The Catalytic Activity of Monomeric Yeast Hexokinase A

By BERWIN P. YIP and FREDERICK B. RUDOLPH

Department of Biochemistry, Rice University, Houston, TX 77005, U.S.A.

(Received 5 October 1976)

It has been suggested [Williams, D. C. & Jones, J. G. (1976) *Biochem. J.* **155**, 661–667] that monomeric hexokinase isoenzyme A is not catalytically active. We here present data from reacting-enzyme sedimentation, dissociation experiments and from previous studies which are consistent with the monomeric form possessing catalytic activity.

Yeast hexokinase exists in a reversible monomer-dimer equilibrium in solution. This equilibrium is dependent on ionic strength, pH, temperature and the presence of substrates (Easterby & Rosemeyer, 1972; Derechin *et al.*, 1966, 1972; Shill *et al.*, 1974). The existence of enzymically active monomeric species of hexokinase isoenzymes B and C has been well documented (Shill *et al.*, 1974). In a report by Williams & Jones (1976) it was suggested that isoenzyme A was active only as a dimer and underwent dissociation only to an inactive monomer. The results of experiments that are in disagreement with their suggestion are presented.

Materials and Methods

Yeast hexokinase isoenzyme A was purified from baker's yeast as described by Rustum *et al.* (1971), with the modification of using phenylmethanesulphonyl fluoride in place of di-isopropyl phosphorofluoridate to prevent proteolysis. The specific activity of the A isoenzyme was 240 units/mg (Rustum *et al.*, 1971), and it exhibited a single protein band on gel electrophoresis (Davis, 1964) and moved as a single species in sedimentation-velocity centrifugation (Schachman, 1959). Kinetic assays were performed with the glucose 6-phosphate dehydrogenase system at pH 8.0 as described previously (Fromm & Zewe, 1962). Protein concentration was determined from the A_{280} (Rustum *et al.*, 1971).

Reacting-enzyme sedimentation studies as initially described by Cohen & Mire (1971) were performed in a Beckman-Spinco model E analytical centrifuge with a RTIC temperature-control unit and photo-electric scanner. The assays were done as described by Shill *et al.* (1974) by observing the change in A_{400} of *p*-nitrophenol as it was titrated by the H^+ formed during centrifugation as the band of active enzymes, initially layered on top, passed through the reaction mixture. The assay mixture contained 0.1 μ M-tri-(hydroxyethyl)ammonium chloride, 0.01 μ M-*p*-nitrophenol, 2 μ M-ATP, 20 mM-glucose and 8 mM-MgCl₂ in 50% ²H₂O at pH 6.75. The pH was corrected for the

presence of the ²H₂O, which is added to stabilize the enzyme boundary. Hexokinase was diluted in a dilution mixture identical with the assay mixture, except for the omission of ATP and ²H₂O, and the pH was adjusted to 6.55. The reacting-enzyme sedimentation studies were done at 20°C at 60000 rev./min in an An-D rotor with a charcoal-filled band-forming double-sector 12 mm-path-length cell with Epon centrepiece and sapphire windows. The double-sector cell allows the high initial absorbance of the dye to be blanked out. Both sectors were filled with 0.29 ml of the assay mixture, and, since the titration of the *p*-nitrophenol produced a decrease in absorbance, the capillary chamber of the blank sector was filled with 10 μ l of enzyme which had been diluted to the desired protein concentration in the dilution mixture. The capillary chamber of the sample sector was filled with 10 μ l of the dilution mixture. This allowed a positive change in absorbance to be recorded on the scanner trace.

The distances of the enzyme band from the axis of rotation (r) were determined from the midpoints of the scanner-traced boundaries at either 4 or 8 min intervals. Observed sedimentation coefficients were calculated from plots of $\log r$ versus time. The $s_{20,w}$ values were calculated by using the correction factor of 1.31 for density, viscosity and partial specific volume as determined by Shill *et al.* (1974) for the same assay system.

Results and Discussion

The association state of the active species of hexokinase A was monitored by reacting-enzyme sedimentation. An example of the reacting-enzyme sedimentation is shown in Fig. 1, with loading concentration of 4 μ g/ml. Results from experiments carried out over a range of initial loading concentrations from 4 to 22 μ g/ml are summarized in Fig. 2. At a loading concentration of 4 μ g/ml the enzyme sediments in a band with $s_{20,w}$ approx. 3.0S. Above this concentration the reacting enzyme associates to the dimer, with $s_{20,w}$ 4.8S. These data are consistent

Fig. 1. Reacting-enzyme sedimentation of yeast hexokinase A

(a) Scanner tracings (change in A_{400} versus the distance from the centre of rotation, r) were taken at 8 min intervals and are shown here superimposed on a common point of reference every 16 min for clarity. The enzyme loading concentration was $4 \mu\text{g}$ of isoenzyme A/ml. Other conditions of centrifugation are described in the Materials and Methods section. (b) Plot of the log of the radius of the boundary midpoint against time (min) from the sedimentation data shown in (a): $s_{20,w} = 3.0\text{S}$.

with a rapid equilibrium between the active dimer and the active monomer and are similar to the results obtained with B and C isoenzymes (Shill *et al.*, 1974). The $s_{20,w}$ values of 3.0S and 4.7S for the monomer and dimer respectively are consistent with previous studies on the sedimentation of the enzyme (Derechin *et al.*, 1966; Easterby & Rosemeyer, 1972; Shill *et al.*, 1974).

The reacting-enzyme sedimentation technique is extremely useful in demonstrating the existence of a

Fig. 2. Plot of $s_{20,w}$ obtained with reacting-enzyme sedimentation as a function of hexokinase A loading concentration

Experimental conditions are described in the Materials and Methods section.

specific aggregate state of an active enzyme, and the present results clearly show that an active monomeric species of hexokinase A exists at low protein concentration. This observation is clearly contradictory to the conclusion by Williams & Jones (1976). Their conclusion was mainly derived from studies on inactivation of enzyme on dilution (shown in Fig. 3 of Williams & Jones, 1976). A small error in the determination of the intercept on the abscissa gives rise to a large difference in the determination of specific activity. Their data points can be fitted approximately equally well to lines going through an intersection point of 0.075 on the abscissa, which would give the monomer an activity of 70 units/mg, about 30% as active as the dimer, assuming 200 units/mg as the activity of the dimer. We have also performed several dissociation experiments at pH 8.0, 8.4 and 9.0 and at 30°C , as described by Williams & Jones (1976), but using the more sensitive glucose 6-phosphate dehydrogenase coupling-enzyme assay. It must be noted that conclusions from our data are also subject to uncertainty intrinsic to the method of analysis mentioned above, but the data when analysed as described by Williams & Jones (1976) are more consistent with the monomer having a specific activity about 25% of that of the dimer. This conclusion is consistent with the results obtained with isoenzyme B, which has been shown to be 50% active in the monomeric state (Shill *et al.*, 1974).

A related study involving the effect of succinylation (3-carboxypropionylation) on hexokinase A has been done by Rossi *et al.* (1975). On succinylation, yeast hexokinase A dissociates into its subunits and the chemically modified subunits could be isolated in a catalytically active form. With three amino groups modified, the enzyme was a mixture of dimer and monomer and had 50% of the activity of the native

protein. With six amino groups modified the enzyme was shown to be completely dissociated, and retained 30% residual activity. As stated by Rossi *et al.* (1975), it is difficult to state with precision whether the loss of activity was due to modification of a residue on the enzyme active site or was related to dissociation of the dimers into monomers, but the conclusion that the enzyme can function either in the dimeric or in the monomeric form was well established.

In summary, the evidence for existence of an enzymically active monomeric species of yeast hexokinase A seems conclusive and is contradictory to the study of Williams & Jones (1976).

This work was supported in part by grants CA-14030 from the National Cancer Institute and C-582 from the Robert A. Welch Foundation. B. P. Y. is a Robert A. Welch Foundation Predoctoral Fellow.

References

- Cohen, R. & Mire, M. (1971) *Eur. J. Biochem.* **23**, 267–275
Davis, B. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
Derechin, M., Ramel, E. H., Lazarus, N. R. & Barnard, E. A. (1966) *Biochemistry* **5**, 4017–4025
Derechin, M., Rustum, V. M. & Barnard, E. A. (1972) *Biochemistry* **11**, 1793–1797
Easterby, J. S. & Rosemeyer, M. A. (1972) *Eur. J. Biochem.* **28**, 241–252
Fromm, H. J. & Zewe, V. (1962) *J. Biol. Chem.* **237**, 3027–3032
Rossi, A., Menezes, L. C. & Pudles, J. (1975) *Eur. J. Biochem.* **59**, 423–432
Rustum, V. M., Ramel, E. H. & Barnard, E. A. (1971) *Prep. Biochem.* **1**, 309–329
Schachman, H. K. (1959) *Ultracentrifugation in Biochemistry*, Academic Press, New York
Shill, J. P., Peters, B. A. & Neet, K. E. (1974) *Biochemistry* **13**, 3864–3870
Williams, D. C. & Jones, J. G. (1976) *Biochem. J.* **155**, 661–667