

# Effect of Temperature and Effectors on the Conformations of Yeast Pyruvate Kinase\*

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**ABSTRACT:** Yeast pyruvate kinase has been shown to be susceptible to inactivation at low temperatures. Addition in micromolar amounts of the allosteric activator, fructose 1,6-diphosphate (FDP), markedly enhances, by as much as 1000-fold, the rate of loss in activity both in the cold, and at room temperature. Addition of  $Mg^{2+}$  or  $Mn^{2+}$  prevents inactivation. A biphasic inactivation results, with the first step dependent on the half-power of the enzyme concentration. This is ac-

companied by a decrease in the sedimentation coefficient of the enzyme from 8.6 to 3 S followed by a slower decrease to 1.7 S. The data are consistent with the binding of a minimum of two molecules of FDP per molecule of enzyme with a geometric average dissociation constant of  $63 \mu M$ . A mechanism is proposed which involves the binding of FDP, followed by dissociation of the enzyme into subunits during inactivation.

The kinetic properties of homogeneous yeast pyruvate kinase (EC 2.7.1.40) have been partially elucidated, after the successful purification of a stable enzyme preparation (Haeckel *et al.*, 1968; Hunsley and Suelter, 1969a). The significant differences between the kinetic properties of this enzyme and the enzyme from rabbit muscle (Reynard *et al.*, 1961) stimulated us to examine the physical characteristics of the former. Our intention was to search for similarities or differences between these enzymes to gain insight into the mechanism of enzyme action.

We recently discovered that the yeast enzyme, which exhibits a cold lability, was markedly destabilized by the allosteric activator, fructose 1,6-diphosphate in the absence of the monovalent and divalent cations required for catalytic activity. This paper reports our efforts to elucidate the mechanism of this inactivation, with the hope that the results would lead to a clearer insight concerning the relationship of protein structure to the effects on enzyme catalysis by allosteric activators.

## Materials and Methods

Pyruvate kinase was isolated from fresh "Budweiser" bakers' yeast (Anheuser-Busch, Inc.) according to the procedure of Hunsley and Suelter (1969a) and stored as a suspension in 90% saturated (3.64 M)  $(NH_4)_2SO_4$ . Prior to use the enzyme was chromatographed at room temperature on a column of Sephadex G-25 (coarse,  $0.8 \times 11.0$  cm). Aliquots of the Sephadex-chromatographed enzyme were tested with  $BaCl_2$  to ensure them free of ammonium sulfate. All experiments were performed in 0.1 M Tris·HCl (pH 7.5) at the indicated temperatures.

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Protein concentrations were estimated using an  $E_{1\text{cm}}^{0.1\%}$  value of 0.653 at 280 nm (Hunsley and Suelter, 1969a). Kinetic assays were performed at 30° by employing a linked lactic dehydrogenase assay in the presence of FDP<sup>1</sup> under the conditions described by Hunsley and Suelter (1969b). All enzyme used had a minimum specific activity of 210  $\mu$ moles/min per mg. FDP concentrations were determined in the presence of excess aldolase by the method of Rutter *et al.* (1966). Ammonium sulfate suspensions of rabbit muscle lactic dehydrogenase and aldolase were obtained from Sigma Chemical Co. Tricyclohexylammonium-PEP, NaADP, and tetracyclohexylammonium-FDP were also Sigma products. All other chemicals were reagent grade.

Sedimentation velocity experiments were performed with a Beckman-Spinco Model E analytical ultracentrifuge equipped with phase-plate schlieren optics, and sedimentation coefficients were calculated using the method described by Schachman (1957) and corrected to 20° and water.

## Results

Figure 1 shows the effect of temperature on the stability of yeast PK at 0.5 mg/ml. Over a period of 72 hr less than 10% of the activity was lost at 23° whereas at 0° over 95% of the activity was lost over the same time interval. Each point represents an assay of an aliquot of enzyme diluted directly into the assay mix at 30°. Assays were linear over the 1–3-min observation period.

Before each stability study, but after treatment with Sephadex, the protein was allowed to stand at 23° for 3 hr. Little or no change in specific activity could be observed over this time period. This preincubation step was introduced in this and all future experiments to eliminate the unusual results obtained without preincubation (Figure 2). Preincubation of the enzyme at 23° for 3 hr eliminates the initial activation observed at 0.05 mg/ml (lower curves) but not at 0.5

<sup>1</sup> Abbreviations used are: FDP, fructose 1,6-diphosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde 3-phosphate.

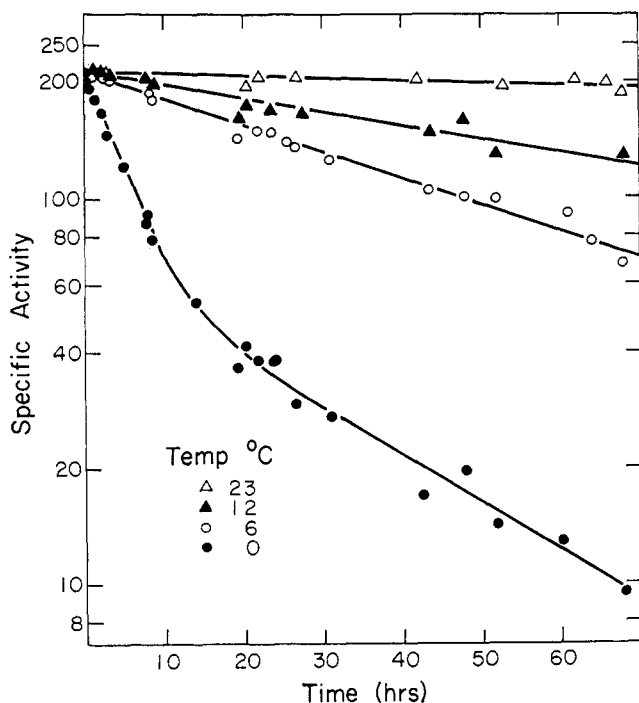


FIGURE 1: Effect of temperature on the stability of yeast pyruvate kinase in 0.1 M Tris·HCl (pH 7.5). Protein concentration, 0.50 mg/ml. Each point represents an assay at 30° of an aliquot of enzyme removed from the incubation mixture. Enzyme was assayed employing a linked lactic dehydrogenase assay in the presence of FDP under the conditions described by Hunsley and Suelter (1969b). Enzyme was chromatographed on Sephadex to remove monovalent cations.

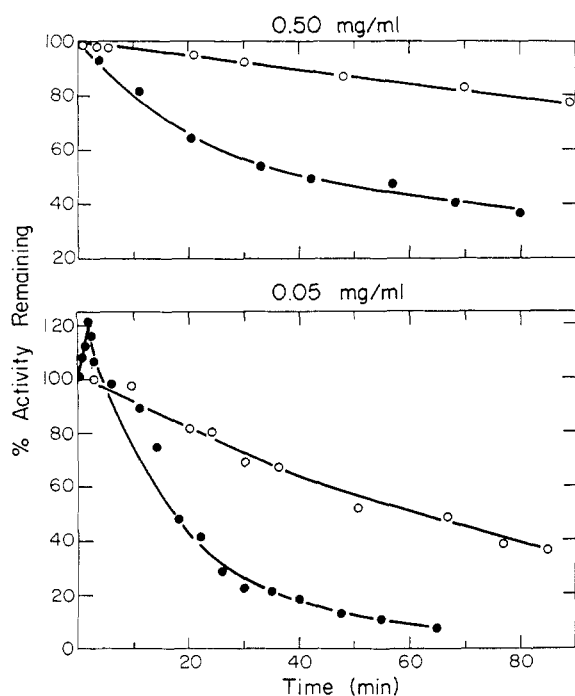


FIGURE 2: Effect of preincubation at 23° on the stability of yeast pyruvate kinase at 0°. (●) Enzyme Sephadexed into 0.1 M Tris·HCl (pH 7.5) at 23°, then immediately diluted to the indicated protein concentration at 0°. (○) Enzyme Sephadexed and allowed to stand for 3 hr at 23°, then diluted to the final concentration at 0°. Assay conditions were as indicated in Figure 1.

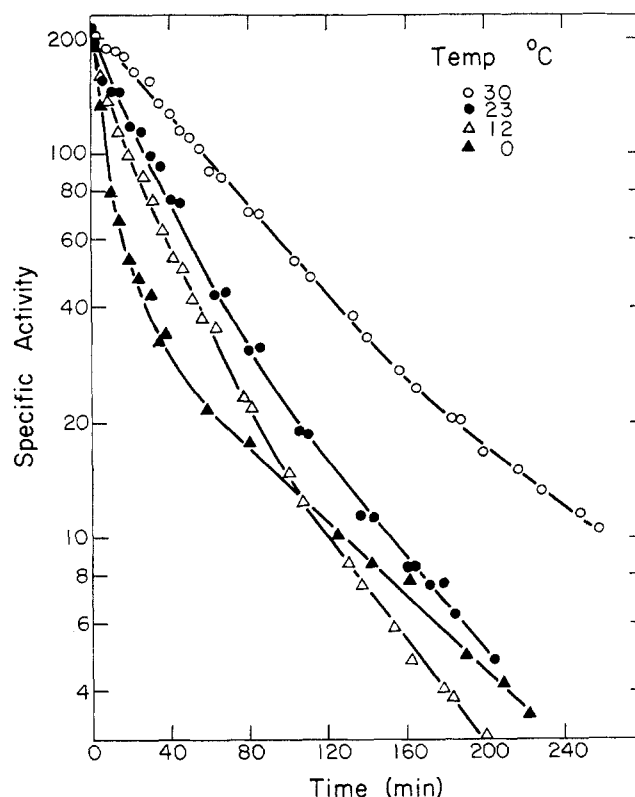


FIGURE 3: Effect of temperature on the FDP-enhanced inactivation of yeast pyruvate kinase. All samples were at 0.50 mg/ml. FDP concentration was  $1.26 \times 10^{-3}$  M. See Figure 1 for conditions and assay.

mg/ml (upper curves). Both samples were eluted from the Sephadex column at about 0.6 mg/ml. The activation observed at low protein concentration was qualitatively reproducible, and was not observed above 0.10 mg/ml. The activation was observed when dilution to 0.05 mg/ml was made at 23 or 0° either from a Sephadex-chromatographed stock solution or from the ammonium sulfate precipitate, and either into a glass or polypropylene reaction vessel. The 23° dilution, however, resulted in a higher activation over a much shorter period of time.

To determine whether any changes in the sedimentation pattern of the enzyme could be observed as a result of the preincubation period, aliquots of the ammonium sulfate precipitate were treated in two ways. One was chromatographed over Sephadex into Tris buffer, diluted to 6.0 mg/ml, and allowed to stand at room temperature for 3 hr. The second was centrifuged in a preparative centrifuge for 10 min at 0°, the precipitate was collected, and at the end of the 3-hr preincubation period for the former sample, was dissolved in 0.1 M Tris buffer. Both samples were immediately centrifuged in the analytical ultracentrifuge at 20°. The sample preincubated after treatment with Sephadex sedimented as a single symmetrical peak (8.0 S), while the other contained three peaks with approximate *s* values of 7.7, 11.5, and 13.6 S, in a ratio of approximately 4:3:3.

**FDP Inactivation.** A search for compounds which might stabilize the enzyme against cold inactivation revealed that fructose 1,6-diphosphate, an allosteric activator of yeast

pyruvate kinase, markedly enhanced the low-temperature inactivation. Figure 3 shows the effect of millimolar concentrations of FDP on this inactivation. The data in Figures 1 and 3 indicate that the rate constant for inactivation is increased as much as 1000-fold over the sample at 23°. To insure that this effect was produced by FDP, PK was incubated at 0° at 1.0 mg/ml under the conditions shown in Table I.

TABLE I: Effect of FDP on Inactivation of Yeast Pyruvate Kinase.<sup>a</sup>

Sample	Addn to a Final Vol of 0.1 ml	Enhanced Inactivn <sup>b</sup>
1	None	—
2	1.1 μg of aldolase	—
3	132 μmoles of DHAP 120 μmoles of G-3-P	—
4	132 μmoles of DHAP 120 μmoles of G-3-P 1.1 μg of aldolase	+
5	126 μmoles of FDP 1.1 μg of aldolase	+
6	126 μmoles of FDP	+

<sup>a</sup> Each sample contained yeast pyruvate kinase at 1.0 mg/ml. Samples were incubated at 0°, and activity was followed with time. <sup>b</sup> (—) indicates that the resultant inactivation curve resembled the 0° curve of Figure 1. (+) indicates that inactivation resembled the 0° curve in Figure 3, both in rate of inactivation, and in extent.

Essentially, two rates of inactivation were obtained: that resembling the 0° curve of Figure 1, (samples 1, 2, and 3), and that resembling the 0° curve of Figure 3 (samples 4, 5, and 6). The inactivation was not produced by metal contaminants of the FDP such as copper (Passeron *et al.*, 1967) since FDP treated with Chelex and untreated FDP produced identical results.<sup>2</sup>

Because FDP acts as a kinetic activator in the presence of Mg<sup>2+</sup> and K<sup>+</sup>, efforts were made to characterize the mechanism by which FDP, in the absence of cations, promoted inactivation of yeast PK. Figure 4 presents the effects of increasing concentrations of FDP on inactivation of pyruvate kinase at 0.5 mg/ml. The curves obtained from the semi-logarithmic plot are biphasic, indicative of two pseudo-first-order inactivation steps. Under this assumption, the data shown in Figure 4 were treated as in Figure 5. Extrapolation of the data of the slow step to zero time yielded intercepts which varied from specific activity 210–39 μmoles/min per mg as FDP concentration was increased from 0 to 1.26 mM. Identical intercepts were obtained for 0.756 and 1.26 mM FDP concentrations, suggesting that the latter concentration was saturating. The difference between the intercept at

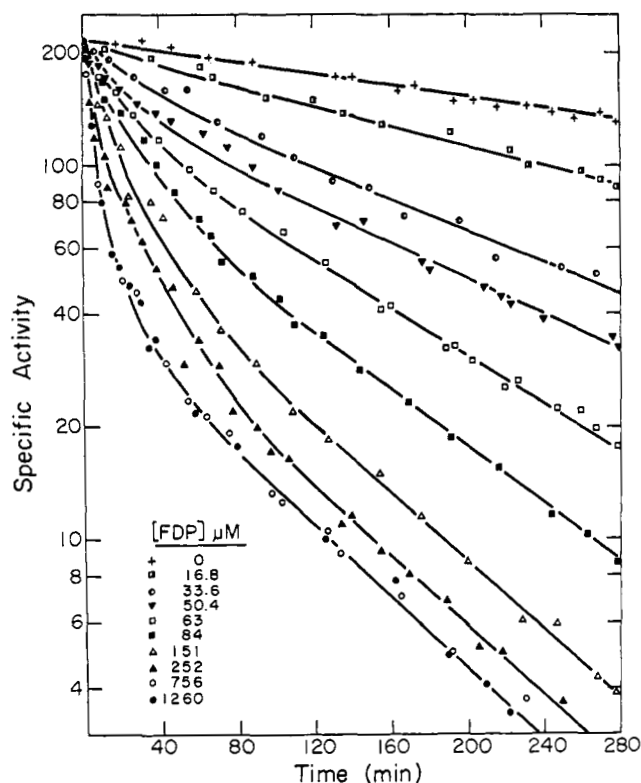


FIGURE 4: Effect of FDP concentration on the stability of yeast pyruvate kinase at 0° in 0.1 M Tris·HCl (pH 7.5). Final protein concentration was 0.50 mg/ml. All samples had an initial specific activity of 214 units/mg. Each point represents an assay of an aliquot removed from the inactivation mixture and diluted directly into the cuvet as in Figure 1.

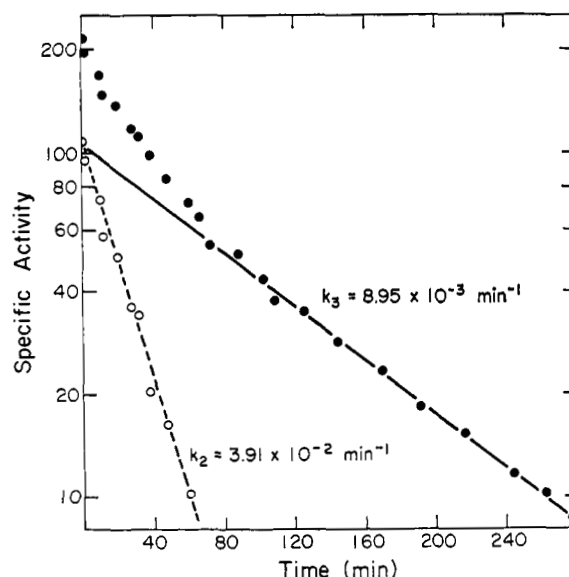


FIGURE 5: Example of the method of treatment of the data from Figure 4. The sample above was inactivated in the presence of 84 μM FDP in 0.1 M Tris·HCl (pH 7.5) at 0.5 mg/ml of pyruvate kinase. Inactivation was allowed to continue until the slow process, labeled  $k_3$ , became linear. This rate was extrapolated to zero time, and specific activity values along this line were subtracted from the experimental points (●). (○) represents the differences so obtained.

<sup>2</sup> Assistance of Mr. George Lorimer in performing these experiments is gratefully acknowledged.

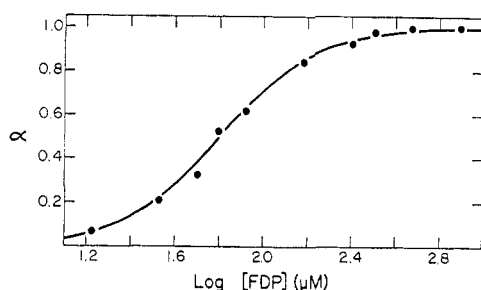


FIGURE 6: Plot of  $\alpha$ , defined in text in eq 1 vs. log FDP. The solid line is calculated assuming  $n = 2$  and  $K_D = 63 \mu\text{M}$ .

saturation concentration of FDP and that obtained in the absence of FDP was equated to unity and  $\alpha$  values, defined as

$$\alpha = \frac{(\text{intercept})_{0[\text{FDP}]} - (\text{intercept})_{X[\text{FDP}]}}{(\text{intercept})_{0[\text{FDP}]} - (\text{intercept})_{1.26 \text{ mM FDP}}} \quad (1a)$$

were calculated for each FDP concentration and plotted vs. log FDP in Figure 6. If the binding is represented by the successive equilibria

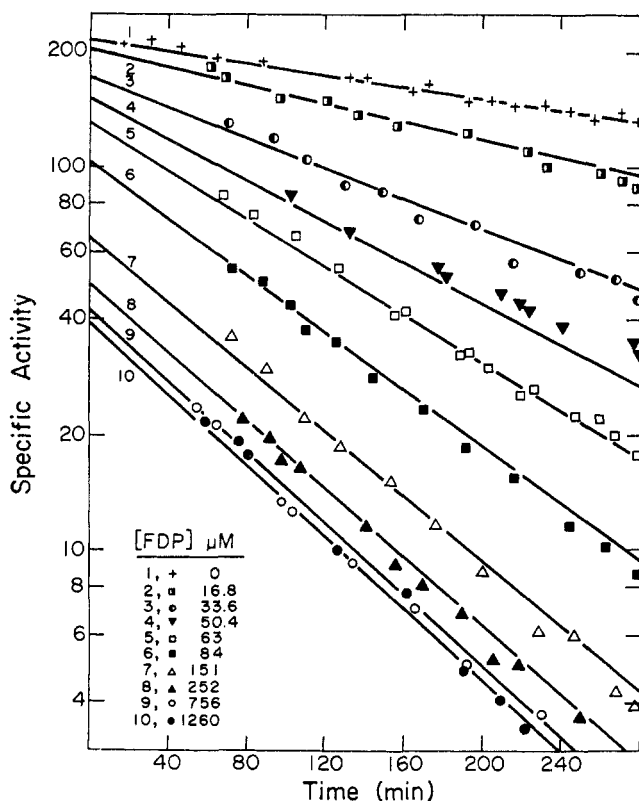


FIGURE 7: Comparison of experimental data with theoretical determinations of the zero time intercept and calculated rate constants. Points are taken from the final half of Figure 4. Intercepts at zero time were calculated using experimental FDP concentrations, and the theoretical curve in Figure 6. Solid lines were drawn using calculated rate constants as described in the text using eq 2.

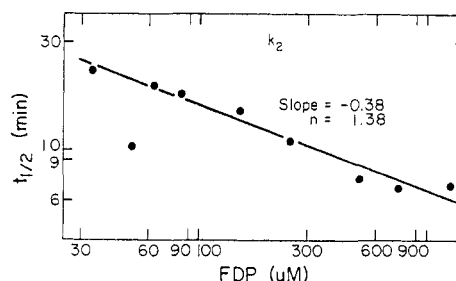


FIGURE 8: Determination of the kinetic interaction constant between FDP and protein using eq 3. The line is a least-squares plot, eliminating the point at  $50.4 \mu\text{M}$  FDP. Details in Results.



and if  $K_I > K_{II} > K_n$ , then

$$pK_I + pK_{II} \dots + pK_n - np[\text{FDP}] = \log \frac{\alpha}{1 - \alpha}$$

or

$$n(pK_{av} - p[\text{FDP}]) = \log \frac{\alpha}{1 - \alpha} \quad (1b)$$

where E is enzyme, and  $\alpha$ , defined in eq 1a, represents the fraction of enzyme-FDP complex. The theoretical line in Figure 6 was calculated assuming  $n = 2$  and the geometrical average  $K_D = 63 \mu\text{M}$ . For similar treatments of data, see Suelter *et al.* (1966) and references therein.

If the variation in extent of the first reaction with increasing FDP concentrations reflects an equilibrium of enzyme with FDP, and if the fraction of enzyme reacting with FDP is equal to  $\alpha$ , the remaining unreacted enzyme would become inactivated at the rate observed in the absence of FDP (defined as  $k_1$  and obtained from Figure 4 at zero FDP concentration). Based on this assumption, theoretical  $k_T$ 's (slow step) were calculated for each FDP concentration with the use of

$$e^{-k_T} = \alpha e^{-k_3} + (1 - \alpha)e^{-k_1} \quad (2)$$

where  $k_T$  represents the sum of the rates of slow inactivation,  $k_1 + k_3$ ,  $\alpha$  is the theoretical fraction of enzyme-FDP complex derived from the solid line in Figure 6,  $1 - \alpha$  is the fraction of free enzyme,  $k_3$  is the rate constant of inactivation for the enzyme-FDP complex, and  $k_1$  represents the rate constant of inactivation of the free enzyme. The theoretical inactivation curves obtained in this manner are plotted as solid lines in Figure 7, along with the experimental points for each FDP concentration as obtained from Figure 4. The theoretical intercepts for 0.756 and 1.26 mM FDP (Figure 7) reflect 98.2 and 99.7% saturation of enzyme with FDP and thus are not distinguishable in the experimental data.

The rate constants for the fast inactivation,  $k_3$ , were treated by the half-life method (Frost and Pearson, 1961) to obtain

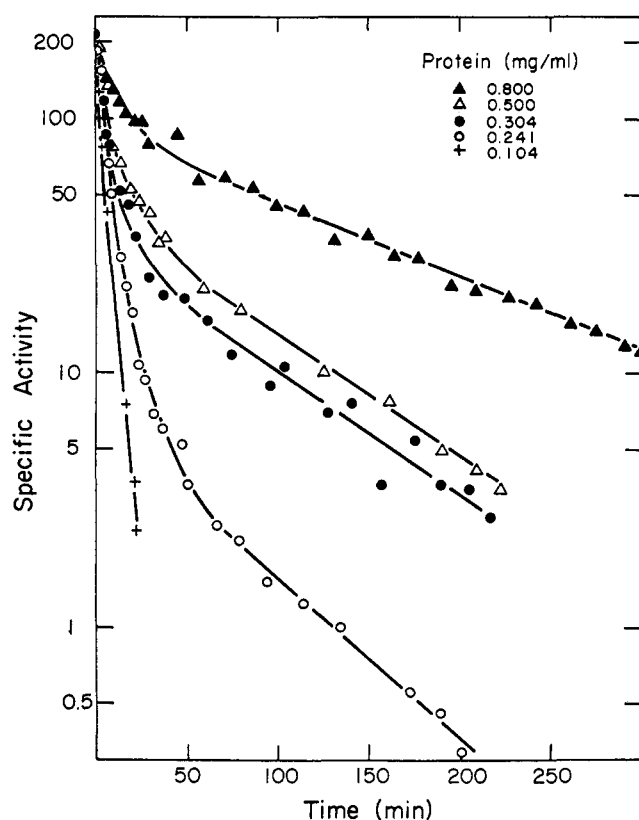


FIGURE 9: Effect of protein concentration on the FDP-enhanced inactivation of yeast pyruvate kinase at 0° in 0.1 M Tris·HCl (pH 7.5). FDP concentration in all cases was  $1.26 \times 10^{-3}$  M.

the order of the reaction with respect to FDP. For any rate expression of the type  $dx/dt = k(a - x)^n$ , the half-life may be defined for all values of  $n$  as  $t_{1/2} = f(n, k)/a^{n-1}$ , where  $f$  is some function of  $n$  and  $k$ , and  $a$  is defined as the initial concentration of the reactant. Placing this equation in logarithmic form yields

$$\log t_{1/2} = \log f - (n - 1) \log a \quad (3)$$

A log-log plot of  $t_{1/2}$  vs.  $a$  should yield a straight line with slope  $(1 - n)$ . Figure 8 is a log-log plot of the  $t_{1/2}$ 's obtained from the experimental  $k_2$ 's vs. FDP concentration from which a value for  $n = 1.4$  was calculated.

FDP inactivation was then examined as a function of five different protein concentrations (Figure 9), each in the presence of 1.26 mM FDP. Again inactivation was biphasic except at very low protein concentrations, where a single-step inactivation was obtained to the extent that the inactivation could be followed. Log-log plots of  $t_{1/2}$  vs. protein concentration are shown in Figure 10a for  $k_2$ 's (fast rate) and in Figure 10b for  $k_3$ 's (slow rate). It should be noted at this point that 1.0 mg/ml of bovine serum albumin in the 0° reaction vessel containing PK at 0.20 mg/ml and FDP at 1.26 mM had no effect on either  $k_2$  or  $k_3$ .

The effect of FDP on the sedimentation coefficient of yeast PK was also examined at 0.8 mg/ml after incubation in the presence or absence of 1.26 mM FDP all at 3.6°. Aliquots of protein were removed from the incubation mixtures before

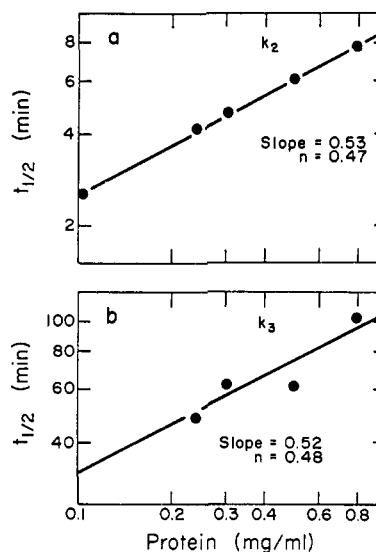


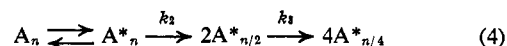
FIGURE 10: Determination of the order with respect to protein of the FDP-enhanced inactivation of yeast pyruvate kinase at 0°. Both lines are least-squares plots. Rate constants were determined from the data in Figure 9, as described in Figure 5. The contribution to the rate of inactivation by  $k_3$  at 0.104 mg/ml was assumed to be negligible, and the  $k_2$  in part a at this concentration was assumed to be the initial rate of inactivation.

loading the centrifuge cell and maintained at 3.6° to be assayed. Activity remaining was determined at the time that the first picture of the sedimenting species was taken. Enzyme minus FDP (95% activity) sedimented as a single peak (8.6 S). In the presence of FDP, with 30% activity remaining, two peaks could be observed with  $s$  values of 3 and 8 S in a ratio of about 80:20. With less than 0.1% activity remaining, in the presence or absence of FDP, a single peak was observed with an  $s$  value of 1.7 S.

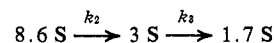
Finally, efforts to reverse the inactivation either by re-warming or by the addition of  $Mg^{2+}$  or glycerol have been unsuccessful. The addition of  $Mg^{2+}$  or  $Mn^{2+}$  to the PK-FDP system at any stage of inactivation, however, stabilizes the enzyme to further inactivation.

## Discussion

A schematic mechanism of inactivation consistent with the data is presented in eq 4.



The rapid ( $k_2$ ) and slow ( $k_3$ ) steps of inactivation involving dissociations of the protein into subunits noted in eq 4 are consistent with the half-power dependence of both steps on protein concentration (Figure 10a,b) and the sedimentation pattern of the enzyme at various stages of inactivation. The 3S



species represents either an unfolded  $A_{n/2}$  or a mixture of  $A_{n/2}$  and  $A_{n/4}$  resulting in an apparent  $s = 3.0$ , and the 1.7S species corresponds to an unfolded  $A_{n/4}$  produced by the slow inactivation,  $k_3$ .

The biphasic inactivation curve obtained at 0° in the presence or absence of FDP (Figure 1 and Figure 4) and the appearance of the 1.7S species after near complete cold inactivation also in the presence or absence of FDP suggests that similar mechanisms of inactivation are operative in both cases. In the presence of saturating FDP the rapid rate of inactivation,  $k_2$  (Figure 10a), is dependent on the one-half-power of protein concentration suggesting that protein dissociation is then the rate-limiting step. The data also suggest that FDP enhances the inactivation by promoting a shift in the equilibrium of  $A_n$  to  $A_n^*$ , and that a minimum of 2 moles of FDP bind/mole of enzyme in shifting this equilibrium. Wieker *et al.* (1969) have shown that 2 moles of FDP bind per mole of native enzyme consistent with this suggestion. The interaction constant of 2 for FDP (Figure 6) in defining the equilibrium following the rapid inactivation is also consistent with the above model. However, since the stoichiometry for binding of FDP under these conditions was not determined an alternative model in which 4 or more moles of FDP are bound per mole of enzyme to achieve the inactivation must be considered.

The kinetic order of 1.4 for interaction of FDP (Figure 8) suggests a complex series of steps involved in establishing the inactivation equilibrium. Yet, the interaction constant of 2 as defined by eq 1b suggests that the concentration of the intermediate complexes with FDP which might occur during the inactivation are insignificant after the equilibrium is established. Such a mechanism is analogous to the dissociation of a diprotic acid in which two protons dissociate with no detectable amount of an intermediate singly dissociated form in the equilibrium mixture (Suelter *et al.*, 1966, and references therein).

In the FDP-promoted inactivation,  $k_2$ , which depends on the half-power of protein concentration, would yield dimers ( $A_{n/2}$ ) and since  $k_T (= k_1 + k_3)$  (eq 2 and Figure 7) depends on the concentration of only two species, the data suggest that the dimer is partially active;  $k_3$  would then represent inactivation of dimer, presumably containing bound FDP while  $k_1$  would represent inactivation in the absence of FDP.

The fact that an allosteric activator of yeast PK promotes inactivation is interesting from the point of view of the mechanism of allosteric interactions. The temperature sensitivity of this alteration in structure is reminiscent of reports of other enzymes which are dissociated by activators or inhibitors. For example, D-fructose 1,6-diphosphatase is dissociated by the allosteric inhibitor, adenosine 5'-phosphate, only at 0° (Rosen *et al.*, 1967). Dissociation is prevented by the presence of substrate. Similarly, yeast glyceraldehyde 3-phosphate dehydrogenase is dissociated by ATP at 0°, but not at 20° (Stancel and Deal, 1968). Carbamoyl phosphate synthetase from rabbit muscle, which requires the presence of acetyl glutamate as cofactor, is reversibly dissociated by this ligand at low temperatures to an inactive form (Guthöhrlein and Knappe, 1968). This same ligand, at 30°, promotes an active, associated form of the enzyme.

A major question is whether FDP under the conditions described here, binds at the same site in the presence and absence of divalent cations. It seems unlikely that two distinct sites exist on the enzyme for FDP, one site promoting an activation and the second site promoting an inactivation. Protection by  $Mg^{2+}$  and  $Mn^{2+}$  *in vivo* would require extensive compartmentalization and/or regulation of the uptake of

these cations for the inactivating role of FDP to be metabolically operative. In addition, the role of FDP activation of PK as a regulatory mechanism for glycolysis makes the second-site alternative highly improbable. If the altered catalytic behavior and increased instability of this enzyme results from ligand binding at the same site, then binding of the divalent cation in conjunction with FDP could promote a conformational change leading to an activated enzyme form. Without divalent cation, the enzyme would respond "incorrectly" or incompletely to the bound FDP and dissociate.

Another alternative attributes a stabilizing function to the cation. This would explain the close agreement between the half-maximal concentrations of FDP required for the inactivation phenomenon ( $K_D = 63 \mu M$ ) and the catalytic effects ( $K_M = 26 \mu M$ ), and further suggests that a divalent cation is not required for FDP binding. Binding of FDP in the presence or absence of metal may produce identical structural effects on PK, but the divalent cation is required to stabilize the newly induced conformation. A combination of these alternatives seems most likely; *i.e.*, binding of FDP might induce only a partial conformational change to a highly unstable form of the enzyme which, in the presence of cation, would be completed to the fully active, stabilized form.

Reports of cold-labile enzymes are appearing with increasing frequency (for a partial review, see Jarabak *et al.*, 1966). Low-temperature instability of proteins indicate that associations between apolar groups, significantly weakened at low temperatures (Kauzmann, 1959; Scheraga *et al.*, 1962), are important in these proteins. The temperature dependence of inactivation both in the presence and absence of FDP suggests a first-step dissociation involving such apolar groups. In the presence and absence of FDP, the initial rates of inactivation,  $k_2$ , decrease as the temperature is raised. Although the data are insufficient to quantitate the effect of temperature on the equilibrium concentration of the dimer, examination of the shapes of the curves suggests that the concentration of the dimer decreases with increasing temperature but that the rate of dissociation of dimer,  $k_3$ , increases. This suggests a heterologous interaction between subunits, *i.e.*, hydrophobic forces predominating between the dimers, and electrostatic forces predominating between the subunits of the dimer.

The temperature dependence of inactivation in the absence of FDP suggests a break in the stability curves between 6 and 0° (Figure 1). Possibly the increased structuring of water, which occurs near 4°, may be influential in creating the sudden instability of the enzyme in this temperature region. Thermodynamic analyses of the interaction of apolar groups with water indicate a large unfavorable entropy loss as the temperature is lowered, which is attributed to a change in the structuring of water (Kauzmann, 1954). The "iceberg" concept of Frank and Evans (1945) is consistent with an ordering of water molecules around the apolar group which would be facilitated by low temperatures as the hydrophobic regions became more accessible to solvent. When inactivation studies are performed at 30°, precipitation of protein is observed in the early stages of the inactivation and continues to increase in amount with time. No precipitation is observed at 0°. This is also consistent with an exposure of apolar surfaces during inactivation, facilitating random aggregation of the inactive protein *via* these regions at 30° and would not be promoted by tight water structures at 0°. The participation of water in the

dissociation phenomenon was suggested by the observation (Hunsley and Suelter, 1969a) that 50% aqueous glycerol solutions completely stabilize the enzyme.

Kayne and Suelter (1968) have described conformational transitions for rabbit muscle pyruvate kinase as observed by ultraviolet difference spectra, changes in optical rotatory dispersion parameters, and changes in sedimentation velocity. Their data also showed structural changes in the protein at lowered temperatures. It would be of interest to know whether this temperature-dependent structural change is observed in other preparations of pyruvate kinase and whether or not such changes are important under physiological conditions.

The dissociation promoted by FDP is direct evidence that this ligand produces an alteration in the quaternary structure of yeast PK, and supports the contention that allosteric ligands affect conformational transitions from one enzyme form to another. Further, if the mechanism of inactivation of yeast PK is identical in the absence and in the presence of FDP, an interesting consequence results. One could conclude that the enzyme exists in solution in an equilibrium between two or more tetrameric forms, and that both forms exist in the absence of FDP. Inactivation in the absence of FDP would depend on the equilibrium distribution and addition of FDP would merely produce a shift in the equilibrium concentrations of the two forms.

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