Fructose 1,6-Diphosphate Enhanced Inactivation of Yeast Pyruvate Kinase at 23°. Evidence for a Stabilized Dimer Intermediate*

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ABSTRACT: Yeast pyruvate kinase, a cold-labile enzyme, which was shown to be further destabilized at 0° by the positive effector, fructose 1,6-diphosphate (FDP) [R. T. Kuczenski and C. H. Suelter (1970), *Biochemistry 9*, 939] is similarly affected by the same ligand at 23°. Although the overall mechanism of inactivation, involving binding of at least 2 moles of FDP, followed by a two-step dissociation of the native tetramer to inactive subunits appears similar at both temperatures, the data suggest that the mechanism of inactivation proceeds by two alternate routes of dissociation, depending on the temperature involved. The first step of inactivation at 23° involves formation of a dimer with $s_{20,w}$ = 4.2 S, with loss of half of the activity of native enzyme, followed by dissociation to inactive subunits. Both steps of the inactivation at 23° are dependent on the FDP concentration. However, above saturating concentrations of FDP and/or increased ionic strength the rate of the second step is markedly inhibited while the rate of the first step is not affected. This inhibition particularly by high concentration of FDP provides evidence for a stabilized dimer with one-half the specific activity of native enzyme. The effects of ionic strength and temperature on the inactivation of yeast pyruvate kinase are consistent with heterologous subunit interactions leading to different dimers at 0 and 23°.

We recently reported (Kuczenski and Suelter, 1970a) that the cold-labile yeast pyruvate kinase (EC 2.7.1.40) was markedly destabilized at 0° in the absence of K^+ and Mg^{2+} by micromolar amounts of the allosteric activator, fructose 1,6-diphosphate. The data suggested a mechanism of inactivation consistent with the binding of at least 2 moles of fructose 1,6-diphosphate, followed by a two-step dissociation of the enzyme into subunits. The inactivation was attributed to a significant weakening of hydrophobic bonding between subunits.

This paper describes the results of a similar study of the effect of fructose 1,6-diphosphate on the stability of yeast pyruvate kinase at 23°. Although the overall mechanism involving binding of FDP[1] and dissociation of the enzyme into subunits appears similar at both temperatures, the data suggest that the mechanism of inactivation from native tetramer to inactive monomer proceeds by two alternate pathways depending on the temperature involved.

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) $(NH_4)_3SO_4$. Prior to use, the enzyme was chromatographed at room temperature on a column of Sephadex G-25 (coarse) equilibrated with 0.1 M Tris·HCl (pH 7.5) unless otherwise indicated. Aliquots of the chromatographed enzyme were tested with saturated $BaCl_2$ to ensure them free of ammonium sulfate. The enzyme was then allowed to stand for 3 hr at 23° before initiation of stability studies (Kuczenski and Suelter, 1970a). Little or no change in specific activity could be observed over this time period.

Protein concentrations were estimated from absorbance at 280 nm ($E_{1\,cm}^{0.1\%}$ 0.653) (Hunsley and Suelter, 1969a). Kinetic assays were performed at 30° with a linked lactic dehydrogenase assay in the presence of FDP under the conditions of saturating substrates described by Hunsley and Suelter (1969b). All enzyme had a minimum specific activity of 210 μmole min^{-1} mg^{-1}. Crystalline rabbit muscle aldolase, lactic dehydrogenase, FDP, PEP, (FDP and PEP were always added as the cyclohexylammonium salts), and NaADP were from Sigma. Rabbit muscle α-glycerophosphate dehydrogenase–triose phosphate isomerase mixed crystals was a Calbiochem product. $(CH_3)_4NCl$ from Aldrich was recrystallized from absolute ethanol and passed over a Chelex-100 in the Tris form to remove contaminating heavy metals. FDP concentrations were determined in the presence of excess aldolase using a modified assay of Rutter *et al.* (1966).

Sedimentation velocity experiments were performed with a Beckman-Spinco Model E analytical ultracentrifuge equipped with phase-plate schlieren optics. Single-sector, capillary-type synthetic boundary centerpieces were employed to facilitate measurements on the slower moving species. Sedimentation coefficients were calculated using the method described by Schachman (1957) and corrected to 20° and water.

Results

Although yeast PK is considerably more stable at 23° than at 0° in the absence of FDP (half-lives differ by nearly an order of magnitude) (Kuczenski and Suelter, 1970a), the addition of FDP to the enzyme in saturating amounts induces

* From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823. *Received February 22, 1971.* Supported in part by Grants GB-7780 and 25116, National Science Foundation, and the Michigan State Agricultural Experiment Station, Michigan State Journal No. 5150.

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[1] Abbreviations used are: FDP, fructose 1,6-diphosphate; CHA, cyclohexylammonium cation; PK, pyruvate kinase; $\langle K_D \rangle_{g\,av}$, geometrical average dissociation constant.

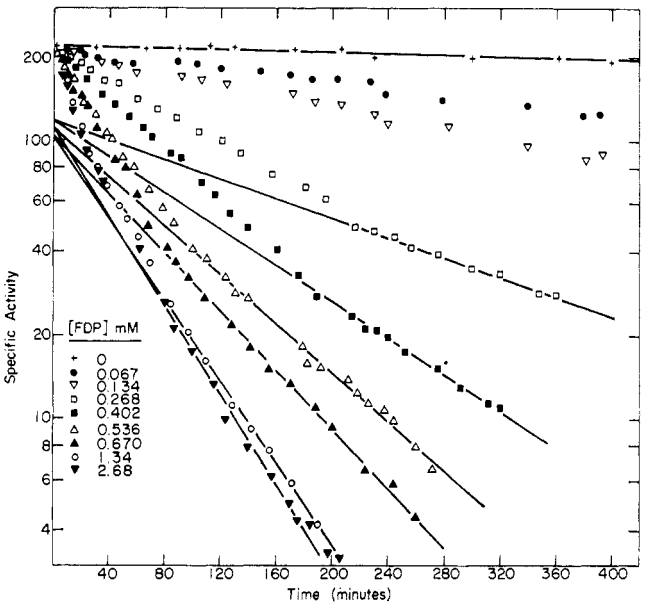

FIGURE 1: Effect of FDP concentration on the stability of yeast pyruvate kinase at 23° in 0.1 M Tris·HCl (pH 7.5). Protein concentration was 0.25 mg/ml. All samples had an initial specific activity of 211 units/mg. The enzyme had been previously chromatographed on Sephadex to remove monovalent cations. Each point represents an assay at 30° of an aliquot removed from the incubation mixture. Enzyme was assayed employing a linked lactic dehydrogenase assay in the presence of FDP (Hunsley and Suelter, 1969b).

an essentially identical lack of stability at the two temperatures. Figure 1 shows the effect of increasing FDP concentration from 0 to 2.68 mM on the stability of PK (0.25 mg/ml) at 23°. 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. The curves obtained from the semilogarithmic plot of specific activity *vs.* time are biphasic, indicative of two pseudo-first-order inactivation steps. Under this assumption, the data shown in Figure 1 were treated as in Figure 2. Extrapolation of the data of the slow step (k_3) to zero time yields essentially identical intercepts of specific activity 110.7 ± 6.5 units/mg representing 52% of the initial activity. As will be shown, increasing the FDP concentration above 2.68 mM has no further effect on the fast step (k_2), suggesting that this concentration of FDP was saturating. The k_2's obtained from Figure 1 were treated according to eq 1, where k_1 represents the observed rate of inactivation

$$\alpha = \frac{k_{2(X\,[\mathrm{FDP}])} - k_1}{k_{2(2.68\ \mathrm{mM\ FDP})} - k_1} \qquad (1)$$

in the absence of FDP (Figure 1); α values were plotted *vs.* log FDP in Figure 3. The theoretical line in Figure 3 was calculated assuming $n = 2$ and the geometrical average dissociation constant $\langle K_D \rangle_{g\ av} = 0.66$ mM (Kuczenski and Suelter, 1970a, and references therein). The rates for the slow

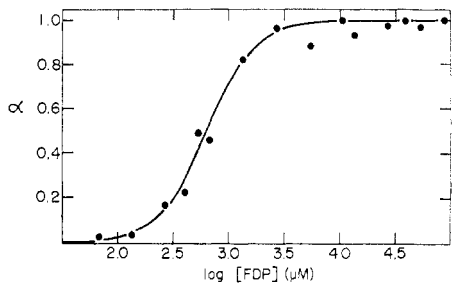

FIGURE 3: Plot of α, determined for the fast step (k_2) in Figure 1 and Figure 7 and defined in the text in eq 1, *vs.* log FDP. The solid line is calculated assuming $n = 2$ and $K_D = 0.66$ mM. The contribution to the rate of inactivation by k_3 at 0.067 and 0.134 mM FDP was assumed to be negligible, and the k_2 at this concentration was assumed to be the initial rate of inactivation.

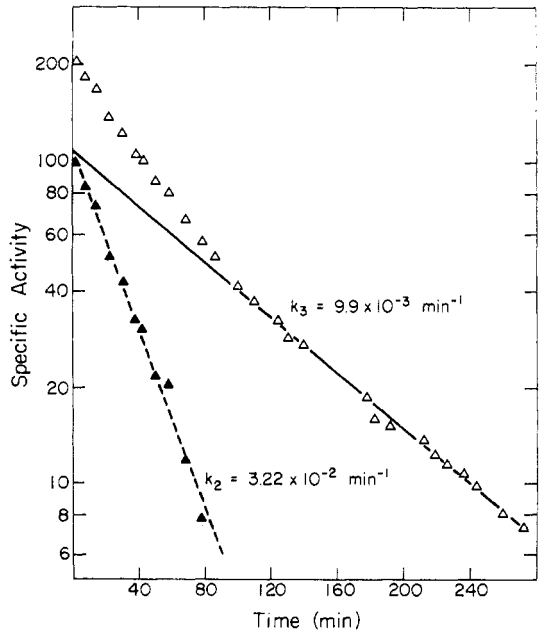

FIGURE 2: Example of the method of treatment of data from Figure 1. The sample above was inactivated in the presence of 0.536 mM FDP in 0.1 M Tris·HCl (pH 7.5) at 0.25 mg/ml of yeast pyruvate kinase. Inactivation was allowed to continue until the slow process, labeled k_3, became linear. This rate was extrapolated to zero time, and specific activity values along this line were substrated from the experimental points to obtain k_2.

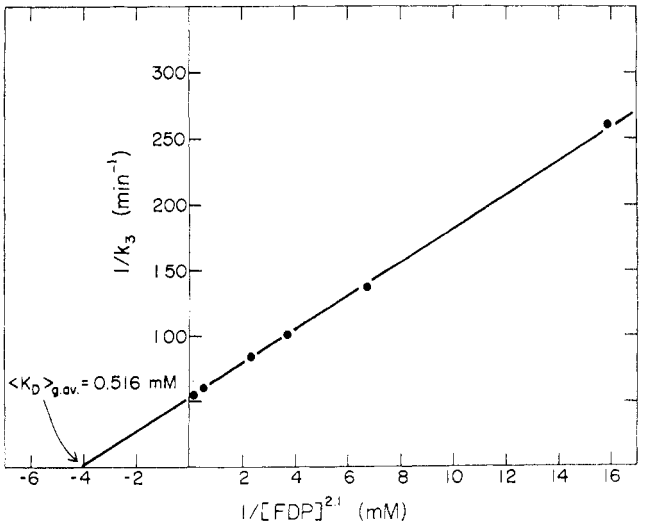

FIGURE 4: Plot of the rate of the slow step from Figure 1 as $1/k_3$ *vs.* $1/[\mathrm{FDP}]^{2.1}$. The apparent dissociation constant for FDP is 0.516 mM, and $k_{3,\max} = 1.9 \times 10^{-2}\ \mathrm{min}^{-1}$.

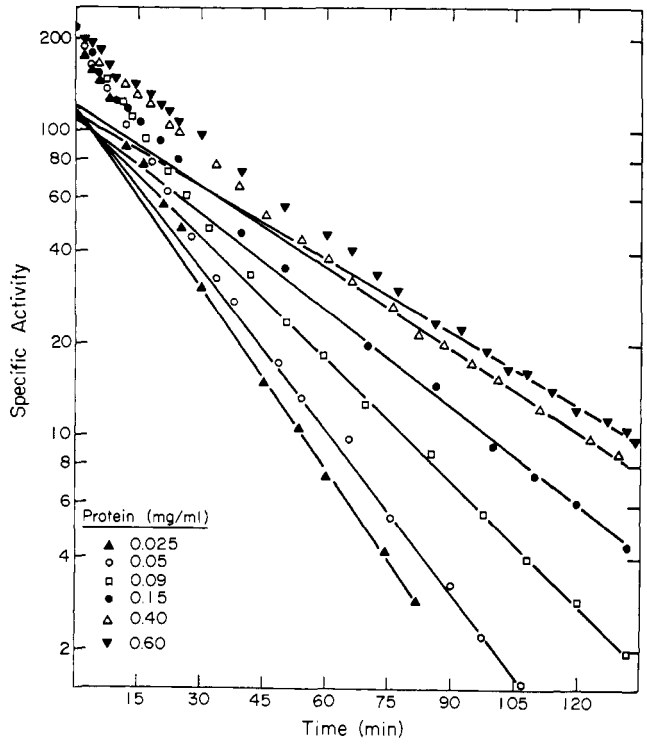

FIGURE 5: Effect of protein concentration on the FDP-enhanced inactivation of yeast pyruvate kinase at 23° in 0.1 M Tris·HCl (pH 7.5). FDP concentration in all cases was 2.68 mM.

step (k_3) were plotted in Figure 4 as $1/k_3$ *vs.* $1/\text{FDP}^n$ with n = 2.1 giving a line which best fits the data. The $\langle K_D \rangle_{g\ av}$ obtained for the slow step was 0.52 mM, with a maximum k_3 of 1.9×10^{-2} min^{-1}.

The FDP-enhanced inactivation was then studied as a function of six different protein concentrations in addition to 0.25 mg/ml used in Figure 1, each in the presence of 2.68 mM FDP. The data are presented in Figure 5. Again, inactivation was biphasic, and the extrapolation of the slow step (k_3) to zero time gave values which, within experimental error, were independent of protein concentration and identical to the values obtained in Figure 1. As at 0°, the rates for both the fast and the slow steps decreased as the protein concentration increased. This decrease in rate was found to be a linear function of the reciprocal of the square root of the initial pyruvate kinase concentration (Figure 6). Furthermore, at $1/[\text{PK}]^{1/2} = 0$, that is, at infinite concentration of pyruvate kinase, the inactivation rates[2] for both the fast and slow steps were identical and equivalent to a half-life of 63 min. A summary of the constants for both steps of the inactivation at 0° and 23° is presented in Table I.

During efforts to determine the maximal rate of inactivation of yeast PK as a function of FDP concentration (Figure 1), it was observed that concentrations above 5.36 mM led to a decrease in the rate of the slow step (k_3). Thus additional data for increased FDP concentrations up to 97.2 mM were obtained as presented in Figure 7. Identical rates of inactivation were observed at 2.68 mM (Figure 1) and 5.36 mM (Figure 7), consistent with the argument that FDP saturation was achieved with respect to inactivation before stabilization began. Although the rate of the second, slow step is markedly decreased by high FDP concentrations, no effect on the first step is observed. (Values for k_2 at high FDP concentrations are incorporated in Figure 3.)

Since high concentrations of FDP, with a net charge near 4 at pH 7.5 (Dawson *et al.*, 1969), markedly increase the ionic strength of the solution over the contribution of the buffer, and since it had been shown that the conformational change induced by FDP as measured by fluorescence of PK is inhibited by increasing the ionic strength of the solution (Kuczenski and Suelter, 1971), the possibility existed that a similar

TABLE I: Summary of the Constants for Both Steps of the Inactivation at 0 and 23°.[a]

Temp (°C)	k_1 (zero FDP), min$^{-1} \times 10^2$	min^{-1} [PK, mg/ml]$^{-1/2} \times 10^2$ Fast Step (Satd FDP)	Slow Step (Satd FDP)
0	0.181 at (0.5 mg/ml)	8.1	0.66
23	0.0235 at (0.25 mg/ml)	2.6	0.78

[a] Rates of inactivation determined in 0.1 M Tris·HCl (pH 7.5). The values given for k_1 at fixed levels of enzyme were obtained in the absence of FDP. The values for the fast and slow step of inactivation in the presence of saturating FDP are obtained from the slopes of the respective data of Figure 6. The values for the 0° data were obtained from the slopes of identical plots of data given by Kuczenski and Suelter (1970a, see Figure 9). In the latter case, the intercept for both steps at $1/[\text{PK}]^{1/2} = 0$ was zero.

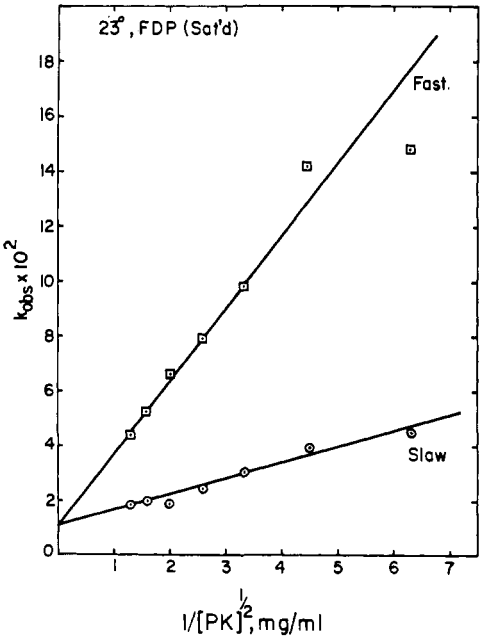

FIGURE 6: Presentation of the rates of the fast (k_2) and slow (k_3) steps of inactivation as a function of $1/[\text{PK}]^{1/2}$. Rates were determined from the data in Figure 5 as described in Figure 2.

[2] A similar relationship for the inactivation at 0° (data from Figure 9 of Kuczenski and Suelter, 1970a) was also obtained. In the latter case the rate of both the fast and slow steps was zero at $1/[\text{PK}]^{1/2} = 0$ indicating complete stability at infinite concentration of yeast pyruvate kinase at 0°.

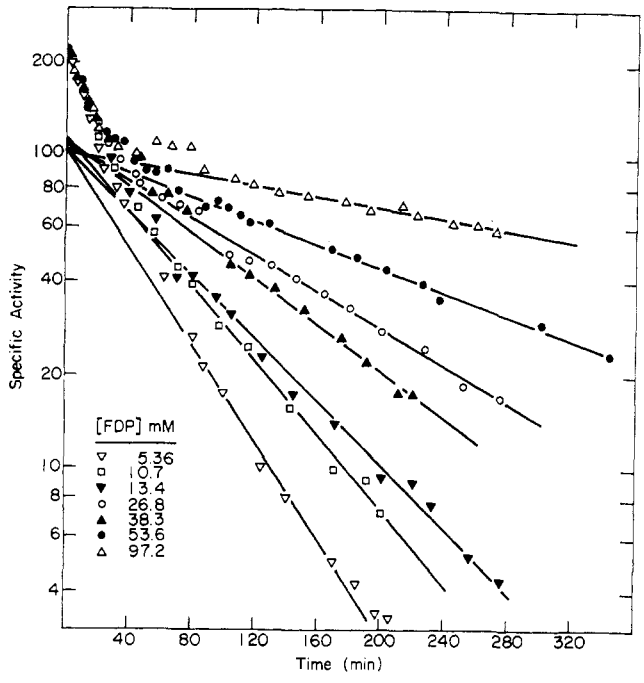

FIGURE 7: Stabilizing effect of FDP above 5.36 mM on k_3, the slow step for inactivation of yeast pyruvate kinase at 23°. Enzyme was incubated in 0.1 M Tris·HCl (pH 7.5) at 0.25 mg/ml.

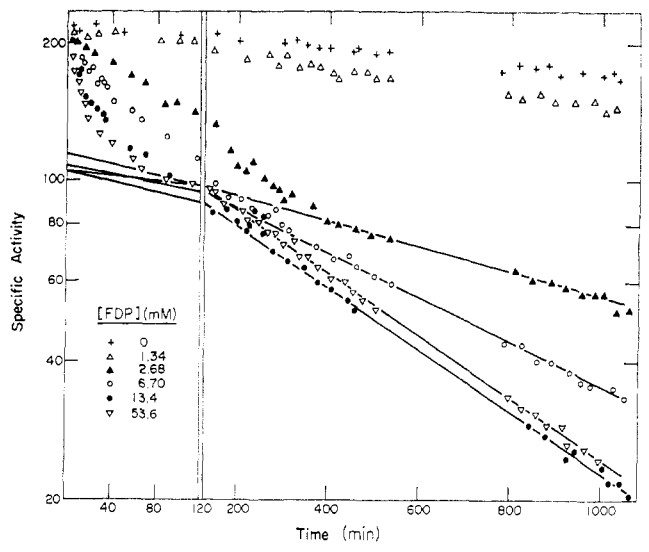

FIGURE 9: FDP-enhanced inactivation of yeast pyruvate kinase at constant ionic strength (0.93 μ). Conditions were: 0.1 M Tris·HCl (pH 7.5), 0.25 mg/ml of enzyme, 23°, and ionic strength maintained with $(CH_3)_4NCl$. The lines extending to the ordinate on the left side of the figure are extensions of the extrapolations of the slow step on the right side of the figure.

ionic strength effect, arising from the highly charged FDP molecule itself, was the potential source of the apparent stabilization. The addition of 0.23 M KCl or $(CH_3)_4NCl$ (Figure 8) in the presence of saturating (2.68 mM) FDP markedly stabilizes the enzyme, although K^+ apparently has a much greater effect. Included in the Figure are data for the inactivation at 26.8 mM FDP in the absence of added cation, a concentration which represents the same ionic strength as 0.23 M monovalent salt plus 2.68 mM FDP.

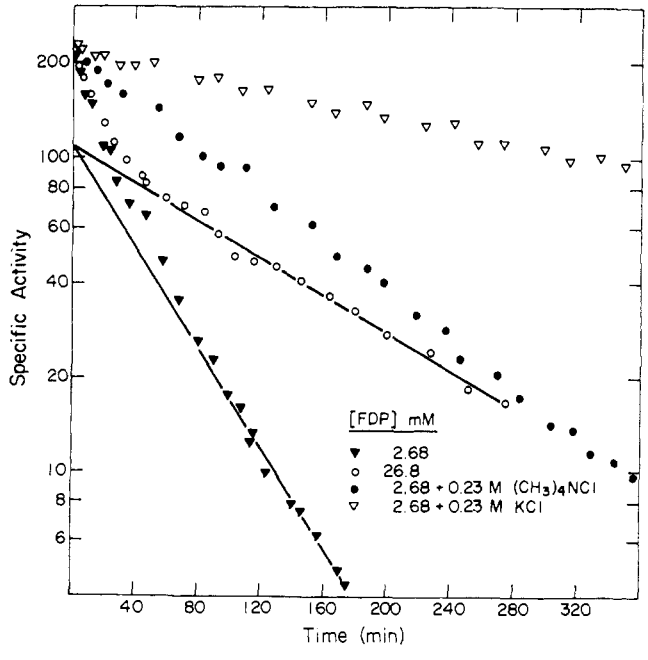

FIGURE 8: Effect of monovalent cations on the FDP-enhanced inactivation of yeast pyruvate kinase at 23°. Enzyme was incubated in 0.1 M Tris·HCl (pH 7.5) at 0.25 mg/ml. KCl (0.23 M) or $(CH_3)_4NCl$ (0.23 M) in the presence of 2.68 mM FDP represents the same ionic strength as 26.8 mM FDP.

In an effort to determine whether the effect of ionic strength was mediated through the rate of inactivation, or through the binding constant for FDP, the effect of increasing FDP concentrations at constant ionic strength was measured, and the results are presented in Figure 9. Ionic strength was maintained with $(CH_3)_4NCl$ at 0.93 μ, the value at 97.2 mM FDP (uppermost curve, Figure 7). Rates for both the fast step (k_2) and the slow step (k_3) at 53.6 mM FDP adjusted to 0.93 μ with $(CH_3)_4NCl$ (Figure 9) are identical as those obtained in Figure 7 at 97.2 mM FDP.

To examine the effect of the FDP-enhanced inactivation on the molecular weight of the enzyme, the following sedimentation experiments were performed. Enzyme was chromatographed on a Sephadex column equilibrated with 0.1 M Tris-HCl (pH 7.5) and 0.5 M $(CH_3)_4NCl$, and then allowed to stand for 3 hr at 23°. The enzyme solution was next divided into two parts. FDP, to give a final concentration of 45.2 mM, was added to the first part, maintaining protein at 5 mg/ml. The second part was adjusted to 5 mg/ml while maintaining the ionic strength with $(CH_3)_4NCl$ equal to that of the first part. Aliquots of the two protein samples were introduced into centrifuge cells and mounted in the centrifuge rotor, all at 23°. After about 30 min, the centrifuge was started, and at the time of the first picture, the remaining portions of the enzyme samples were assayed. The sample in the absence of FDP, with 98% of the original activity remaining, contained a major component (85%) with an $s_{20,w} = 8.5$ S, and minor components (10 and 5%) with sedimentation coefficients of 12.6 and <8.5 S, respectively. On the other hand, the sample in the presence of FDP, with 65% of the original activity remaining, contained two species with $s_{20,w}$ values of 8.1 S (25–30%) and 4.0 S (70–75%) which is consistent with the 4S species having a specific activity 50% of native enzyme. The 12.6S species in the former sample might be expected, since we had previously shown (Kuczenski and Suelter, 1970a) that high salt concen-

trations led to aggregation of the enzyme. Addition of FDP and Mg^{2+} to the protein under those conditions completely eliminated the aggregate (R. T. Kuczenski and C. H. Suelter, unpublished observation).

To determine whether the 4S species could be stabilized or possibly reversed to the 8S species by removal of the FDP, enzyme was prepared as in the previous experiment, and activity was measured as a function of time after addition of FDP. When activity reached a level of 110 units/mg, an aliquot was removed and chromatographed on a Sephadex column into the Tris buffer containing 0.5 M $(CH_3)_4NCl$ to remove the FDP. Enzyme in the absence of FDP was similarly treated. Fractions with the highest protein concentration (2 mg/ml) were immediately introduced into centrifuge cells, and sedimentation coefficients were determined. Enzyme not treated with FDP again contained species with $s_{20,w}$ values of 8.3 S (90%) and 12.9 S (10%). On the other hand, enzyme treated with FDP contained species with $s_{20,w}$ values of 4.2 S (50%) and 2.3 S (50%). Specific activity of the preparation at the time of the first picture was 40 units/mg, and continued to decrease linearly. Again the data are consistent with the dimer (4.2 S) having 50% of the activity of native enzyme.

Finally, in an effort to reverse the FDP-enhanced inactivation, aliquots of enzyme inactivated in the presence of 2.68 mM FDP in Tris buffer at 23°, were diluted at various stages of inactivation into solutions whose composition was known (M. Tobes, R. T. Kuczenski, and C. H. Suelter, unpublished results) to yield about 70% reactivation of yeast PK which had previously been dissociated to subunits with 6 M guanidine hydrochloride containing 0.15 M mercaptoethanol (Kuczenski and Suelter, 1970b). Although the diluted aliquots were stable to further inactivation for at least 12 hr, in no case was any additional activity recovered.

Discussion

A considerable literature exists which shows that the interaction of FDP with yeast pyruvate kinase in the presence or absence of K^+ and Mg^{2+} promotes an alteration in the quaternary structure of this enzyme. For example, in the presence of K^+ and Mg, interaction of the enzyme with FDP results in changes in catalytic properties (Haeckel *et al.*, 1968; Hunsley and Suelter, 1969b) and in quenching of the intrinsic fluorescence (Kuczenski and Suelter, 1971). Interaction of FDP in the absence of K^+ and Mg^{2+}, both at 0 and 23°, results in changes of the quaternary structure as evidenced by inactivation. The mechanism of this inactivation including the change in conformation following the interaction of FDP which is consistent with the sedimentation data for the inactivated enzyme and the inverse dependence of both steps of the inactivation on protein concentration is depicted in eq 2. As

$$A_4 \rightleftarrows A_4^* \longrightarrow 2A_2^* \longrightarrow 4A^* \qquad (2)$$

noted, the scheme[3] depicts two consecutive dissociations of native tetramer (Kuczenski and Suelter, 1970b) to inactive monomers. The sedimentation constant (s = 2.1 S) for the monomer suggests an unfolded configuration for a molecular weight of 42,000: a globular protein of this molecular weight would be expected to sediment with an s = 2.5–3.5 S (Holleman, 1966). The species with s = 4 S supports the formation of a partially unfolded dimer as an intermediate in the inactivation.

Although a similar two-step dissociation was proposed as the mechanism of FDP-enhanced inactivation of 0° (Kuczenski and Suelter, 1970a), a comparison of the characteristics of the effects of FDP on the course of the inactivation at each temperature suggests alternate pathways of subunit dissociation. The low-temperature instability of yeast pyruvate kinase (Kuczenski and Suelter, 1970a) indicates that apolar interactions, which are significantly weakened at low temperature (Kauzmann, 1959; Scheraga *et al.*, 1962), are important in the subunit associations of this enzyme. The lack of any stabilizing effect of high ionic strength on the maximal value obtained for k_2 at 23° (Figures 1, 7, and 9) is consistent with the suggestion that electrostatic forces play a major role in the association of the intermediate dimers at the higher temperature. Stabilization of the dimer formed at 23° by high ionic strength (Figure 9 *vs.* Figure 1) which is known to favor association of apolar groups by a salting-out effect (von Hippel and Schleich, 1969) is consistent with a dimer having predominantly nonpolar interactions between the subunits. Therefore, if the inactivation from tetramer to monomer, facilitated by the presence of FDP, proceeds by alternate pathways of cleavage of the tetramer at the 2 temperatures examined, heterologous interactions (Monod *et al.*, 1965; Hanson, 1966) between the subunits of yeast PK, as previously suggested (Kuczenski and Suelter, 1970a) would seem likely.

While the data at 0° are consistent with the establishment of an inactivation equilibrium between tetramer and dimer as reflected in the dependence of the extent of the rapid step of inactivation on both FDP and protein concentrations, the extent of this step at 23°, proceeding to 50% inactivation, appears independent of either of these parameters (Figures 1 and 5). The corresponding changes in sedimentation coefficient suggest that the 50% remaining activity may be primarily associated with the 4S species, since no 8S species was observed after removal of FDP by treatment with Sephadex, even though approximately 25% of the initial activity remained. Although it was previously suggested that the dimer obtained at 0° could be partially active (Kuczenski and Suelter, 1970a), attributing a specific activity of 50% of the native enzyme to the 0° dimer would not be consistent with the data and suggests that the dimers at 0° and 23° are different.

While the extent of the first step of inactivation at 23° is independent of FDP concentration, the rate of inactivation of both steps at 23° increases with increased FDP. Removal of FDP after completion of the first step of inactivation at 23°, however, led to a more rapid inactivation of the dimer at 0.5 μ than that observed at a comparable ionic strength maintained with FDP (53.6 mM FDP, Figure 6). Thus it would appear that FDP at high ionic strengths contributes some stability to the dimer. The data in Figure 7 suggest, in addition, that a stabilizing effect is also exerted by K^+ as opposed to $(CH_3)_4N^+$, particularly on the first step of the inactivation. A similar result was obtained in measuring the effects of monovalent cations on the FDP-induced fluorescence change of yeast PK (Kuczenski and Suelter, 1971), confirming the existence of both specific cation and general ionic strength effects.

The physiological significance of the effects of FDP at 23° as described here are not appreciated. Inactivation of enzymes as a significant physiological control mechanism is one mode by which enzyme levels can be regulated (Grisolia, 1964; Schimke and Doyle, 1970). On the other hand, for significant rates of inactivation of yeast pyruvate kinase to be observed,

[3] A more detailed model for the mechanism of inactivation at 0° and 23° is the subject of a future communication.

the concentration of free K^+ and Mg^{2+} must be low enough to prevent protection. How other cell constituents effect the rate of inactivation at 23° is also not known.

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Oxidized Triphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase from *Azotobacter vinelandii*. Modification of a Reactive Sulfhydryl Group with Cyanide*

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ABSTRACT: A thiocyano derivative of *Azotobacter vinelandii* isocitrate dehydrogenase has been prepared. The modified enzyme is prepared by displacing a thionitrobenzoate group from monothionitrobenzoate derivative of the enzyme with cyanide. The replacement of the thionitrobenzoate group by cyanide results in the regeneration of catalytic activity in the enzyme. The results indicate that the most reactive thiol group of the enzyme is not essential for catalytic activity although its modification by iodoacetic acid, *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, and 5,5′-dithiobis(2-nitrobenzoic acid) results in almost complete loss of catalytic activity. The Michaelis constants for both TPN^+ and isocitrate were unchanged by the modification of the native enzyme. The pH stability of the thiocyano derivative was determined, the enzyme is stable at pH 8 and 9.2 but becomes increasingly unstable at lower pH values. In the thiocyano enzyme a second thiol residue previously inaccessible to Ellman's reagent becomes accessible for titration.

The TPN^+-specific isocitrate dehydrogenase from *Azotobacter vinelandii* (ATCC 9104) contains three cysteine residues which exhibit different reactivities toward thiol reagents (Chung and Franzen, 1969). One of the thiol groups of the enzyme is readily titrated with DTNB.[1] This titration results in an almost complete loss of catalytic activity. The enzymatic activity is restored by treatment of the modified enzyme with DTT; this restoration of activity is accompanied by a stoichiometric release of the TNB chromophore and regeneration of the free thiol group of the enzyme. The reactive thiol group is also titrated by iodoacetic acid with complete loss of catalytic activity. Concomitantly one carboxymethyl group is bound per mole of enzyme. Treatment of the enzyme with HMB results in the rapid titration of two thiol groups with complete loss of catalytic activity and the slow titration of the third thiol group. The proximity of the most reactive thiol to the active site of the enzyme is indicated by the observation that substrates of the enzyme decrease the reactivity of the thiol with respect to DTNB. The thiol groups of the enzyme exist in an interesting steric relationship. Titration of the reactive thiol with DTNB and subsequent exposure of the mono-TNB derivative of the enzyme to mild denaturing conditions results in the formation of an enzyme species which contains an

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[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; HMB, *p*-hydroxymercuribenzoate; TNB, thionitrobenzoate; IDH, isocitrate dehydrogenase.