis of relaxation times

Values of the rate constants $k_{diss.}$ and $k_{ass.}$ ($= k_{diss.}/K_{ass.}$) can readily be determined from eqn. (5) and the values of $K_{ass.}$ and the concentration of free monomer [M] at given concentrations of glucose and total enzyme (1.75 mg/ml or 34 μM-monomer subunits). Rate constants derived in this way from the data of



Scheme 2

Table 2. Rate constants and association constants for the individual mechanistic steps of the monomer–dimer reaction

Reaction step	$k_{diss.}$ (s^{-1})	$10^{-5} \times k_{ass.}$ ($M^{-1} \cdot s^{-1}$)	$10^{-5} \times K_{ass.}$ (M^{-1})
$UU \rightleftharpoons 2U$	0.21 (k_{21})	1.2 (k_{12})	5.7 (5.45)*
$UB \rightleftharpoons U + B$	1.70 (k'_{21})	2.0 (k'_{12})	1.18
$BB \rightleftharpoons 2B$	25 (k''_{21})	2.7 (k''_{12})	0.110 (0.112)*

* The $K_{ass.}$ values were determined from the quotient of the corresponding rate constants $k_{ass.}/k_{diss.}$; for comparison the numbers in parentheses are the values obtained from the ultracentrifuge data in the absence and presence of glucose (Fig. 4).

Fig. 2 are shown in Table 1 and Fig. 5; it is evident that the rate constant for dissociation of the dimer ($k_{diss.}$) increases markedly (by a factor of about 70) as the glucose concentration is increased towards saturating levels, but the rate constant of monomer association ($k_{ass.}$) is very insensitive to changes in glucose concentration.

Evaluation of microscopic rate constants for the monomer–dimer reaction

The apparent rate constants $k_{diss.}$ and $k_{ass.}$ evaluated above are average rate constants for the individual association–dissociation steps shown in Scheme 2 weighted according to the occupancy of the various states. With the notation that UB represents the sum of both species of dimer containing a single bound glucose molecule, and, on the basis that relaxation processes in the vertical direction in this scheme (i.e. glucose binding, not involving the association–dissociation reaction) are fast, the dependence of the apparent rate constants on glucose concentration are given by the following equations:

$$k_{diss.} = \frac{[k_{21} + (k'_{21}[G]/K_4) + (k''_{21}[G]^2/K_4K_5)]}{[1 + ([G]/K_4) + ([G]^2/K_4K_5)]} \quad (7)$$

$$k_{ass.} = \frac{[k_{12} + k'_{12}[G]/K_1) + (k''_{12}[G]^2/K_1^2)]}{[1 + (2[G]/K_1) + ([G]^2/K_1^2)]} \quad (8)$$

The values of $k_{diss.}$ and $k_{ass.}$ derived from the relaxation times were fitted separately by a Marquardt non-linear least-squares procedure to the above equations using the known values of the glucose-binding parameters (K_1, K_4 and K_5) given above. The best-fit curves for $k_{diss.}$ and $k_{ass.}$ are shown in Fig. 5 together with

the predicted dependence of τ^{-1} on glucose concentration obtained from the equation:

$$\tau^{-1} = k_{diss.} + 4[M]k_{ass.}$$

in all three cases the agreement between the experimental and fitted values is within the limits of error over the whole range of glucose concentration studied. The values of the rate constants of the individual association–dissociation reactions obtained from these fits are shown in Table 2 together with the corresponding monomer–dimer association constants derived from the relationship $k_{ass.} = k_{ass.}/k_{diss.}$.

DISCUSSION

The relaxation process observed with hexokinase can be attributed to a glucose-dependent monomer–dimer equilibrium, and the results are in good accord with a simple mechanism in which the monomer–dimer reaction equilibrates relatively slowly compared with the glucose-binding steps which are known to be fast [19,25]. Although the bulk of the results were analysed using values of the monomer–dimer association equilibrium constant previously established using analytical ultracentrifugation, the kinetic data also provide an independent determination of the rate and equilibrium constants. Thus, at 1 mM-glucose, where the proportions of monomer and dimer are comparable under the present conditions, the values of $k_{diss.}$ and $k_{ass.}$ determined from the dependence of τ^{-2} on enzyme concentration (3.1 s^{-1} and 2.5×10^5 $M^{-1} \cdot s^{-1}$ respectively, Fig. 3), are in good agreement with the corresponding values determined using the ultracentrifuge result (4.0 s^{-1} and 2.3×10^5 $M^{-1} \cdot s^{-1}$ respectively). The independently determined value of the equilibrium constant from the kinetic data (8.4×10^4 M^{-1}) is within 50% of the ultracentrifuge result (5.5×10^4 M^{-1}), which is a satisfactory measure of agreement given the very different methods used. This agreement, coupled with the observation that the monomer–dimer reaction is relatively fast ($\tau < 5$ s even in the absence of glucose) on the time-scale of sedimentation-velocity experiments, supports the view that $K_{ass.}$ values can be properly determined from the weighted sedimentation coefficients on the basis of a rapidly re-equilibrating reaction between monomer and dimer. A similar observation of rapid equilibration between monomer and dimer relative to the separation time of size-exclusion chromatography has been reported for the hexokinase PII isoenzyme [26].

The influence of glucose on the monomer–dimer reaction arises almost entirely from its effect on the rate of dissociation of the dimer ($k_{diss.}$), which increases by a factor of more than 100 at saturating concentrations of glucose (Fig. 5). The second-order rate constant for association ($k_{ass.}$) was about 2×10^5 $M^{-1} \cdot s^{-1}$ and varied little with glucose concentration. For comparison, $k_{ass.}$ for the association of haemoglobin dimers to form the tetramer is 4.3×10^5 $M^{-1} \cdot s^{-1}$ [27]. Information on the rates of protein–protein interactions in the literature is relatively sparse [22,28], but this value is probably close to the upper limit for a diffusion-controlled association for protein subunits of this size [22]. This consideration further supports the idea that the reaction can be treated as a simple association not involving any isomerization steps.

Analysis of the glucose-dependence of the observed (weighted-average) rate constants yields a set of rate constants for the individual mechanistically occurring steps (Table 2) which reproduces within experimental error the variation of τ^{-1} with glucose concentration (Fig. 5 and Table 1). The equilibrium association constants derived from these rate constants are also consistent with the ultracentrifuge data (Table 2) and with the glucose-binding parameters for the monomer and dimer. Thus, from equilibrium data, binding of the first and second molecules of glucose would be expected to decrease $K_{ass.}$ by factors of 3.6 and 49 respectively; the corresponding factors derived from the rate constants were 4.5 and 52. When the appropriate statistical factor is taken into account, these values imply that each glucose bound causes a sevenfold weakening in the strength of the subunit contacts. The kinetic results show that these differences in stability reflect variation in the 'off' or dissociation rates, which is usual in protein–protein and protein–ligand interactions [29].

Although the monomer–dimer equilibrium is established rapidly relative to the separation time of sedimentation experiments, the rate of the association–dissociation reaction is much lower than the rate of enzymic turnover of hexokinase. The intracellular pH of the yeast cytoplasm as revealed by n.m.r. studies is lower than that used in the present study, varying within the range 6.5–7.6, depending on the carbon source, oxygen concentration and state of catabolite repression of the cells [30,31]. The formation of dimers is favoured at lower pH, and the association constant for the monomer–dimer equilibrium of hexokinase is approximately 10–20-fold greater at pH 7 than at pH 8 [6,18]. However, the relatively high ionic strength of the yeast cytoplasm (about 0.25–0.30 M [31]) would favour dissociation compared with conditions of the present study. On the basis that the association constant for the monomer–dimer reaction decreases by a factor of about 7 when the ionic strength is increased by 0.1 M [18], it can be concluded that the association constant at pH 7, under conditions of physiological ionic strength, will be similar in magnitude to that observed in the present study. The intracellular concentrations of hexokinase isoenzymes in yeast are relatively high (in the micromolar region [15,16]), making it likely that both monomer and dimer forms are present under these conditions. Steady-state kinetic studies have established that, for both the PI and PII isoenzymes, the monomer and dimer show the same $V_{max.}$ at saturating concentrations of glucose, but K_m for the dimer is higher than that for the monomer [15]. Thus, at the subsaturating concentrations of glucose which exist in the yeast cell, variation in the extent of the monomer–dimer reaction would be in a position to influence the activity of the enzyme, and so may be physiologically important. The kinetic evidence reported here implies that changes in the extent of this reaction arising from variation in glucose concentration or intracellular pH would occur with a significant lag (in the region of seconds) generating hysteretic effects. Such effects would not be expected in the usual assays *in vitro* where the concentration of enzyme is very low and the monomer form predominates.

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