Dissociation and Catalysis in Yeast Hexokinase A

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1. The specific activity of yeast hexokinase A depends on the concentration of the protein in the solution being assayed. When a solution containing 13.5 mg of hexokinase A/ml is diluted 10–100-fold at various values of pH and temperature, there is a gradual decline in the specific activity of the enzyme until an equilibrium value is reached, which varies with the chosen experimental conditions. 2. The catalytic activity lost when hexokinase A (1 mg/ml) is incubated at 30°C is recovered by lowering the temperature to 25°C. 3. These concentration- and temperature-dependent phenomena are consistent with the existence of a monomer–dimer equilibrium in which the dimer alone is the catalytic form of the enzyme. 4. Glucose alone prevents the decline in specific activity of hexokinase A after dilution, but it does not re-activate dilute solutions of the enzyme. It is concluded that glucose binds to both the dimer and the monomer and prevents both association and dissociation. 5. The progress curve describing the phosphorylation of glucose catalysed by hexokinase A does not attain a steady state. It is possible that dissociation of catalytically active dimers in a ternary complex with glucose and ATP (or glucose 6-phosphate and ADP) could explain the non-linearity of this progress curve.

All native isoenzymes of yeast hexokinase exist in solution as a monomer–dimer equilibrium, the position of which is affected by the addition of one or both of the substrates (Easterby & Rosemeyer, 1972; Derechin *et al.*, 1972). Hexokinase B exhibits regulatory properties of co-operativity (Kosow & Rose, 1971) and hexokinases A and B show a slow transient phase in the progress curve describing the phosphorylation of glucose (Shill & Neet, 1971). The contribution, if any, that the degree of dissociation of the protein makes to these important kinetic properties remains uncertain. Current evidence suggests that hexokinases B and C exist largely as monomers under conditions used to assay hexokinase activity *in vitro* (Derechin *et al.*, 1972; Shill *et al.*, 1974), and the regulatory properties mentioned above may be due to the monomer alone. However, under some conditions, the specific activity of hexokinase B depends on the concentration of the protein and it appears that the dimer may be as much as ten times as active as the monomer (Shill & Neet, 1975). This fact may well be significant in the yeast cell, which also shows transient kinetics and negative co-operativity when assayed for hexokinase activity as whole cells which were rendered permeable to substrates by treatment with protamine (Reitzer & Neet, 1974).

The present work was undertaken to determine the specific activity of the monomeric and dimeric forms of hexokinase A by measuring changes in the catalytic activity of the enzyme during experimen-

tally induced dissociation of the dimer. Hexokinase A was chosen because the transient seen in the progress curve during its assay is slower than that observed for the other isoenzyme, hexokinase B (Shill & Neet, 1975). The initial rate of the reaction can be measured with reasonable accuracy and taken to represent the specific activity of the preparation as introduced into the assay system.

Materials and Methods

Enzyme

Hexokinase A was prepared from baker's yeast by the method of Rustum *et al.* (1971). The preparation was examined by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969) and found to have a mol.wt. approx. 55000. The preparation contained a single contaminant (approx. 10% of the protein), which could not be removed by repeated chromatography on DEAE-cellulose as described by Rustum *et al.* (1971).

Assay of hexokinase activity

The enzyme was assayed as a routine by following the release of H⁺ by using Cresol Red as indicator (Lazarus *et al.*, 1966). One unit of enzyme activity is defined as 1 μ mol of product formed/min at 25°C and pH 8.4. The preparation of hexokinase used had a specific activity of 210 units per mg when assayed from a stock solution containing 13.5 mg of protein/ml.

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In some experiments the release of H^+ was followed titrimetrically under N_2 by using a Radiometer pH-meter and Titrigraph (TT1 and SBR2; Radiometer, Copenhagen, Denmark). The incubation mixture contained 5 mM-glucose, 5 mM-ATP, 12.5 mM- $MgCl_2$, 40 mM-NaCl and appropriate amounts of enzyme in a final volume of 2.0 ml. The number of H^+ ions released at pH 7.0 and 25°C per mol of ATP was calculated by allowing one reaction (with a large amount of enzyme) to go to completion.

Determination of protein

The concentration of protein in solution was measured spectrophotometrically at 280 nm. An extinction of 1.0 in a 1 cm cuvette was taken to represent a protein concentration of 1 mg/ml.

Buffers

All Tris buffers were prepared by titrating the appropriate solution of HCl to the required pH with Tris.

Experimental and Results

Effect of protein concentration on enzyme activity

Samples of a stock solution of hexokinase A (13.5 mg/ml in 50 mM-HCl/Tris buffer, pH 7.3) were diluted with a large excess of 0.1 M-HCl/Tris buffer, pH 8.4, to give protein concentrations in the range 0.05–0.52 mg/ml. These were immediately incubated at 30°C and samples (25 μ l) removed at time-intervals and assayed for hexokinase activity by the spectrophotometric method. The initial rate of the reaction was taken to represent the activity of the enzyme prepara-

Fig. 1. Dilution-induced inactivation of hexokinase A

Enzyme was incubated at 30°C in 0.1 M-Tris/HCl, pH 8.0. The concentration of protein was 0.05 mg/ml (Δ), 0.11 mg/ml (□), 0.22 mg/ml (○) and 0.52 mg/ml (■).

tion at the time of sampling. The results (Fig. 1) show a progressive loss of enzyme activity down to a finite value. The fraction of the initial activity which remains at the end of the process decreases with decreasing concentration of protein. Similar results were obtained at 25°C (pH 8.0, pH 8.4), 30°C (pH 8.0, pH 9.1) and 35°C at pH 8.4. At the highest temperature, however, a second phase of inactivation becomes significant. This effect, which is also apparent to a lesser extent at the lowest concentration of protein in Fig. 1, is prevented by the inclusion of 2-mercaptoethanol in the incubation mixture (Fig. 2). The thiol does not affect the rate of the first phase of the inactivation which was slightly variable in extent.

These data can be interpreted in terms of an equilibrium between catalytically active dimers, the form of the enzyme assumed to be in the concentrated stock solution, and less active monomers. Accepting that the monomers are chemically identical (Rustum *et al.*, 1971) the reaction can be written as

and the following equation would describe the difference between the catalytic activity of the dimer at the beginning of the experiment and the equilibrium

Fig. 2. Effect of 2-mercaptoethanol on the inactivation of hexokinase

Enzyme (1 mg/ml) was incubated at 35°C in 0.1 M-Tris/HCl, pH 8.4 (●) or under the same conditions with the addition of 70 mM-2-mercaptoethanol (○).

Fig. 3. Relationship between ΔA and $e/\Delta A$ for various concentrations of protein under a variety of experimental conditions

25°C, pH 8.0 (●), pH 8.4 (▲); 30°C, pH 8.0 (■), pH 8.4 (□), pH 9.1 (○); 35°C, pH 8.0 (△). See the text for details.

mixture of monomer and dimer which is eventually attained:

$$(eKC^2/\Delta A) - KC = 4\Delta A \quad (1)$$

where e is the concentration of protein (mg/ml), K is the dissociation constant, ΔA is the difference in enzyme activity between the initial dimer and the equilibrium mixture (units/ml) and C is the difference in specific activity between the dimer and the monomer (units/mg) (for derivation see Williams, 1974). A plot of ΔA against $e/\Delta A$ for different experimental conditions is shown in Fig. 3. The relationship is linear, as predicted by eqn. (1), and the value for C (the reciprocal of the intercept on the $e/\Delta A$ axis) is the same for all experiments and is 200 units/mg. The value of C , of course, relates to the conditions of the assay solution and not the conditions of the incubation mixture and should therefore be constant for all experiments. The value of 200 units/mg for C means that the monomer has negligible hexokinase activity under the conditions of the assay. The difference between the reciprocal of 210 units/mg (the specific activity of the dimer) and the reciprocal of 200 units/mg would not be easily measured on Fig. 3. It is reasonable to accept 210 units/mg as the activity of the

Table 1. Dissociation constants for hexokinase A under different experimental conditions

Enzyme was incubated at appropriate concentrations in 0.1 M-HCl/Tris buffer at the pH and temperature indicated. Dissociation constants were calculated from eqn. (1) by using the data in Fig. 3.

Temperature (°C)	pH	$10^3 \times K$ (M)
25	8.0	8
25	8.4	70
30	8.0	52
30	8.4	88
30	9.1	170
35	8.0	140

dimer, as this value is unaltered when a solution containing 1 mg of the enzyme/ml is incubated at 25°C and pH 8.4. Values for the dissociation constant for the dimer-monomer equilibrium have been calculated from eqn. (1) by using the data in Fig. 3, and these are collected in Table 1.

The rate of dissociation of the dimer is given by the expression:

$$-d\Delta D/dt = k_1(\bar{D} + \Delta D) - k_2(\bar{M} - 2\Delta D)^2 \quad (2)$$

where $\bar{D}$ and $\bar{M}$ are the equilibrium concentrations (mol/litre) of the dimer and the monomer respectively. $\Delta D = (D - \bar{D})$, where D is the concentration of the dimer in mol/litre at any time t . k_1 is the first-order constant for dissociation and k_2 is the second-order rate constant for association.

Eqn. (2) can be rearranged to give

$$-d\Delta D/dt = \Delta D(k_1 + 4k_2\bar{M} - 4k_2\Delta D)$$

which is of the form $dx/dt = x(a - bx)$

Integration and letting ΔD_0 be ΔD when $t = 0$ gives

$$t(k_1 + 4k_2\bar{M}) = \frac{\ln \Delta D_0(k_1 + 4k_2\bar{M} - 4k_2\Delta D)}{\Delta D(k_1 + 4k_2\bar{M} - 4k_2\Delta D_0)} \quad (3)$$

where $\Delta D_0 = (D_0 - \bar{D})$ and D_0 is the concentration of dimer at zero time. A fuller derivation of this and subsequent equations is given by Williams (1974). Eqn. (3) is simplified under certain conditions. Thus if the concentrations of monomers is zero at the beginning of the experiment, eqn. (3) becomes

$$k_1 t \frac{(2D_0 - \Delta D_0)}{\Delta D_0} = \ln \frac{\Delta D_0(2D_0 - \Delta D_0) - \Delta D(D_0 - \Delta D_0)}{D_0 \Delta D} \quad (4)$$

which is the expression originally derived by Frost & Pearson (1953) and enables k_1 to be determined from a linear plot. However, eqn. (3) is a general expression which predicts the change in concentration of the dimer from any equilibrium position when that is

Fig. 4. Dilution-induced inactivation of hexokinase A at 25°C and pH 8.4

Concentration of protein was 0.27 mg/ml (a) and 0.14 mg/ml (b). The points are experimental and the lines are calculated from eqn. (3) with values for k_1 and k_2 of 0.015 min⁻¹ and 2.14×10^4 litre·mol⁻¹·min⁻¹ respectively.

perturbed by changes in physical conditions such as pH, temperature or concentration of protein. Another simplified form of eqn. (3) can be obtained by using the known dissociation constant for the reaction under investigation. Substituting the dissociation constant $K(k_1/k_2)$ into eqn. (3) gives

$$k_1 t \frac{(K + 4\bar{M})}{K} = \ln \frac{\Delta D_0(K + 4\bar{M} - 4\Delta D)}{\Delta D(K + 4\bar{M} - 4\Delta D_0)} \quad (5)$$

Eqn. (5) was used to obtain preliminary estimates of k_1 from a linear plot by using the measured dissociation constant (Table 1) and accepting that the monomer is catalytically inactive.

By using this value for k_1 , theoretical curves describing the loss of enzyme activity with time were

Fig. 5. Inactivation of hexokinase A at 35°C followed by re-activation at 25°C

Enzyme (1 mg/ml) was incubated at 35°C and pH 8.4 for 10 min and then incubated at 25°C. The points are experimental and the solid line was calculated from eqn. (4) by using values for k_1 and k_2 of 0.003 min⁻¹ and 0.48×10^4 litre·mol⁻¹·min⁻¹ respectively.

calculated from eqn. (5) and the value of k_1 modified slightly where necessary to give the best fit between experimental points and theoretical curve. Comparisons between calculated and measured progress curves for one set of experimental conditions are shown in Fig. 4. The values for k_1 and k_2 (k_1/K) were 0.015 min⁻¹ and 2.14×10^4 litre·mol⁻¹·min⁻¹ respectively. Rate constants calculated in this way were independent of the concentration of protein within the range 0.1–0.52 mg/ml.

Effect of temperature on enzyme activity

Hexokinase A (1 mg/ml) in 0.1 M-HCl/Tris, pH 8.4, and containing 70 mM-2-mercaptoethanol was incubated at 35°C. After 10 min, when the enzyme activity had decreased by 70%, the enzyme was transferred to a water bath maintained at 25°C. The sample attained the new temperature within 1 min and was subsequently assayed periodically for hexokinase activity. The results (Fig. 5) show that the activity is recovered at the lower temperature and reaches the value expected at 25°C and pH 8.4 when the protein concentration is 1 mg/ml.

The rate of increase in the concentration of dimer following the decrease in temperature is given by:

$$-d\Delta D/dt = k_2(\bar{M} + 2\Delta D)^2 - k_1(\bar{D} - \Delta D) \\ = \Delta D(k_1 + 4k_2\bar{M} + 4k_2\Delta D)$$

Integration and simplification gives

$$k_1 t \frac{(K + 4\bar{M})}{K} = \ln \frac{\Delta D_0(K + 4\bar{M} + 4\Delta D)}{\Delta D(K + 4\bar{M} + 4\Delta D_0)} \quad (6)$$

Table 2. Rate constants for association and dissociation of hexokinase A

Rate constants were calculated from data obtained by following the rate of change in catalytic activity of the enzyme after dilution of the protein and/or changes in temperature.

Experimental conditions	Reaction followed	$10^2 \times k_1$ (min^{-1})	$10^{-4} \times k_2$ ($\text{litre} \cdot \text{mol}^{-1} \cdot \text{min}^{-1}$)
Stock solution at 4°C diluted and incubated at 25°C	Inactivation	1.5	2.14
Stock solution at 4°C diluted and incubated at 37°C for 10 min and then at 25°C	re-activation	0.3	0.48
Stock solution at 4°C diluted and incubated at 37°C for 10 min and then 25°C for 80 min and finally diluted tenfold and kept at 25°C	Inactivation	0.8	1.4

Fig. 6. Effect of substrates on the dilution-induced inactivation of hexokinase A

(a) Enzyme (0.9 mg/ml) was incubated alone at 30°C and pH 8.4 (○) and in the presence of ATP and Mg²⁺ (△). (b) Enzyme was incubated as in (a) (○) and with the addition of glucose (●). Glucose was either added at the start of the experiment or after 40% of the enzyme activity had been lost. The times of addition of glucose are marked by (⊙). See the text for details.

Again k_1 was calculated from the linear plot corresponding to eqn. (6) and used to construct the theoretical progress curve for the recovery of activity at 25°C which is shown in Fig. 5. The values for k_1 and k_2 were 0.003 min^{-1} and $4.8 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{min}^{-1}$ respectively.

In another experiment, enzyme (1 mg/ml) was incubated at 35°C and pH 8.4 for 20 min, then at 25°C for 85 min, when equilibrium was re-established at the lower temperature. The enzyme was then diluted to 0.09 mg/ml and the loss of catalytic activity was followed at 25°C and pH 8.4. The best fit between experimental and theoretical curves for the inactivation were obtained with values for k_1 and k_2 of 0.008 min^{-1} and $1.4 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{min}^{-1}$ respectively. The values for the two rate constants obtained from the three different experiments are collected in Table 2.

Effect of substrates on enzyme activity

(a) *Glucose or ATP.* Hexokinase A (0.9 mg/ml) was incubated at 30°C in 0.1 M-HCl/Tris, pH 8.4, in the presence of either 13.5 mM-glucose or 8.3 mM-ATP with 20.8 mM-MgCl₂. The results (Fig. 6a) show that glucose but not ATP prevents inactivation under these conditions. In another experiment glucose was added to the enzyme during the course of dilution-induced inactivation. Under these conditions further loss of enzyme activity is prevented by the addition of the substrate, but no activity is recovered (Fig. 6b).

(b) *Glucose and ATP.* For this experiment the phosphorylation of glucose was followed over a prolonged period of time with various amounts of hexokinase A. The reaction was followed titrimetrically as described above. The results (Fig. 7a) show that the slope of the progress curve gradually declines during the course of the reaction. The rate of decrease in the slope is a

Fig. 7. Inactivation of hexokinase A in the presence of glucose and ATP

(a) Phosphorylation of glucose by hexokinase A at 0.17 μg/ml (●), 0.34 μg/ml (○) and 0.69 μg/ml (□). The reaction was followed at 25°C by continuous titration with NaOH to maintain the pH at 7.0. (b) Time-dependent change in the slopes of the curves in (a). The slope at time t is the average rate of production of H^+ during the 5 min period with t as the mid-point.

first-order process and the rate constant is independent of the concentration of the enzyme (Fig. 7b). It therefore cannot be due to depletion of substrates or accumulation of products, but must represent a change in the enzyme from an active to an inactive form with a rate constant of $0.011\text{--}0.016\text{ min}^{-1}$.

Discussion

Dilution of hexokinase A under a variety of experimental conditions leads to a decrease in specific activity, to a value which depends on the concentration of protein. The extent of this change and the kinetics of the process are both consistent with a dissociation of an active dimer to a virtually inactive monomer. As expected, the position of the equilibrium is temperature-dependent and therefore a preparation of low specific activity when kept at 35°C is activated by

incubation at 25°C. Again the kinetics of this activation are consistent with an increase in the dimerization of the protein at the lower temperature. However, the rate constants measured by following the inactivation process at 25°C and pH 8.4 are some fourfold larger than those calculated from following the reactivation of a higher concentration of the enzyme under the same condition of temperature and pH. As the calculated rate constants are independent of the concentration of the protein, another reason must be sought for this discrepancy. It is possible that the protein undergoes a conformational change as the temperature is raised and this structural alteration is reflected in lower rates of association and dissociation when the temperature is subsequently decreased. It is likely that this conformational change is reversed at the lower temperature, but this process appears to be slow compared with the rates of association and dissociation. Hence when the enzyme is incubated for 10 min at 35°C followed by 85 min at 25°C and then diluted, the rate constants calculated by following the subsequent inactivation have values which are between those obtained when the enzyme is brought directly from 35° to 25°C and those obtained when the enzyme is kept at 4°C and then diluted at 25°C.

The addition of glucose but not ATP prevents the dilution-induced dissociation of hexokinase A. Further, when glucose is added after the diluted enzyme has been incubated for a short time at 25°C, the decline in enzyme activity is stopped but not reversed. Therefore glucose must bind to both the dimer and the monomer, and equilibration between enzyme-glucose complexes is much slower than that between free enzyme species. In contrast, Easterby & Rosemeyer (1972) reported partial dissociation of hexokinase A in the ultracentrifuge at relatively high concentrations of protein (2.5–5.0 mg/ml) and the degree of dissociation was increased by the addition of glucose. The reason for this discrepancy is not clear, but may be due to the different buffers used for the two different kinds of experiments or to different procedures used for the initial extraction of the enzyme from the yeast cell. The above authors autolysed yeast cells in phosphate buffer at 37°C, whereas we homogenized frozen yeast cells in the presence of solid CO_2 . In the presence of both glucose and ATP there is a progressive loss of catalytic activity, as seen by a gradually changing progress curve for the phosphorylation of glucose. The rate of this decline is independent of the concentration of protein and cannot be due to deleterious changes in the environment due to the accumulation of products. Hence the loss of enzyme activity during the catalytic event is due to a change from an active to an inactive form of the enzyme and is probably due to complete dissociation of the dimer to monomers at the very low concentration of protein used for the assay. This would be analogous

to the situation with other isoenzymes of yeast hexokinase, where evidence suggests that hexokinases B and C exist as monomers under assay conditions (Derechin *et al.*, 1972; Shill *et al.*, 1974). These latter experiments were done in an ultracentrifuge, when there would be sufficient time for equilibrium between monomer and dimer to be established. The difference noted here between the behaviour of the enzyme-glucose complex and either the free enzyme or the ternary catalytic complex is in keeping with other evidence showing conformation differences between enzyme, enzyme-glucose complex and reacting enzyme-substrate complexes of hexokinase B (Shill & Neet, 1975).

With hexokinase B, the progress curve describing the phosphorylation of glucose, is characterized by a rapid transient phase leading to a steady-state rate of reaction (Shill & Neet, 1971, 1975). These authors argued that the transient phase cannot be due to dissociation of the protein, as the substrates have an associating effect on the enzyme (Shill *et al.*, 1974). However, this latter paper also shows that, even with this associating effect of substrates, the enzyme exists eventually as a monomer at the low concentration of protein used for the assay. It therefore appears quite possible that dissociation of dimers to less active

monomers is partly responsible for the transient phase observed with hexokinase B.

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