Activation of Yeast Pyruvate Kinase by Natural and Artificial Cryoprotectants*

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SUMMARY

The inactivation of yeast pyruvate kinase at temperatures near 0°C and the stabilization achieved by addition of 50% glycerol or 0.1 M KCl and 24 mM MgCl₂ recently documented (Kuczenski, R. T., and Suelter, C. H., Biochemistry, 9, 939, 1970) prompted an examination of the effects of glycerol and other cryoprotectants on the kinetic activity of this enzyme. We now report that addition of glycerol, dimethyl sulfoxide, trimethylphosphine oxide, or dextran to an assay mixture containing less than \( K_m \) levels of substrates activates yeast pyruvate kinase to the same extent as that observed by addition of the positive modifier, fructose 1,6-diphosphate, in the absence of the cryoprotectant. Maximum effects were observed with 33% glycerol, 20% dimethyl sulfoxide, and 14% trimethylphosphine oxide. The activation by glycerol and dimethyl sulfoxide at 30°C results from a lowering of the \( K_m \) for phosphoenolpyruvate from 1.1 mM to 0.3 to 0.4 mM and the Hill slope \( (n_H) \) from 3.1 to 2.1. Kinetic analysis at 0°C in the absence of the cryoprotectant gave a \( K_m \) of 0.13 mM and \( n_H = 0.94 \). These changes in the kinetics of yeast pyruvate kinase are discussed in terms of the effect of temperature and solvent on the structure of both water and enzyme.

Yeast pyruvate kinase because of its cold lability (1) must be stabilized during purification at 4°C. Glycerol, a natural cryoprotectant, was used for this purpose and which is also required for renaturation of guanidine·HCl denatured enzyme. Also protects many biological materials against freeze-thaw damage (2). Other compounds such as dimethyl sulfoxide have been used extensively in low temperature work (a), and dextran has been shown to protect rat hearts (3), erythrocytes (4, 5), and bone marrow (6) from extreme freeze-thaw damage. Trimethylphosphine oxide was predicted to have cryoprotective properties for red blood cells, but had not yet been tested (7).

This paper reports the results of our study of the effects of these compounds on the catalytic activity of yeast pyruvate kinase examined at 30 and 0°C.

MATERIALS

Yeast pyruvate kinase was prepared by the method of Hunse and Suelter (1). Ammonium sulfate suspensions of rabbit muscle lactic dehydrogenase, NaADP, tricyclohexylammonium-P-enolpyruvate, and tetracyclohexylammonium-fructose-1,6-di-P were obtained from Sigma. The Grade A dithiothreitol was purchased from Calbiochem.

Dimethyl sulfoxide (Aldrich, industrial grade) was shaken with \( \text{NaHCO}_3 \) and distilled in vacuo at approximately 90°C. Between experiments it was stored frozen. Dextran was purchased from K and K Laboratories, Inc., Plainview, New York (average mol wt 86,000). Reagent grade glycerol was a product of J. T. Baker Chemical Company, Phillipsburg, New Jersey. Trimethylphosphine oxide was synthesized by the method of Burg and McKee (8) with a bromide Grignard instead of the iodide Grignard.

METHODS

Yeast pyruvate kinase was desalted by passing a solution of the enzyme over a column of Sephadex G 25 (coarse, 0.8 × 11.0 cm) equilibrated with 0.1 M Tris, pH 7.5, and eluted with the same buffer. Aliquots were tested with saturated \( \text{BaCl}_2 \) solution to insure them free of ammonium sulfate. Protein concentrations were estimated with the absorption coefficient \( E_{1\text{cm}}^{280} = 0.653 \) at 280 nm (1). For the kinetic studies, the enzyme at 0.25 mg per ml was incubated for 3 hours at 23°C in 0.1 M Tris, pH 7.5, 0.23 mM KCl, 25 mM MgCl₂, and 50 mM dithiothreitol to avoid the superactivation phenomena reported by Kuczenski and Suelter (9). Dithiothreitol, K⁺ and Mg²⁺ were required to prevent loss in the catalytic activity of the enzyme when assayed at suboptimal concentrations of substrate.

Yeast pyruvate kinase was assayed according to the method of Hunse and Suelter (10) with the following modifications. Yeast pyruvate kinase was assayed according to the method of Hunse and Suelter (10) with the following modifications.

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‡ The abbreviations used are: fructose-1,6-di-P, fructose 1,6-diphosphate; P-enolpyruvate, phosphoenolpyruvate.

§ M. J. Ruwart and C. H. Suelter, unpublished observation.
Phosphoenolpyruvate was 0.4 mM; ADP, 5 mM; and MgCl₂, 17 mM, as compared with 5 mM, 10 mM, and 24 mM, respectively. The concentration of NH₄⁺ introduced with the lactate dehydrogenase was calculated to be 12 mM, while the concentration of Na⁺ from the NADH was approximately 30 mM. In all cases the enzyme solution was maintained at 23° and the assays were run at 30° in a total volume of 1.00 ml, except in the assays containing (CH₃)₂PO where the volume was 0.25 ml.

For assays at 0°, the temperature of the Beckman DB was monitored with a calibrated thermistor. The cuvette chamber was dehumidified with heat-activated silica gel (Sigma). The lactic dehydrogenase concentration was increased 7-fold to compensate for its lower catalytic rate at 0°.

RESULTS

Effect of Cryoprotectants—In order to examine the effect of glycerol and other cryoprotectants on the kinetic parameters of yeast pyruvate kinase, the enzyme was assayed at suboptimal concentrations of substrates, particularly P-enolpyruvate, in the presence and absence of fructose-1,6-di-P at increasing concentrations of the test compounds. Suboptimal concentrations of substrates were chosen with the hope that either an activation or an inhibition would be readily discerned. Fig. 1A shows the effect of increasing concentrations of glycerol on the activity of the enzyme. The activation factor shown on the ordinate is the ratio of the catalytic activity observed under each condition divided by the activity obtained in the absence of fructose-1,6-di-P at 0% glycerol. O-0, assayed without fructose-1,6-di-P; •---•, assayed without fructose-1,6-di-P. The observed ratios were similar to those observed with glycerol but the peaks in the activation ratios were at 2.9 M (20%) (CH₃)₂SO in the absence of fructose-1,6-di-P and 2.4 M (15%) (CH₃)₂PO in the presence of fructose-1,6-di-P. The increased ratio observed at high concentrations of (CH₃)₂SO is not due to inhibition of lactate dehydrogenase since increasing lactate dehydrogenase had no effect. The reported interaction of (CH₃)₂SO with pyruvate to produce a 340-nm adsorbing material (11) was not observed.

The maximum activation ratio for (CH₃)₂PO as the concentration of (CH₃)₂PO is increased in the assay in the absence of fructose-1,6-di-P occurs at 1.4 M (14%) (CH₃)₂PO; the activation ratio in the region of (CH₃)₂PO concentrations at which a peak in the activation ratio was observed with fructose-1,6-di-P plus other activators was not determined (Fig. 1C). The activation ratios in (CH₃)₂PO were not as great as those observed in (CH₃)₂SO and glycerol. Dextran also produced an activation ratio of 4 in the absence of fructose-1,6-di-P which was still increasing at 37% w/v dextran, but because of viscosity effects, completion of the study was not possible.

Phosphoenolpyruvate Kinetics—In an effort to understand the activation observed in the binary solvents, the saturation kinetics of P-enolpyruvate were examined under several conditions noted in Table I. In each case the apparent $K_m$ and Hill slope ($n_H$) was obtained from a standard Hill plot (12) of log[($V_{max}/v$) - 1] plotted versus log[P-enolpyruvate]. It was previously shown that P-enolpyruvate saturation kinetics were sigmoid with $n_H$ = 3, $K_m$ = 1 mM; in the presence of fructose-1,6-di-P, saturation was normally hyperbolic with $n_H$ = 1.0 and $K_m$ = 0.1 mM (10, 13). Assaying at 0° (Table I) gave similar results to those observed with fructose-1,6-di-P, $n_H$ = 1.33, $K_m$ = 0.12 mM.
Effect of various conditions on $K_a$ and Hill slope ($n_H$) for P-enolpyruvate saturation kinetics

Yeast pyruvate kinase was assayed as previously described (10) at 5 mM ADP, 17 mM MgCl$_2$, 0.23 mM KCl, varying P-enolpyruvate, 0.05 mM Tris-HCl, pH 7.5, with enzyme diluted and stored at 23°C in 0.23 mM KCl, 25 mM MgCl$_2$, 50 mM dithiothreitol, and 0.1 mM Tris-HCl, pH 7.5.

| Additive      | Temperature of assay | $K_a$ | $n_H$ |
|---------------|----------------------|-------|-------|
| None          | 30°C                 | 1.12  | 3.10  |
| None          | 0°C                  | 0.13  | 1.28  |
| Fructose-1,6-di-P | 30°C             | 0.13  | 0.94  |
| Glycerol 33%  | 30°C                 | 0.27a | 2.08  |
| (CH$_3$)$_2$SO | 30°C                 | 0.30b | 2.13  |

* Average of three determinations.

a Average of four determinations.

Further, inclusion of fructose-1,6-di-P in the assay at 0°C has no effect on kinetics.

Although the concentrations of glycerol and (CH$_3$)$_2$SO used in the kinetic studies gave similar activation ratios as fructose-1,6-di-P alone at 0.4 mM P-enolpyruvate, the same kinetic parameters for P-enolpyruvate kinetics were not observed in glycerol and (CH$_3$)$_2$SO as with fructose-1,6-di-P. The $n_H$ values were decreased slightly but cooperativity was not abolished. However, the $K_a$ for P-enolpyruvate was lowered significantly when assayed in 33% glycerol and 20% dimethyl sulfoxide.

**DISCUSSION**

Generally, the cryoprotective solvents tested in this study produced the same qualitative effects as the normal physiological activator fructose-1,6-di-P (10, 13) on the catalytic properties of yeast pyruvate kinase (Table I). Further an interaction between the activation by fructose-1,6-di-P and the cryoprotectant was observed since a maximum in the activation is observed at lower concentrations when fructose-1,6-di-P is included in the binary solvent. It is noteworthy that the extent of activation observed was greatly dependent on P-enolpyruvate concentration. In 20% (CH$_3$)$_2$SO, for example, activation ratios as used in this study. In this case, addition of the cryoprotectants allowed the formation of an enzyme configuration such as pyruvate kinase, which are catalytically sensitive to allosteric effectors, might then be expected to display altered catalytic properties when assayed under other solvating conditions as used in this study. In this case, addition of the cryoprotectants allowed the formation of an enzyme configuration with a greater affinity for P-enolpyruvate; cooperativity in the P-enolpyruvate saturation kinetics was still observed. The decrease in the activation factor as the concentration of the cryoprotectant is increased beyond the 33% glycerol (0.12-mole fraction), 20% (CH$_3$)$_2$SO (0.06-mole fraction) or 14% (CH$_3$)$_2$PO (0.03-mole fraction) may be due to a "falling apart" of the structure, a term previously suggested by Arnett and McKelvy (26).

Evidence for alterations in the structure and activity of water due to the addition of hydrogen-binding solvents has been observed not only by infrared spectroscopy and freezing point depression, but also by examination of a wide range of other physical processes such as solvation of ions and nonelectrolytes, hydrolytic reactions, oxidation-reduction reactions, and ionization of weak electrolytes (23, 24). Consideration of these observations in light of the structured and nonstructured two-state model for water structure (25) suggests that addition of 0.1- to 0.2-mole fraction of glycerol, (CH$_3$)$_2$SO or (CH$_3$)$_2$PO, or lowering the temperature of water are perturbations that participate in the water structure-making process. Enzymes, such as pyruvate kinase, which are catalytically sensitive to allosteric effectors, might then be expected to display altered catalytic properties when assayed under other solvating conditions as used in this study. In this case, addition of the cryoprotectants allowed the formation of an enzyme configuration with a greater affinity for P-enolpyruvate; cooperativity in the P-enolpyruvate saturation kinetics was still observed. The decrease in the activation factor as the concentration of the cryoprotectant is increased beyond the 33% glycerol (0.12-mole fraction), 20% (CH$_3$)$_2$SO (0.06-mole fraction) or 14% (CH$_3$)$_2$PO (0.03-mole fraction) may be due to a "falling apart" of the structure, a term previously suggested by Arnett and McKelvy (26).

Material that free amino groups are important in the association phenomenon (18). Another perturbation in the balance of forces dictating protein structure may result from an interaction of these compounds with the solvent water, per se. For example, polyvinylpyrrolidone, an effective cryoprotectant, was shown to enhance the structure of water as evidenced by infrared spectroscopy (19) and to deviate considerably from ideality in freezing point depression experiments, i.e. a much greater freezing point depression of water is observed than one would expect from its osmolarity (20, 21). Physiologically analogous compounds, glycoproteins of molecular weight between 10,000 and 20,000 isolated and characterized by DeVries, Komatsu, and Feeny (22) from antarctic fishes, also depress the freezing point of water much more than one would calculate from the number of particles in solution. This excess freezing point depression activity which was destroyed by acetylation of three amino groups per 10,000 g of protein or by cleavage of a few peptide bonds was suggested to be due to its expanded structure and the presumed immobilization of water (22).
to explain the position and shape of peaks in plots of heats of solution of salts versus mole fraction of water in a binary water alcohol solution. A reversible disruption of the protein structure at these solvent concentrations would be a reasonable alternative. An irreversible loss in activity cannot account for the decreased activity since storage of yeast pyruvate kinase in 30% (CH₃)₂SO or 50% glycerol does not result in irreversible denaturation. Thus, as previously stated by Yielding (16), compounds such as glycerol and dimethyl sulfoxide may prove useful in studying enzyme solution properties and structure transitions.

Lowering the temperature to 0°C allows the formation of an enzyme configuration with a greater affinity for P-enolpyruvate; in this case, cooperativity in the P-enolpyruvate saturation kinetics is abolished. Somero (27) also observed a decreased affinity for P-enolpyruvate and loss of cooperativity in the pyruvate kinase measured by fluorescence quenching in the presence of K⁺ and Mg²⁺ at 0°C (KD = 0.36 mM) is also substantially smaller than that measured at 30°C (KD = 3.3 mM) (28). Cooperativity in the binding of fructose-1,6-diphosphate to yeast pyruvate kinase from Alaskan King Crab at 0°C (KD = 0.86 mM) is essentially abolished. Somero (27) also observed an increased affinity for P-enolpyruvate and loss of cooperativity in the pyruvate kinase measured by fluorescence quenching in the presence of K⁺ and Mg²⁺ at 0°C (KD = 0.36 mM) is also substantially smaller than that measured at 30°C (KD = 3.3 mM) (28). Cooperativity in the binding of fructose-1,6-diphosphate was also abolished at 0°C. Whether this loss in cooperativity observed at 0°C is due to a change in the solvent structure or in some property of the protein which may be important in mechanisms of allosteric regulation or both is still an open question.

It is tempting to suggest, moreover, that these same solvent effects are physiologically significant. For instance, the ability of certain insects to accumulate glycerol in their bodies when subjected to low temperature stress is now well established. In hibernating adults of the order Hymenoptera, glycerol accumulation can be induced at any time of the year simply by temperature change (29). Since the cold-induced glycerol accumulation was also observed in fourteen species of ichneumon wasps (29), the data are consistent with the suggestion that glycerol accumulation in vivo plays an important role in the overwintering process of this group of insects. Furthermore, the abolition of cooperativity in yeast pyruvate kinase at low temperatures produces a greater affinity for substrate, resulting in greater activity at substrate levels of substrate than would otherwise be possible. In this manner, an adapting organism may partially compensate for the decrease in enzyme activity which accompanies a temperature drop. In addition, as pointed out in the introduction, these binary solvents have provided a satisfactory medium for low temperature storage of many tissues (11, 17, 30).

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