Activation of yeast hexokinase PI1 at low pH by citrate or ATP has been shown to occur at the monomer level, without requiring association to the dimer level. Thus, the previous suggestion (Steitz, T. A., Anderson, W. F., Fletterick, R. G., and Anderson, C. M. (1977) J. Biol. Chem. 252, 4494-4500) that anionic activators of hexokinase, such as citrate and ATP, act by binding to the intersubunit site of the symmetric dimer (BII) is unlikely. ATP also promotes formation of an inactivated dimer, and this inactivation can be reversed by citrate. The fact that ATP is not an activator at the high enzyme concentrations found in yeast suggests that the high rate of glycolysis is dependent on other positive effectors such as citrate. On the other hand, sulfonated buffers such as Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in the same pH range are shown to promote formation of an elongated dimer that may correspond to the BI crystal form described by Steitz et al. (reference cited above). Upon activation by citrate, this dimer is partially dissociated to the active monomer. However, citrate must interact with the dimer as well as the monomer, because at high protein concentrations, saturating citrate cannot fully activate or dissociate the dimer stabilized by Hepes. These results suggest that the activation event is a conformational change that can occur at the monomer level and is stabilized by citrate or ATP. Sulfonated buffers inhibit by binding preferentially to the less active monomer conformation and prompting dimerization. At low pH and ATP concentration, the Hepes-stabilized dimer cannot exist in the activated form, although it may under other conditions. A model is presented to describe these interactions.

Yeast hexokinase (EC 2.7.1.1) isozyme PI1 was shown by Kosow and Rose (1) to have apparent negative cooperativity for ATP at pH <7. The activation at low ATP was also brought about by a number of polyamionic metabolites, such as citrate and phosphoglycerate, suggesting a role in regulation at this point in fermentation. In this pH range, the enzyme was turning over. Shill et al. (6) demonstrated that the presence of both substrates favored dimerization, whereas either ATP or glucose alone did not. They concluded, however, that this association was not sufficiently strong to account for the hysteretic loss of activity during assay conditions. Because their studies were performed at 2 mM ATP which is sufficient to partially activate hexokinase, it is not clear whether allosteric activation by ATP is coupled to dimer formation.

In considering the nature of the several kinetically demonstrable forms of hexokinase, it will be important to refer to the three crystal structures that are known. These are BIII, a monomer (7), BI, an elongated dimer formed by a "head to head" association (8), and BII, a globular asymmetric dimer which has a noncatalytic intersubunit binding site for 1 ATP molecule (9, 10). It has been reported that at partially activating concentrations of ATP, the dimer is apparently more active than the monomer and that the rate of the hysteretic transition increases with increased protein concentration (6). On this basis, the intersubunit site has been postulated to be the allosteric activator site, capable of binding nucleotides or anions, resulting in dimerization and activation of the enzyme (11).

The present paper attempts to determine whether the hysteretic loss of activity and activation are associated with conformational changes or dimerization (or both). Steady state rates, i.e. after the hysteretic transition, have been determined as a function of enzyme concentration at low pH and high glucose, conditions which lead to the largest difference between initial and steady state velocities. The influence of citrate, ATP, and sulfonated buffer on activity and state of association have been correlated.

The results suggest that the basic event involved in the allosteric behavior is a conformation change that occurs at the monomer level. Effectors such as citrate, ATP, or Hepes shift the various equilibria involved and thus affect the active monomer/inactive monomer/inactive dimer equilibria. As a result, the degree of activation of the steady state velocity can depend strongly on the protein concentration.

MATERIALS AND METHODS

The enzyme used in this study was a generous gift of S. P. Colowick, Vanderbilt University School of Medicine, Nashville, Tenn. Protein concentration was determined by absorbance at 280 nm (12). Based on this, the specific activity at 22°C and saturating substrates at pH 8.0 was 400 units/mg and at 30°C, 720 units/mg. Glucose-6-phosphate dehydrogenase and pyruvate kinase were obtained from Boehringer Mannheim. Sucrose, glucose, and MgCl2 were reagent grade from Baker Analytical. All other chemicals and proteins were from Sigma Chemical Co. Imidazole was recrystallized from benzene prior to use. Assays were conducted at 22°C as described in the figure legends.
Linearity of the coupled assay system with hexokinase concentration was established by adding increasing amounts of trypsin-treated yeast hexokinase PI. This isozyme does not dimerize readily and showed that we have not exceeded the capacity of the coupling system. Exact conditions are given in the figure legends. Hexokinase activity in column fractions was assayed at saturating substrate levels after using glucose as a substrate in some of the assays confirmed that anomeration of glucose 6-phosphate was not rate-limiting or contributing to lower specific activities at high protein concentrations.

The solid line of Fig. 4 was generated by assuming an \( M_{app} \) and solving Equation 4 for the specific activity using the limits described above.

**RESULTS**

**Activation by Citrate and ATP in Imidazole Buffer**—It has been reported (5) that the steady state specific activity of hexokinase PII at pH 6.5 in the presence of 2.0 mM ATP increases with increasing protein concentration, when the reaction is monitored by acid production. Because the level of ATP used in those studies was in the region where ATP in a good activator, it is not clear whether this evidence for a greater activity in the multimer form applies to the unactivated state. Furthermore, the titrimetric method of assay is unfavorable because of the accumulation of reaction products to progressively larger concentrations, especially in experiments done with higher amounts of enzyme.

To avoid this source of uncertainty and to provide greater flexibility in the concentrations of ATP that could be employed, the studies to be reported were conducted in the presence of traps for both products: pyruvate kinase and phosphoenolpyruvate, which serve to regenerate ATP, thus keeping it constant, and glucose-6-phosphate dehydrogenase and TPN which provide a sensitive optical assay at 340 nm.

Fig. 1 shows the specific activity as a function of protein concentration under a variety of conditions at pH 6.7 with 50 mM imidazole HCl as buffer. Using ATP at 50 \( \mu \)M, the steady state specific activity was about 20 units/mg (5% of \( V_{max} \) at pH 8.0) and independent of protein concentration. Addition of saturating citrate (1.5 mM compared with \( K_d = 0.06 \text{ mM} \) (1)) leads to an 8-fold enhancement of this rate throughout the protein concentration range studied. In contrast to this, with 2 mM ATP, there is a large decrease in specific activity as protein concentration increases. At 1 \( \mu \)g of enzyme/ml, more than 90% of the activation effect is lost. It was shown that addition of citrate under these conditions fully activates the enzyme to a specific activity of 235 units/mg, at hexokinase

The following equations have been derived for determining \( n \) and \( K_d \) of the polymerization reaction, neglecting intermediates (Fig. 5) where \([E]\) is the total protein concentration; \([E]\), monomer concentration; \( n \), degree of polymerization; \([E]\), polymer concentration; and \( K_d \), dissociation constant for polymerization.

\[
E_r = [E] + n[E]\tag{1}
\]

\[
K_d = \frac{[E]^n}{[E]}	ag{2}
\]

Combining Equations 1 and 2 gives Equation 3:

\[
\frac{[E]^\prime}{n} = \frac{K_d([E] - [E])}{[E]}	ag{3}
\]

Rearranging, one gets:

\[
-\log ([E] - [E]) = -\log K_d/n + n(-\log [E])	ag{4}
\]

With the approximation that the activity of monomer is much greater than that of other forms, and extrapolating the specific activity to zero protein concentration to determine the monomer specific activity (see insets to Figs. 1 and 2), one can determine \([E] \) at any \([E]\). A subsequent plot according to Equation 4 yields \( n \) from the slope and \( -\log K_d/n \) from the y-intercept.

For Fig. 4, a theoretical relationship between specific activity versus \( M_{app} \) (apparent molecular weight) has been generated assuming a monomer-dimer equilibrium where the monomer is fully active and the dimer about 5% active. We have used the \( M_{app} \) in Hepes as the dimer \( M_{app} \) (\( M_{app2} \)) and that in imidazole as the monomer \( M_{app} \) (\( M_{app1} \)). In the case where \( M_{app2} \neq 2 M_{app1} \), the relationship between specific activity (SA) and \( M_{app} \) is not linear. This is because gel filtration measures a weight average \( M_{app} \) (13) and can be seen as follows:

\[
M_{app} = \frac{[E]M_{app1} + [E]M_{app2}}{[E] + [E]}	ag{5}
\]

\[
SA = \frac{[E]}{[E]} S_{A(E)} + \frac{n [E]}{[E]} S_{A(E)} \tag{6}
\]

After solving Equations 5 and 6 for \([E]\) and combining these two expressions, rearrangement yields Equation 7.

\[
SA = \left[S_{A(E)} - S_{A(E)}\right] \frac{M_{app} - M_{app2}}{2M_{app1} - M_{app2} - M_{app1}} + S_{A(E)}\tag{7}
\]

Fig. 1. Specific activity of yeast hexokinase PII (HK) as a function of protein concentration in 0.05 M imidazole/chloride. Assays were initiated by the addition of the indicated concentrations of enzyme. All assays were 5 mM in glucose, 5 mM MgCl\(_2\), 0.5 mM TPN, 0.5 mM phosphoenolpyruvate, and 1.75 units/ml each of glucose-6-phosphate dehydrogenase and pyruvate kinase, pH 6.7. A, 50 \( \mu \)M ATP; V, 50 \( \mu \)M ATP plus 1.5 mM citrate; and O, 2.0 mM ATP. The inset shows the specific activity in 2 mM ATP versus the square root of the protein concentration.
concentrations up to 0.36 μg/ml. As shown by the linearity of a plot of specific activity versus the square root of the protein concentration (see inset, Fig. 1), this result can be explained by association to a much less active dimer when ATP is high. The $K_d$ for dimerization at 2 mM ATP appears to be about 0.4 μg/ml (7.5 × 10⁻⁹ M based on a molecular weight of 63,000) (12). This dissociation constant is in good agreement with the active enzyme centrifugation studies (6) in which it was shown that a monomer to dimer association was occurring in the same range of protein concentrations used in Fig. 1. Since the measurement of absolute specific activity in the centrifugation experiments is difficult (6), it is doubtful that differences in specific activity we see would have been detected.

In separate experiments, it was shown that a similar protein concentration dependence is observed in the range 0.1 to 13 mM ATP (data not shown). With the assumption that dimer is inactive, the dissociation constant for dimerization was calculated over this range of ATP concentrations. It was found that increasing ATP decreased the apparent dissociation constant for dimerization to a limiting value of 0.1 μg/ml with half-saturation in the range of 6 mM ATP. This can be compared to the experimentally determined $K_d$ for activation by ATP of 5 mM at 0.05 μg/ml enzyme concentration (data not shown). It thus appears that ATP activates the monomer and permits dimerization with about the same affinity.

In order to assess the aggregation state of hexokinase PII at 50 μM ATP under reaction conditions, the apparent molecular weight was determined by gel filtration. When hexokinase PII is applied at 0.25 or 2.5 μg/ml concentration to a Sephacryl S-200 column equilibrated with the complete assay mixture in imidazole buffer, pH 6.5, the apparent molecular weight was 64,000 in the presence or absence of citrate. It is thus apparent that citrate can activate the H⁺-inhibited enzyme without causing dimerization (see below). It may therefore be assumed that activation, and possibly the hysteretic adjustment to the less active form, represents the conformational interconversion of catalytically functioning monomers (16).

In considering these results, it is useful to consider which catalytic complex(es) are involved in the kinetic measurements. Previous work has shown that saturating glucose is necessary for maximal activation by citrate (1). It is also apparent that low ATP is required to see the largest effect of citrate, since saturating ATP itself activates the monomer. At low ATP and high glucose, it would seem that the steady state activity is a measure of $V/K$ for ATP adding to the $E\cdot$glucose complex. The fact that preincubation with either substrate does not abolish the initial phase of high activity, but incubation of the complete system (3, 5) does, has been interpreted to mean that the kinetic pathway for the slow transition from the active to the less active state is through the substrates ternary complex (5). Since, at the low ATP concentration, we are measuring activities after attainment of steady state and under approximately $V/K$ conditions, the active and less active conformations indicated, should correspond to $E\cdot$glucose binary complexes. It is assumed then that the transition from inactive to active forms requires binding of ATP. One of the conformations, induced or stabilized by citrate, is more active at low ATP. The residual activity of the monomer in the absence of citrate may then be due to an inherent activity of the less active conformation or to the equilibrium amount of the more active conformation.

At high levels of ATP (●, Fig. 1), it is apparent that at sufficiently high protein concentrations, ATP will not be an activator. However, the enzyme can be completely activated by citrate at $K_d$ levels of protein (data not shown). In analogy to the results presented below, it is likely that citrate activates by binding preferentially to the active monomer (see "Discussion").

**Activation by Citrate in Hepes Buffers**—Previous workers (2, 3) have shown that at low pH and low ATP, hexokinase is inhibited further by the presence of sulfonated buffers such as Hepes. Preliminary studies have also indicated buffer-dependent differences in the pH profile at low ATP and in the apparent molecular weight of the native enzyme. In light of the observation that the dimer form (with ATP) was of low activity, it was of interest to determine whether the sulfonated buffers were exerting their inhibitory effect by causing dimerization.

Fig. 2 demonstrates that in 50 mM Hepes, pH 6.3, and 50 μM ATP, the specific activity is independent of protein concentration at about 12 units/mg. This is about 60% of the rate seen in imidazole and is constant up to pH 6.8 (data not shown). In the presence of 1.5 mM citrate, however, there is a strong protein concentration dependence in which the dimer is again relatively inactive. The level of citrate used is saturating for activation as shown by the failure of additional citrate to stimulate the rate at any of these protein concentrations. This is a striking difference from the behavior in citrate and imidazole buffer (Fig. 1) where no protein concentration dependence is seen at low or high ATP. It is interesting to note that Hepes plus citrate has the same effect on activity as just raising the ATP level. The inset to Fig. 2 demonstrates that specific activity is inversely proportional to the square root of the protein concentration over most of the range tested. In the case of a monomer-dimer equilibrium, plots such as the insets to Figs. 1 and 2 would be linear for 50 to 60% of the activity loss if the dimer is relatively inactive. For this equilibrium, one can calculate an apparent dissociation constant of 0.4 μg/ml in the presence of Hepes and citrate at both pH values.

**$M_{app}$ Determinations by Gel Filtration**—In order to confirm association to multimeric form as indicated kinetically, the apparent molecular weight of hexokinase PII was determined using gel filtration under the conditions of Figs. 1 and 2. A Sephacryl S-200 superfine column was equilibrated with solutions containing 0.05 mM imidazole Cl, pH 6.5, or 0.05 mM sodium Hepes, pH 6.5, and various combinations of 50 μM ATP, 5 mM MgCl₂, 5 mM glucose, and 1.5 mM citrate (final concentrations). Fig. 3 shows the elution profiles of several
Thus, in the absence of substrates, \( n/r_{\text{app}} = 235,000 \); in the presence of both substrates, \( n/r_{\text{app}} = 168,000 \); and in the presence of both substrates plus citrate, \( n/r_{\text{app}} = 126,000 \).

It is clear from Figs. 2 and 3 that association is occurring in Hepes buffer. The molecular weight data do not, however, define the extent of this association (dimer, tetramer, etc.) since the gel filtration experiments measure both shape and molecular size (17). Also, this polymerization must be rapid compared to the rate of separation of monomer and polymer, since only a single symmetrical peak of enzyme activity is observed (18).

Fig. 4 shows an attempt to correlate apparent molecular weights with solution specific activities found at concentrations comparable to the average concentration during the column runs. Data from experiments like Figs. 1, 2, and 3, at initial enzyme concentrations of 0.25 and 2.5 \( \mu \text{g/ml} \), are presented here. As already noted, in imidazole buffer, no association was detected under any conditions even though citrate can only activate the monomer form. It must, however, bind to the dimer form also since additional citrate did not shift the equilibrium completely to monomer.

Because Fig. 4 indicates that the dimer is relatively inactive, one can readily use the data from Fig. 2 to calculate an equilibrium constant and \( n \) value for this association (see “Materials and Methods”). Fig. 5 shows this determination and yields an \( n = 2.0 \) and \( K_d = 0.5 \mu \text{g/ml} \) at pH 6.7 and 6.3. When the molecular weights obtained on the columns are used to calculate a \( K_d \), it is also apparent that an \( n = 2 \) is a much better fit than either \( n = 3 \) or 4. (Assuming an \( n \) of 3 or 4 leads to \( K_d \) values 25- and 200-fold different, respectively, for the two protein concentrations of Fig. 4). The basic agreement between all of these data argue that the same dimerization event is being detected by solution specific activity and column elution profiles.

Correlation of \( M_{\text{app}} \) for Frictional Ratios—If, as indicated above, the high molecular weight species is a dimer, it must be a very asymmetric dimer to give such a high molecular weight on gel filtration. For this reason, determinations were carried out with much smaller dimeric species as standard, such as the monomer-dimer equilibrium where the dimer \( M_{\text{app}} \) is 235,000 (see “Materials and Methods”). It thus appears that citrate can only activate the monomer form. It must, however, bind to the dimer form also since additional citrate did not shift the equilibrium completely to monomer.

Because Fig. 4 indicates that the dimer is relatively inactive, one can readily use the data from Fig. 2 to calculate an equilibrium constant and \( n \) value for this association (see “Materials and Methods”). Fig. 5 shows this determination and yields an \( n = 2.0 \) and \( K_d = 0.5 \mu \text{g/ml} \) at pH 6.7 and 6.3.

When the molecular weights obtained on the columns are used to calculate a \( K_d \), it is also apparent that an \( n = 2 \) is a much better fit than either \( n = 3 \) or 4. (Assuming an \( n \) of 3 or 4 leads to \( K_d \) values 25- and 200-fold different, respectively, for the two protein concentrations of Fig. 4). The basic agreement between all of these data argue that the same dimerization event is being detected by solution specific activity and column elution profiles.

Correction of \( M_{\text{app}} \) for Frictional Ratios—If, as indicated above, the high molecular weight species is a dimer, it must be a very asymmetric dimer to give such a high molecular weight on gel filtration. For this reason, determinations were carried out with much smaller dimeric species as standard, such as the monomer-dimer equilibrium where the dimer \( M_{\text{app}} \) is 235,000 (see “Materials and Methods”). It thus appears that citrate can only activate the monomer form. It must, however, bind to the dimer form also since additional citrate did not shift the equilibrium completely to monomer.

Because Fig. 4 indicates that the dimer is relatively inactive, one can readily use the data from Fig. 2 to calculate an equilibrium constant and \( n \) value for this association (see “Materials and Methods”). Fig. 5 shows this determination and yields an \( n = 2.0 \) and \( K_d = 0.5 \mu \text{g/ml} \) at pH 6.7 and 6.3. When the molecular weights obtained on the columns are used to calculate a \( K_d \), it is also apparent that an \( n = 2 \) is a much better fit than either \( n = 3 \) or 4. (Assuming an \( n \) of 3 or 4 leads to \( K_d \) values 25- and 200-fold different, respectively, for the two protein concentrations of Fig. 4). The basic agreement between all of these data argue that the same dimerization event is being detected by solution specific activity and column elution profiles.

Correction of \( M_{\text{app}} \) for Frictional Ratios—If, as indicated above, the high molecular weight species is a dimer, it must be a very asymmetric dimer to give such a high molecular weight on gel filtration. For this reason, determinations were carried out with much smaller dimeric species as standard, such as the monomer-dimer equilibrium where the dimer \( M_{\text{app}} \) is 235,000 (see “Materials and Methods”). It thus appears that citrate can only activate the monomer form. It must, however, bind to the dimer form also since additional citrate did not shift the equilibrium completely to monomer.

Because Fig. 4 indicates that the dimer is relatively inactive, one can readily use the data from Fig. 2 to calculate an equilibrium constant and \( n \) value for this association (see “Materials and Methods”). Fig. 5 shows this determination and yields an \( n = 2.0 \) and \( K_d = 0.5 \mu \text{g/ml} \) at pH 6.7 and 6.3. When the molecular weights obtained on the columns are used to calculate a \( K_d \), it is also apparent that an \( n = 2 \) is a much better fit than either \( n = 3 \) or 4. (Assuming an \( n \) of 3 or 4 leads to \( K_d \) values 25- and 200-fold different, respectively, for the two protein concentrations of Fig. 4). The basic agreement between all of these data argue that the same dimerization event is being detected by solution specific activity and column elution profiles.

Correction of \( M_{\text{app}} \) for Frictional Ratios—If, as indicated above, the high molecular weight species is a dimer, it must be a very asymmetric dimer to give such a high molecular weight on gel filtration. For this reason, determinations were carried out with much smaller dimeric species as standard, such as the monomer-dimer equilibrium where the dimer \( M_{\text{app}} \) is 235,000 (see “Materials and Methods”). It thus appears that citrate can only activate the monomer form. It must, however, bind to the dimer form also since additional citrate did not shift the equilibrium completely to monomer.

Because Fig. 4 indicates that the dimer is relatively inactive, one can readily use the data from Fig. 2 to calculate an equilibrium constant and \( n \) value for this association (see “Materials and Methods”). Fig. 5 shows this determination and yields an \( n = 2.0 \) and \( K_d = 0.5 \mu \text{g/ml} \) at pH 6.7 and 6.3. When the molecular weights obtained on the columns are used to calculate a \( K_d \), it is also apparent that an \( n = 2 \) is a much better fit than either \( n = 3 \) or 4. (Assuming an \( n \) of 3 or 4 leads to \( K_d \) values 25- and 200-fold different, respectively, for the two protein concentrations of Fig. 4). The basic agreement between all of these data argue that the same dimerization event is being detected by solution specific activity and column elution profiles.

Correction of \( M_{\text{app}} \) for Frictional Ratios—If, as indicated above, the high molecular weight species is a dimer, it must be a very asymmetric dimer to give such a high molecular weight on gel filtration. For this reason, determinations were carried out with much smaller dimeric species as standard, such as the monomer-dimer equilibrium where the dimer \( M_{\text{app}} \) is 235,000 (see “Materials and Methods”). It thus appears that citrate can only activate the monomer form. It must, however, bind to the dimer form also since additional citrate did not shift the equilibrium completely to monomer.
made of the sedimentation coefficient of the enzyme in Hepes (or imidazole) by sucrose density gradient centrifugation (13) and the apparent molecular weight by gel filtration in Hepes (or imidazole) buffer plus 7.5% sucrose (the average sucrose concentration encountered by the enzyme during centrifugation). This information allows one to make unique determinations of the true molecular weight and shape factors for the species involved (16).

Table I summarizes these sedimentation and gel filtration data in sucrose. The standards used were yeast alcohol dehydrogenase \( (s_{20, w} = 7.4 \text{ (13)}) \) and beef heart cytoplasmic malate dehydrogenase \( (s_{20, w} = 5.1 \text{ (14)}) \). The “high molecular weight species” is shown here to be a dimer with a fairly high frictional ratio. The lower \( M_{app} \) species is a monomer with a much more “usual” frictional coefficient. These data may be explained if Hepes binds more tightly to the low activity conformation and citrate binds to both low activity monomer and buffer-dimer complex equally but to activated monomer preferentially (see “Discussion”). At low protein concentration and infinite citrate, the enzyme would exist as the active monomer. Thus, either Hepes does not bind to this form or, if it does, it has no effect on activity. We have no data which bear on this point.

Gel filtration was used to determine whether the dimer formed by ATP is similar to that formed in Hepes. The apparent molecular weight of PII, applied at 2.5 \( \mu \text{g/ml} \) in imidazole buffer, pH 6.5, and in the presence of 5 mM glucose and 560 \( \mu \text{M} \) ATP with no added magnesium, was measured. These conditions were chosen to prevent depletion of substrate during the column run, and are reported to lead to the establishment of the slow steady state rate (3). No hysteresis is observed on completing the assay mixture with magnesium and the steady state rate is identical with the final rate observed in the normal assay (3). The value determined under these conditions, \( M_{app} = 140,000 \), is greater than that calculated for dimer BI. The conditions are such that a significant amount of enzyme would be in the monomer form because of the low protein and ATP concentrations. One expects between 20 and 40% monomer (using \( K_d \) from Fig. 1 and the initial and final protein concentrations on the column). Thus, the fully formed dimer must have a \( M_{app} \) much higher than the observed 140,000. This dimer then must have a high frictional ratio, as does the Hepes dimer, and the simplest conclusion is that they are quite similar. Since the intersubunit site described previously (9, 10) is not intact in the BI dimer, we can conclude that ATP is acting at some other site, as it and citrate must be doing to activate the monomer. A similar determination with high ATP in Hepes buffer gives an \( M_{app} \) of 240,000. This is to be contrasted with a value of 200,000 at the same protein concentration with 50 \( \mu \text{M} \) ATP plus magnesium, Fig. 4. It is thus shown that high levels of ATP cause dimerization to a form with the same apparent molecular weight as the dimer stabilized by Hepes.

**DISCUSSION**

In considering a physical model to explain the observations, it is of interest to compare the solution behavior found in this study to the solution equivalents of the known crystal structures for hexokinase PII. Four different structures have been studied, three of which are listed in Table II.

The monomer structure (BIII) has two lobes of electron density connected by a hinge region. Binding of glucose at its catalytic binding site in the hinge region causes a large shift in electron density of one of these lobes (7). An even larger shift of the lobe has been observed with the glucose complex of isozyme PI (26). These two conformational extremes are probably related to those detected during glucose binding in solution (19, 26).

In addition to these crystal forms, two dimers are known. BI is composed of two monomeric units very similar to BIID and related to each other by a rotation and translation. This geometry generates asymmetric interactions between the subunits. In light of the observed sedimentation coefficient of 5.6 S for dimer in the absence of substrates (6), it seems likely that a form similar to the BI crystal form predominates in the absence of substrates (see Table II). The BI crystals (9, 10) and independent solution studies (27) show negative cooperativity in binding of sugar substrates. An additional feature of this crystal form is the presence of an intersubunit nucleotide binding site, where ATP analogue binds. It has been postulated that this is the allosteric activator site for ATP and polyionic activators (11) and that binding of activators to this site stabilizes a dimer form of increased activity. To the contrary, the stabilization of a dimer by ATP is deactivating and only at low protein concentrations in solution does ATP give maximum activation. The result directly contradicts the

---

**Table I**

**Determination of \( M \) and frictional ratio for yeast hexokinase PII**

The sedimentation coefficient was determined by sucrose density gradient centrifugation. \( M_{app} \) was determined on calibrated Sephacryl S-200 columns equilibrated with 7.5% sucrose (see “Materials and Methods” for details). The Stokes radius was calculated from the relation \( r_s = [3 \times M_{app} / 4 \pi N^2]^{1/3} f/f_0 \) (standards) (17). The value determined under these conditions, \( M_{app} = 140,000 \), is greater than that calculated for dimer BI. The conditions are such that a significant amount of enzyme would be in the monomer form because of the low protein and ATP concentrations. One expects between 20 and 40% monomer (using \( K_d \) from Fig. 1 and the initial and final protein concentrations on the column). Thus, the fully formed dimer must have a \( M_{app} \) much higher than the observed 140,000. This dimer then must have a high frictional ratio, as does the Hepes dimer, and the simplest conclusion is that they are quite similar. Since the intersubunit site described previously (9, 10) is not intact in the BI dimer, we can conclude that ATP is acting at some other site, as it and citrate must be doing to activate the monomer. A similar determination with high ATP in Hepes buffer gives an \( M_{app} \) of 240,000. This is to be contrasted with a value of 200,000 at the same protein concentration with 50 \( \mu \text{M} \) ATP plus magnesium, Fig. 4. It is thus shown that high levels of ATP cause dimerization to a form with the same apparent molecular weight as the dimer stabilized by Hepes.

**DISCUSSION**

In considering a physical model to explain the observations, it is of interest to compare the solution behavior found in this study to the solution equivalents of the known crystal structures for hexokinase PII. Four different structures have been studied, three of which are listed in Table II.

The monomer structure (BIII) has two lobes of electron density connected by a hinge region. Binding of glucose at its catalytic binding site in the hinge region causes a large shift in electron density of one of these lobes (7). An even larger shift of the lobe has been observed with the glucose complex of isozyme PI (26). These two conformational extremes are probably related to those detected during glucose binding in solution (19, 26).

In addition to these crystal forms, two dimers are known. BI is composed of two monomeric units very similar to BIID and related to each other by a rotation and translation. This geometry generates asymmetric interactions between the subunits. In light of the observed sedimentation coefficient of 5.6 S for dimer in the absence of substrates (6), it seems likely that a form similar to the BI crystal form predominates in the absence of substrates (see Table II). The BI crystals (9, 10) and independent solution studies (27) show negative cooperativity in binding of sugar substrates. An additional feature of this crystal form is the presence of an intersubunit nucleotide binding site, where ATP analogue binds. It has been postulated that this is the allosteric activator site for ATP and polyionic activators (11) and that binding of activators to this site stabilizes a dimer form of increased activity. To the contrary, the stabilization of a dimer by ATP is deactivating and only at low protein concentrations in solution does ATP give maximum activation. The result directly contradicts the

---

**Table II**

**Comparison of observed hydrodynamic properties and the calculated properties of known crystal structures of hexokinase PII**

Crystal data and the photographs of space-filling models (7-11) were examined to determine the overall shape and dimensions. \( f/f_0 \) (shape) is the ratio of frictional coefficients of the given ellipsoid to that of a sphere of equal volume, and was estimated from the axial ratio (21). \( f/f_0 \) was estimated from the relationship \( f = 6 \pi n \sigma f/f_0 \) (shape) \( (3 M_r / (6 + 8) \pi N^2)^{1/3} \) and \( f_0 = 6 \pi n (3 M_r / 4 \pi N^2)^{1/2} \) was taken as 0.74 ml/g (19) and \( (6 + 8) \) was estimated as 1.67 ml/g from the measured intrinsic viscosity of 4.6 ml/g (23) and a viscosity shape factor of 2.7 (24). Other equations used were: sedimentation coefficient \( s = M(1 - \delta / N \gamma) \), Stokes radius \( r_s = (6 \pi N r_s / (1 - 6 \pi \sigma )) \) (17), \( \delta \), the partial specific volume of hexokinase was taken as 0.74 g/ml (19). N is Avogadro’s number, \( p \), the density of the media, and \( f/f_0 \) (standards) is the frictional ratio of the proteins used in calibrating the columns (same as in Fig. 3) and was taken as 1.3 (20).

<table>
<thead>
<tr>
<th>Property</th>
<th>Monomer BI</th>
<th>Monomer this study</th>
<th>Dimer BI</th>
<th>Dimer this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape</td>
<td>Prolate ellipsoid</td>
<td>Oblate ellipsoid</td>
<td>Prolate ellipsoid</td>
<td></td>
</tr>
<tr>
<td>Axial ratio</td>
<td>1.6</td>
<td>1.6</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>( f/f_0 ) (shape)</td>
<td>1.02</td>
<td>1.02</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>( f/f_0 )</td>
<td>1.33</td>
<td>1.33</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>( s \times 10^2 )</td>
<td>3.6</td>
<td>3.6</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Stokes radius</td>
<td>34</td>
<td>32</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>( M_{app} )</td>
<td>66,000</td>
<td>64,000</td>
<td>115,000</td>
<td>210,000</td>
</tr>
</tbody>
</table>
previous finding that the dimer is more active in the proton release assays (5). Under those conditions, one can predict that between 80 and 250 μM ADP would be produced before the steady state rate was achieved. In order to see if such low concentrations of ADP could account for these differences, we have determined the effect of added ADP at 2.0 mM ATP, 0.22 μg/ml of hexokinase in the absence of pyruvate kinase. It was found that the rate was inhibited 35% by 80 to 100 μM ADP (calculated from the absorbance at the time the rate was measured). This confirms a previous finding that ADP is inhibitory at low concentrations (2). If only the monomer were significantly inhibited by ADP, this would have the effect of obscuring the protein concentration dependence seen in Fig. 1 and would explain the apparent discrepancy between these and the previous results (5).

A similar argument applies to activation by citrate. Since only the monomer can be activated by citrate and activation results in net monomerization, the intact intersubunit site is not required for activation by citrate.

The third crystal structure (BI) is a very asymmetric dimer consisting of two monomer units condensed in a "head to head" fashion. This structure is reported not to bind substrates (6). It is this structure which may have now been detected in solution in the presence of Hepes or high ATP (see Table II). Both dimers show heterologous (asymmetric) subunit interactions. In the case of extreme asymmetry of this type, higher polymers could form, but with hexokinase the interactions are such that this cannot occur. Both dimers form a closed association which cannot lead to polymerization using either mode of subunit interactions known (9).

Table II shows the calculated hydrodynamic properties of the conformations represented by the crystal forms. Although it is considered difficult to make predictions of this type, a considerable amount of the necessary solution data exists for hexokinase with which to attempt such calculations. These can be considered rough estimates, but the agreement with our measured values reinforces their validity. Also included in this table are our measured values for "monomer" and "dimer." There is a very good correlation between the expected and observed properties. Thus, it is clear that the dimer that is stabilized by ATP or Hepes and the BI crystal structure are quite similar. Similarly, our monomer and the BI structure seem related. Under no conditions have we found evidence of significant amounts of BI in solution.

Model for Inhibition by Hepes and Activation by Citrate and ATP—When we integrate the data presented thus far, a schematic model results (Fig. 6).

Our results are probably best explained if the steady state contains two distinct conformations of enzyme, an active and a somewhat less active (or inactive) conformation. Citrate activates by binding preferentially to the active form. Effectors like Hepes act by binding preferentially to the inactive form of the enzyme resulting in dimerization. In the presence of such effectors and citrate, full activation can be achieved only at low protein concentrations. Under our conditions, we have not seen evidence for active dimers and thus, for simplicity, have not included such forms in our model. However, the results of Shill et al. (6) indicate that the active species at high protein, pH, and ATP has a sedimentation coefficient of 4.2 S. This is taken to indicate that under the proper conditions BI-type dimers are present and may be fully active.

These findings are consistent with the previous postulate that the hysteretic transition seen under assay conditions is caused by the slow interconversion of two differentially active conformations (5, 16). These two conformations have been detected by the present steady state measurements. They differ in their activity at low ATP, ability to bind positive and negative effectors and proclivity toward dimerization. Because various effectors change the steady state equilibria of the conformations, the apparent rate of the hysteretic transition will depend on the presence of such effectors. This clarifies the effect of substrates, pH, citrate, and Hepes on the rate of transition (5), since this rate will be a sum of the forward and reverse rates for conformational interconversion.

The top row of structures represents the active conformation of the enzyme and it is presumably these forms that are present when substrate-free enzyme is added to the assays at pH < 7. The hysteretic transition to a slower final rate would establish the multiple equilibria shown here. As less active forms accumulate, the various effectors such as Hepes, citrate, and ATP become important in determining the steady state concentrations of species. At very low concentrations of enzyme, no dimer would be present under any of the conditions. At concentrations above 0.1 to 0.5 μg/ml of enzyme, Hepes or high ATP favor dimerization to what may be the same, less active dimer form.

It should be noted that the dissociation constants measured here are in good agreement with previous determinations. At 2.0 mM ATP in imidazole buffer, pH 6.7, activity measurements (Fig. 1) indicate that \( K_d = 0.5 \) μg/ml, while centrifugation studies under similar conditions give a very comparable value (6). At zero or 50 μM ATP in imidazole buffer, pH 6.7, there is no detectable dimer at hexokinase concentrations as high as 25 μg/ml. This can be compared to the value in the presence of glucose (6), where no dimer is observed below approximately 10 μg/ml.

While one may describe the activation by citrate and ATP in terms of their binding to enzyme, the question of discrete allosteric sites is a confusing one. In the case of citrate, the activation occurs at very low concentrations (1) and our results indicate that under reaction conditions, monomer and dimer can bind citrate. There is, however, no report of citrate binding to the enzyme and several attempts to demonstrate this binding have failed (1). Similarly, the failure to show the BI dimer in either the high specific activity (monomer) or low specific activity (dimer) forms in solution raises the question of the role of those ATP binding determinants that were identified by Stetzel et al. (11).

Possible Physiological Role of Inhibition and Activation—It is interesting to ask if the physiological levels of isozyme PII are high enough to permit the polymerization. A minimum value for its concentration can be obtained from the purification reported when care is taken to prevent proteolysis (28, 29). Approximately 15 μg of pure PII is obtained from 0.9 kg of yeast paste. Assuming that most of that weight is cell water

![Fig. 6. Model depicting low pH conformation and aggregation changes of yeast hexokinase PII, where A = ATP, C = citrate, and H = Hepes.](image-url)
Yeast Hexokinase PII

leads to a minimum concentration of >16 μg/ml of cell water. Of course, if the hexokinase is compartmentalized, this concentration will be much higher. Since yeast hexokinase is thought not to be membrane-bound (29), the concentration cannot be lower.

At this concentration of hexokinase and physiological levels of ATP, the enzyme would be predominantly present as the ATP stabilized inactive dimer. In the absence of other effectors, our data can be extrapolated to estimate that hexokinase PII would be only about 7.5% active at pH 6.5 compared to pH 8.5. Using this number, one can ask if this rate is sufficient to account for glucose utilization in yeast cells containing only isozyme PII. Such mutants are known and they utilize glucose at a rate which is at least 50% that of the total hexokinase activity present (30). This comparison indicates that there must be a physiological activator like citrate in order to account for this flux.

The role of a Hepes-like inhibitor is very speculative at the moment. No physiological equivalent is known. It is clear that if one exists in the cells, its action would be to reverse citrate stimulation. One can conjecture that citrate interaction serves to maintain glucose utilization for the pentose pathway and polysaccharide storage, even when ATP is high and glucose is not needed for glycolysis. Hepes-like inhibitors would allow the cell to modulate glucose utilization if it became necessary.

Note Added in Proof—Two recent papers have appeared which bear on this work (Womack, F., and Colowick, S. P. (1978) Arch. Biochem. Biophys. 191, 742-747; 748-755). Kinetic studies at pH 7 in Tris buffers indicate that at 1 mg/ml, PII is primarily a dimer under assay conditions. Also, they find that ADP or GDP are inhibitors in the reaction catalyzed by PII and are only partially reversed by citrate. This partial reversal to about 85% of full activity at 0.1 μg/ml of PII is comparable to our value of 75% in Hepes buffer at both pH 6.3 and 6.7 (Fig. 2) at the same protein concentration. Thus, in vivo ADP may be the physiological equivalent of Hepes and exert a control similar to that suggested by the above authors.

REFERENCES

Activation of yeast hexokinase PII. Changes in conformation and association.

K D Wilkinson and I A Rose


Access the most updated version of this article at http://www.jbc.org/content/254/6/2125.citation

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/6/2125.citation.full.html#ref-list-1