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THE EFFECTS OF ^2H_2O ON MAMMARY GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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SUMMARY

The NADP-linked and NAD-linked reactions catalyzed by rat mammary gland glucose-6-phosphate dehydrogenase are differentially affected by ^2H_2O. The enzyme is stabilized by ^2H_2O against inactivation in the cold at alkaline and neutral pH. These effects of ^2H_2O resemble those produced by glycerol and are attributed to the stabilization of Monomer X, the enzyme form possessing higher NAD-linked and lower NADP-linked activity than Monomer Y.

Rat mammary gland glucose-6-phosphate dehydrogenase (D-glucose 6-phosphate:NADP oxidoreductase, EC 1.1.1.49) catalyzes the oxidation of glucose 6-phosphate by either $NADP^+$ or NAD^+ under appropriate conditions [1]. Although the NAD-linked reaction is not thought to have physiological significance, it is of interest because it responds differently to various reagents and conditions than does the NADP linked reaction [1, 2]. This has provided some insight into the relationship between the structure and function of the enzyme. Evidence was adduced for the existence of two catalytically active, isomeric forms of the enzyme, X and Y. The NAD-linked activity of X is greater than that of Y whereas Y has higher NADP-linked activity than X. Under suitable conditions Y can aggregate to a dimer and X can dissociate to inactive subunits; both processes can be reversed [2].

In recent studies on the molecular weight of mammary glucose-6-phosphate dehydrogenase (unpublished results) sedimentation equilibrium experiments were conducted which necessitated using buffers prepared in ^2H_2O. This prompted us to examine the effects of ^2H_2O on the enzyme which are reported here.

The enzyme was prepared by a modification (unpublished results) of the procedures described earlier [1] and assayed as detailed elsewhere [2] in a Zeiss PMQ II spectrophotometer with a thermostatted cell compartment maintained at 25 °C. NAD^+, $NADP^+$, NADPH and glucose 6-phosphate were purchased from Sigma Chemical Co. ^2H_2O was obtained from Bio-Rad Laboratories. In solutions containing ^2H_2O the p^2H was taken as the observed pH + 0.4 [3]. When the ^2H_2O concentration was less than 99% it was assumed that a linear relationship exists between the ^2H_2O concentration and the magnitude of this correction factor [4].

At pH 8.6 the NAD-linked reaction is not materially affected by increasing concentrations of ^2H_2O but the NADP-linked reaction is inhibited (Fig. 1). This inhibition is not markedly altered between pH 7 and 9. In contrast, the NAD-linked reaction is stimulated by ^2H_2O at high p^{2}H. This stimulation results from lowering the K_m for NAD^+. At pH (p^{2}H) 9.1 the apparent K_m for NAD^+ was reduced from 0.036 M in H_2O solution to 0.01 4M in 88% ^2H_2O; the maximum velocity was decreased by 33%. Thus, at high pH the NADP-linked reaction is inhibited and the NAD-linked reaction is stimulated in ^2H_2O.

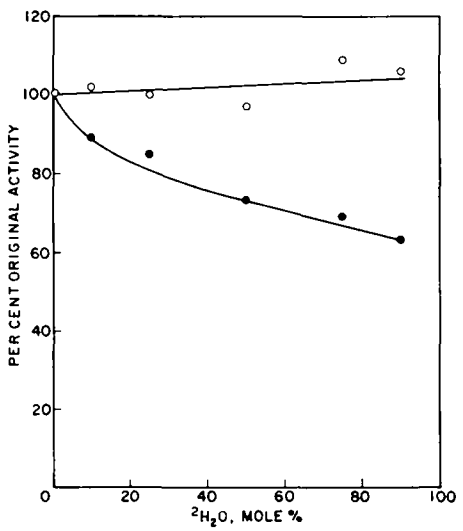

Fig. 1. The effect of ^2H_2O concentration on glucose-6-phosphate dehydrogenase. ○, NAD-linked reaction; ●, NADP-linked reaction. Assays were conducted at pH (p^{2}H) 8.6.

We showed previously that glycerol stimulates the NAD-linked and inhibits the NADP-linked reaction [1]. Glycerol was postulated to act on this enzyme by promoting the formation of Monomer X from Monomer Y [2]. In the presence of NADPH, non-linear Lineweaver–Burk plots resulted when the NAD^+ concentration was varied, but these plots became linear when the reaction was conducted in 40% glycerol. This was explained in terms of opposing effects of glycerol and NADPH on the X–Y equilibrium [2]. We therefore examined the effect of ^2H_2O on the kinetics of the NAD-linked reaction in the presence of a fixed concentration of NADPH. Exactly the same result is produced by 88% ^2H_2O (Fig. 2) as by 40% glycerol (Fig. 3 in [2]); the non-linear Lineweaver–Burk plot in the presence of 3 μM NADPH becomes linear in ^2H_2O, and under these conditions, as in 40% glycerol, NADPH acts as a competitive inhibitor with respect to NAD^+.

When molecular weights were calculated from H_2O- and ^2H_2O-containing cells in the sedimentation equilibrium experiments for determining $\bar{v}$ and φ', no significant differences could be detected; plots of ln c vs r^2 were linear, both in H_2O-containing and ^2H_2O-containing buffers (unpublished results). ^2H_2O protects the enzyme against inactivation under conditions known [5] to dissociate it into inactive subunits (Fig. 3).

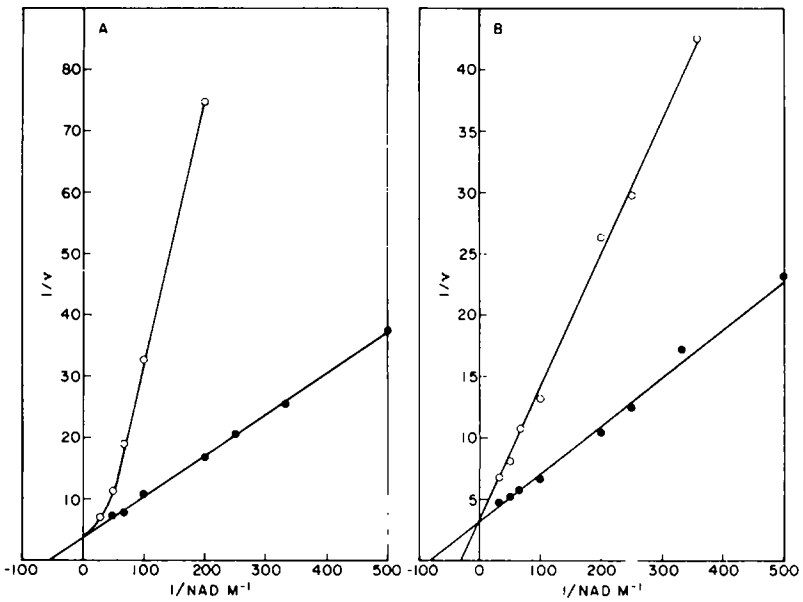

Fig. 2. The effect of ^2H_2O on NADPH inhibition of NAD-linked reaction. (A) Solutions in H_2O in the absence (●) and presence (○) of 3.0 μM NADPH. (B) Solutions in 88% ^2H_2O in the absence (●) and presence (○) of 3.0 μM NADPH.

Sucrose gradient centrifugation of the enzyme in buffers containing 0.1 mM $NADP^+$ and 0.2 M NaCl, prepared in H_2O or ^2H_2O, yielded the same molecular weights within experimental error (unpublished results). We conclude, therefore, that ^2H_2O causes neither aggregation nor dissociation of the enzyme.

Glycerol is an excellent stabilizing agent for mammary glucose-6-phosphate dehydrogenase [1]. ^2H_2O also protects the enzyme (Fig. 3), providing marked stabilization although, in this experiment, 20% glycerol was more effective. When the enzyme was incubated in 0.04 M phosphate buffer (pH 7.2), prepared in H_2O, it lost 41% of its activity at 25 °C, and 71% at 0 °C, in 40 min. Inactivation was more rapid at 0 than at 25 °C. Complete retention of activity was observed at both temperatures for 80 min if the buffer contained 0.1 mM $NADP^+$, or 20% glycerol, or if it was prepared in ^2H_2O.

Several other proteins are affected by ^2H_2O. Stabilization by ^2H_2O was reported for pyruvate carboxylase [6], glutamate dehydrogenase [7], lactate dehydrogenase [8], and fatty acid synthetase [9]. All these enzymes are protected by ^2H_2O against dissociation or denaturation. For α-chymotrypsin [10], α-chymotrypsinogen [11], and β-lactoglobulin in A [12] ^2H_2O promotes aggregation. In rabbit muscle glyceraldehyde 3-phosphate dehydrogenase ^2H_2O prevents dissociation into inactive subunits followed by aggregation [13]. The binding characteristics of NAD^+ for this enzyme, including negative cooperativity, are preserved in ^2H_2O, but the specific activity is reduced [13]. ^2H_2O inhibits the Mg^{2+}-dependent, (Na^+–K^+)-stimulated ATPase from rat brain microsomes while it stimulates the K^+-stimulated p-nitrophenyl phosphatase activity of the same enzyme [14].

The various effects of ^2H_2O on protein structure have generally been inter-

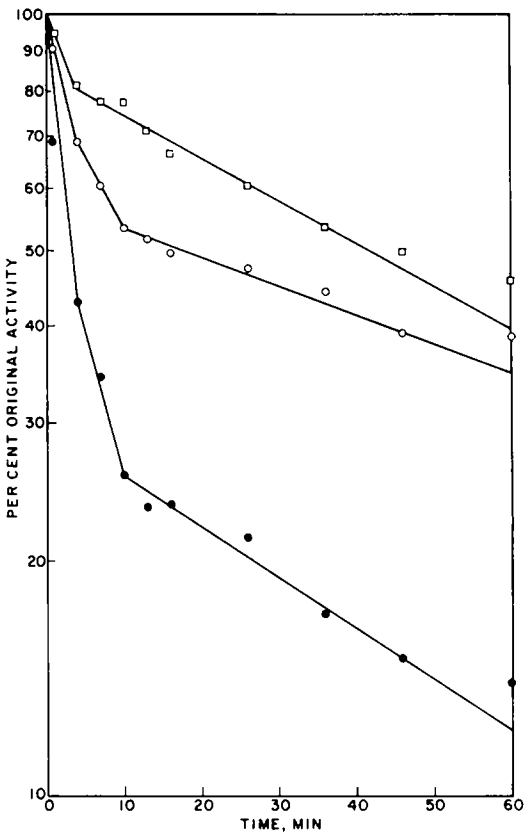

Fig. 3. Stability of glucose-6-phosphate dehydrogenase incubated at 0 °C in 0.04 M Tris–HCl buffer, pH (p^{2}H) 9.2, containing 7.0 mM mercaptoethanol and prepared in H_2O (●), 99% ^2H_2O (○) and 20% glycerol (□). Protein concentration was 51 μg/ml. Assays for NADP-linked activity were performed on aliquots removed at the times indicated.

preted as resulting from the stabilization of hydrophobic [15] or hydrogen [16] bonds in this solvent. Although our results do not permit a definite conclusion concerning the mechanism of the effects observed, they are consistent with stabilization of hydrophobic bonds. ^2H_2O appears to affect mammary glucose-6-phosphate dehydrogenase in the same way as glycerol. Both solvents protect the enzyme against inactivation at alkaline or neutral pH, processes which occur more rapidly at 0 than 25 °C. Hydrophobic bonds, but not hydrogen bonds, are more stable at 25 than at 0 °C [17]. The evidence suggests that ^2H_2O and glycerol stabilize the enzyme by preventing dissociation of Monomer X and, therefore, that hydrophobic bonds are involved in the interaction between the subunits within the monomer. By contrast, the interactions between monomers in the formation of dimer involve ionic forces (unpublished results). These conclusions are similar to those of Cohen and Rosemeyer [18] concerning the forces stabilizing the structure of human erythrocyte glucose-6-phosphate dehydrogenase.

ACKNOWLEDGEMENTS

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