as attempting to distinguish between the substrate and coenzyme sites, and the essential and nonessential tyrosyl residues.

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Interactions of Fructose 1,6-Diphosphate, Substrates, and Monovalent Cations with Yeast Pyruvate Kinase Monitored by Changes in Enzyme Fluorescence*

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ABSTRACT: The tryptophyl fluorescence of yeast pyruvate kinase [like the rabbit muscle enzyme (Suelter, C. H. (1967), *Biochemistry* 6, 418)] is quenched by the addition separately or together of the activating cations, K^+ and Mg^{2+}. The quenching is minimal, however, even in the presence of the substrate, phosphoenolpyruvate (PEP), when compared to the quenching observed in the presence of the activator, fructose 1,6-diphosphate (FDP), either in the presence or absence of the cations or PEP. Titration of the enzyme with FDP in the presence of Mg^{2+} monitored by the fluorescence change reveals a marked dependence of the FDP binding constant on the nature of the effectors present. Addition of 0.23 M K^+ increases the apparent K_D for FDP from 0.48 to 3.1 mM. $(CH_3)_4N^+$ has a similar though smaller effect. On the other hand, the addition of PEP markedly reduces the apparent K_D for FDP to 0.069 mM. K^+ is required to obtain the reduced K_D; $(CH_3)_4N^+$ will not function. Adenosine 5′-diphosphate also promotes a decrease in the apparent K_D for FDP, but no monovalent cation requirement is observed. Changing the temperature from 30 to 0° in the presence or absence of K^+ or PEP decreases the apparent K_D for FDP by an order of magnitude. The data suggest a conformational transition favored by FDP or lowered temperature, which differs from the cation-promoted transition.

Yeast pyruvate kinase (EC 2.7.1.40), like the rabbit muscle enzyme, requires both monovalent and divalent cations for catalytic activity (Boyer *et al.*, 1942; Washio and Mano, 1960; Hunsley and Suelter, 1960b). Aside from this similarity, the two enzymes appear to differ in molecular weight (Kuczenski and Suelter, 1970b; Bischofberger *et al.*, 1970), temperature stability (Kuczenski and Suelter, 1970a), and kinetic properties (Reynard *et al.*, 1961; Hess *et al.*, 1966; Hunsley and Suelter, 1969b). Fructose 1,6-diphosphate, which activates the yeast enzyme, functions by decreasing the substrate K_m's; the same V_{max} is obtained in the absence of fructose 1,6-diphosphate by increasing K^+ from 0.1 to 0.23 M (Hunsley and Suelter, 1969b). The enzyme from *Streptococcus carlsbergenesis* required FDP[1] to achieve optimum V_{max} (Hess and Haeckel, 1967).

Suelter (1967) has shown that the interaction of cations with rabbit muscle pyruvate kinase results in a quenching of the tryptophyl fluorescence of the protein. Since the yeast enzyme exhibits the same cation requirements, we reasoned that a study of the fluorescence properties of the yeast pyruvate

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[1] Abbreviations used are: FDP, fructose 1,6-diphosphate; PEP, phosphenolpyruvate; PK, pyruvate kinase.

kinase could either expose subtle similarities of the yeast and muscle enzymes mediated through the cation activation, or reveal additional differences between the two. This paper reports the results of such a study, with particular emphasis on the effect of fructose 1,6-diphosphate on the fluorescence of yeast pyruvate kinase.

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 was stored at 0–4° as a suspension in 90% saturated (3.6 M) $(NH_4)_2SO_4$. Protein concentrations were estimated from absorbance at 280 nm ($E_{1\text{ cm}}^{0.1\%}$ 0.653) (Hunsley and Suelter, 1969a). Kinetic assays were performed under the conditions and with reagents described by Hunsley and Suelter (1969b). All enzyme preparations had a minimum specific activity of 210 μmoles/(min mg) at 30°. Prior to use, stock enzyme was chromatographed on Sephadex G-25 (coarse) equilibrated with the appropriate buffer at room temperature. Aliquots of the protein were tested for complete removal of $(NH_4)_2SO_4$ with saturated $BaCl_2$. The enzyme, after passage over Sephadex, was allowed to stand at room temperature for at least 3 hr before use in any studies (Kuczenski and Suelter, 1970a).

Crystalline rabbit muscle aldolase, FDP, PEP (FDP and PEP were always added as cyclohexylammonium salts), and NaADP were from Sigma. NaADP was converted into the Tris salt by treatment with Dowex 50W-X8 in the Tris form. A rabbit muscle α-glycerophosphate dehydrogenase–triose phosphate isomerase mixture was a Calbiochem product. $(CH_3)_4NCl$ from Aldrich was recrystallized from absolute ethanol and passed over a column of Chelex-100 in the Tris form to remove contaminating heavy metals. ADP and PEP concentrations were estimated by a modification of the Bücher and Pfleiderer (1953) pyruvate kinase assay in the presence of excess pyruvate kinase (Hunsley and Suelter, 1969b). FDP was estimated in the presence of excess aldolase, using a modified assay from Rutter *et al.* (1966).

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

Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer equipped with an X–Y recorder. Fluorescence at right angle to the illumination was measured with the indicated temperature maintained using a constant-temperature accessory. Several determinations were checked using front-face illumination and emission, but this technique was discarded since results were identical with those obtained using right-angle illumination and emission.

Titrations of fluorescence changes were routinely begun with an initial volume of 2.0 ml of sample in a fused quartz cell (capacity 4.8 ml). Small aliquots of the titrant, which was contained in a solution of identical composition as the sample (including protein concentration), were added and mixed with a magnetic stirrer.

Results

As in the case of the enzyme isolated from rabbit muscle, the fluorescence of yeast pyruvate kinase is quenched by the addition of the cations K^+ or Mg^{2+} but not $(CH_3)_4N^+$. However, the extent of the quenching produced by these cations individually (2–3%) or together (4–5%) is minimal when compared to the quenching of muscle PK (14–16%) (Suelter, 1967), or of yeast PK brought about by the addition of FDP (12–14%). The extent of the quenching produced by FDP was independent of K^+ and/or PEP, although, as will be seen, the concentration of FDP required to bring about the fluorescence change depends on the effectors present. (Because of the instability of yeast PK in the presence of FDP alone (Kuczenski and Suelter, 1970a, 1971) a reproducible quantitative determination of the interaction of enzyme with FDP alone could not be made, although the quenching was greater than 6%.) Phosphoenolpyruvate in the presence or absence of saturating KCl and/or $MgCl_2$ caused a 4–5% quenching of the yeast enzyme.

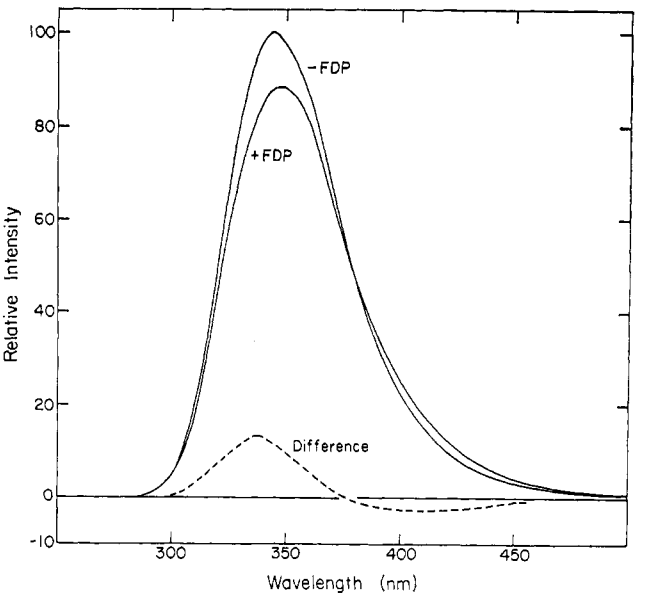

FIGURE 1: Fluorescence emission spectra of yeast pyruvate kinase in 0.1 M Tris·HCl (pH 7.5) and 0.025 M $MgCl_2$ at 23°. Protein concentration was 0.30 mg/ml for both curves, and the FDP concentration was 5×10^{-3} M. Excitation wavelength was 280 nm.

The fluorescence emission spectrum of yeast PK at 0.30 mg/ml in 0.1 M Tris·HCl (pH 7.5) excited at 280 nm is presented in Figure 1. Addition of 5 mM FDP and 25 mM $MgCl_2$ reduced the intensity at 344 nm by 12%, and shifted the peak position slightly to 347 nm. The algebraic difference emission spectrum with a maximum in the range of 330–340 nm is typical of tryptophyl emission. The origin of the enhanced fluorescence at 400–420 nm, while presumably from tryptophyl residues, is not understood. Your attention is directed to unusual difference spectra obtained with tryptophyl-containing proteins (Ananthanarayanan and Bigelow, 1969a,b).

Because the effect of FDP on the fluorescence of PK was sufficiently large to allow for a determination of dissociation constants from titrations of the fluorescence change, we proceeded to examine the extent of interaction of the enzyme with cations and substrates as reflected in the binding of FDP. Except as noted, the excitation wavelength was 280 nm and the emission was monitored at 344 nm. (Identical K_D's and n_H's were obtained when fluorescence differences were determined at 355 nm and when the excitation wavelength was 295 nm.) The data were treated as shown in Figure 2 as a Hill plot (Atkinson, 1966) of the fluorescence of PK as a function of the concentration of FDP at various KCl concentrations. The addition of 0.10 M KCl (the optimal KCl concentration for catalytic activity of PK in the presence of FDP) increases

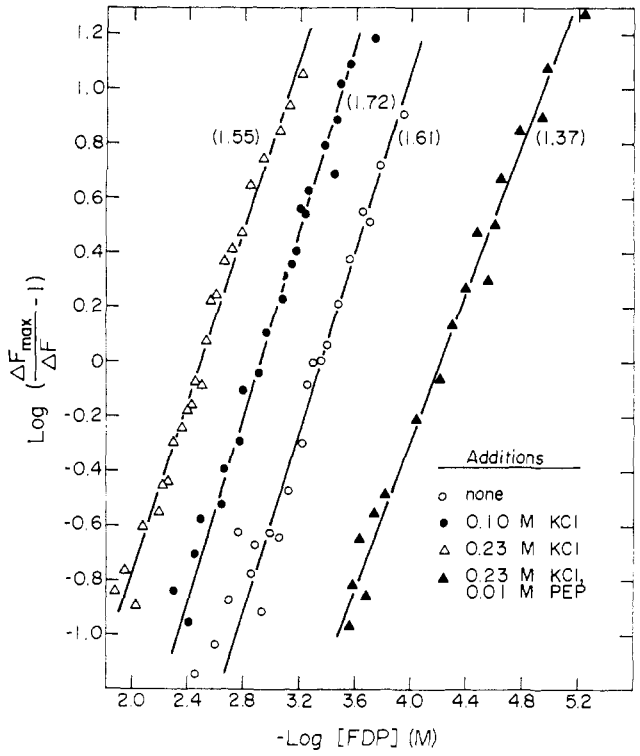

FIGURE 2: Presentation of the log $((\Delta F_{max}/\Delta F) - 1)$ as a function of $-\log$ FDP (M). Protein was maintained at 0.30 mg/ml, temperature 23°. Excitation wavelength was 280 nm, emission wavelength, 344 nm.

the apparent K_D for FDP from 0.49 to 1.3 mM with little effect on the Hill slope (n_H) (Figure 2 and Table IA). Increasing the KCl to 0.23 M (optimal for catalytic activity of PK in the absence of FDP) increases the apparent K_D for FDP even further to 3.1 mM. On the other hand, addition of

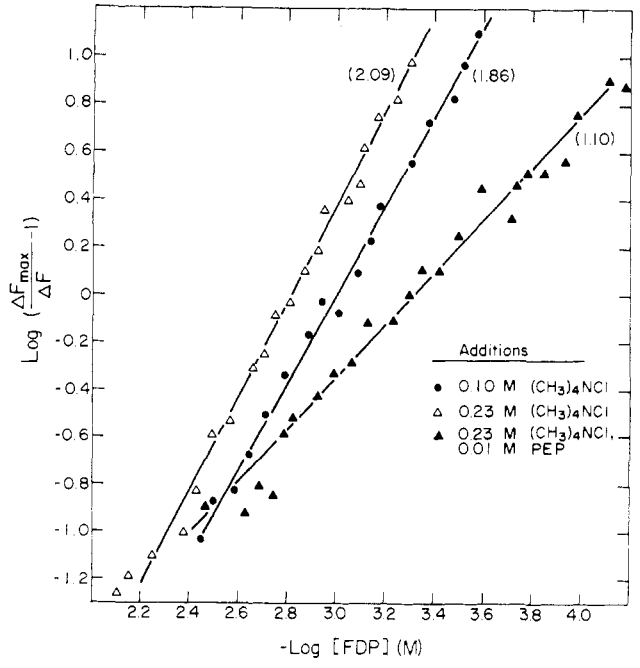

FIGURE 3: Plots of the binding of FDP to yeast pyruvate kinase as monitored by fluorescence changes. See legend to Figure 2 for conditions.

TABLE I: Apparent Dissociation Constants of FDP and Yeast Pyruvate Kinase Obtained by Titration of Fluorescence Changes.

Additions[a]	Temp (°C)	K_D (mM)[b]	n_H[b]
		Part A	
None	23	0.49 ± 0.029	1.58 ± 0.03
$(CH_3)_4NCl$ (0.1 M)	23	1.0	1.86
KCl (0.1 M)	23	1.3 ± 0.08	1.68 ± 0.04
$(CH_3)_4NCl$	23	1.2 ± 0.25	2.03 ± 0.07
KCl	23	3.1 ± 0.15	1.63 ± 0.08
PEP	23	0.48	1.20
$(CH_3)_4NCl$ + PEP	23	0.48 ± 0.005	1.15 ± 0.05
KCl + PEP	23	0.069 ± 0.003	1.37 ± 0.06
ADP	23	0.20	1.16
$(CH_3)_4NCl$ + ADP	23	0.17 ± 0.017	1.02 ± 0.01
KCl + ADP	23	0.19 ± 0.007	1.05 ± 0.07
		Part B	
None	0	0.12 ± 0.009	1.71 ± 0.07
	30	0.91	1.38
KCl	0	0.36	1.13
	30	3.3	2.07
KCl + PEP	0	<0.03	
	30	0.45	1.57

[a] All titrations were done in 0.10 M Tris·HCl (pH 7.5) containing 25 mM $MgCl_2$. Concentrations of salts or substrates when added were: KCl, 0.23 M except when indicated; $(CH_3)_4NCl$, 0.23 M except when indicated; PEP, 0.01 M; ADP 5 mM. Protein concentration in all cases was maintained at 0.30 mg/ml. [b] Values for which a range is given are averages of two determinations.

10 mM PEP (kinetically saturating) at 0.23 M KCl markedly increases the affinity of the enzyme for FDP yielding an apparent K_D of 0.069 mM which agrees with a previously reported value of 0.1 mM (Wieker *et al.*, 1969).

In an effort to separate specific monovalent cation effects from general ionic strength effects, dissociation constants for FDP and PK were determined in the presence of the same concentrations of $(CH_3)_4N^+$, a catalytically nonactivating cation (Figure 3 and Table IA). The effect of $(CH_3)_4N^+$ on the dissociation constant for FDP is analogous to the effect of K^+, although the extent of the increase in the apparent K_D is somewhat lessened. On the other hand, a considerable increase in the Hill slope was obtained with $(CH_3)_4N^+$ which was not observed with K^+. In addition, the combined presence of PEP and $(CH_3)_4N^+$ does not yield the marked decrease in apparent K_D for FDP as was seen with PEP and K^+. In fact, the apparent K_D for FDP in the presence of 10 mM PEP with or without $(CH_3)_4N^+$ is identical with the apparent K_D for FDP obtained in the absence of PEP, that is, in the presence of Mg^{2+} alone, although the Hill slope is considerably reduced.

The effect of the second substrate of the pyruvate kinase reaction, ADP, is also presented in Table IA. For these titra-

tions, an excitation wavelength of 295 nm was used to minimize absorption due to the ADP. In the presence of ADP and either K^+ or $(CH_3)_4N^+$ the apparent K_D for FDP is reduced by an order of magnitude from the value obtained in the absence of ADP; the Hill slope in both cases is reduced to unity from values near 2.

Under each of the three conditions in which effects of temperature were examined as noted in Table IB, *i.e.*, no K^+, 0.23 M K^+, and 0.23 M K^+ plus 0.01 M PEP, the apparent K_D for FDP increases by an order of magnitude as the temperature is raised from 0 to 30°. In contrast, no consistent trend for n_H could be seen, with slopes decreasing in the absence of K^+ as the temperature was raised from 0 to 30°, and increasing in the presence of K^+ and K^+ plus PEP as the temperature was increased from 0 to 30°.

Finally, in considering the possibility that a molecular weight change in the enzyme accompanied the changes in fluorescence, the sedimentation coefficient for yeast PK was determined under several sets of conditions. The values for $s_{20,w}$, extrapolated to zero protein concentration, are presented in Table II. The only observable changes in $s^0_{20,w}$ occur in the presence of FDP with no K^+, and in the presence of FDP, K^+, and PEP. In both cases, slight increases in $s^0_{20,w}$ are obtained.

TABLE II: Sedimentation Coefficients of Yeast Pyruvate Kinase.

Expt	Additions[a]	$s^0_{20,w}$ (S)	$[\Delta s_{20,w/mg}]^b$ (S)	Concn Range (mg/ml)
1	None	8.35 ± 0.04	−0.081	1.3–12.5
2	KCl, $MgCl_2$	8.35 ± 0.04	−0.086	2.0–6.0
3	KCl, $MgCl_2$, FDP	8.32 ± 0.06	−0.073	2.0–7.6
4	$MgCl_2$, FDP	8.45 ± 0.05	−0.061	2.0–7.5
5	KCl, $MgCl_2$, FDP, PEP	8.53 ± 0.04	−0.065	2.0–10.1

[a] All runs were performed at 20° in 0.1 M Tris·HCl (pH 7.5). Concentrations of salts or substrates when added were as follows: KCl, 0.02 M; $MgCl_2$, 25 mM; FDP, 1 mM; PEP, 10 mM. Extrapolations to zero protein concentration were least-squares plots plus and/or minus standard deviations. [b] The Δ values represent the slopes of the least-squares plots of sedimentation coefficient *vs.* protein concentration.

Discussion

Fluorescence Quenching. The quenching of the fluorescence of yeast pyruvate kinase by K^+ and Mg^{2+} suggests that yeast PK undergoes a small conformational transition as a result of interaction with the activating cations. However, the fluorescence quenching data suggest that a more profound effect is produced by FDP than by the cations taken either separately or together. The sedimentation data in Table II, while not conclusive when taken alone, are consistent with this fluorescence data. That is, a significant increase in sedimentation coefficient suggestive of a conformational transition is observed only under those conditions which promote a large fluorescence change (expt 4 and 5, Table II; FDP is not saturating in expt 5). The possibility that the increased $s^0_{20,w}$ values represent a molecular weight change is excluded by the published report (Kuczenski and Suelter, 1970b) that the molecular weight is not changed by the two extreme sets of conditions reported here (expt 1 and 5, Table II). This conformational change reflected in the changes in fluorescence and sedimentation coefficients after addition of FDP are analogous to the transition to a kinetically activated conformer also induced by FDP, and characterized by a lower K_m for PEP.

Effect of K^+. The apparent antagonistic effect of K^+ on the binding of FDP (Figure 2) is totally unexpected in light of the fact that this same cation is required, at the concentrations used in this report, for catalytic activity both in the presence and absence of FDP. The effect appears to be due to both an increase in ionic strength since $(CH_3)_4N^+$ (Figure 3) yields the same increasing trend in apparent K_D's for FDP although it does not function kinetically, and a specific K^+ effect, since the extent of the increase in apparent K_D for FDP produced by $(CH_3)_4N^+$ is not as great as by K^+. A specific K^+ effect is also supported by the report (Kuczenski and Suelter, 1971) that K^+ and $(CH_3)_4N^+$ do not exert equal stabilizing effects during the FDP-enhanced inactivation of the enzyme; K^+ is a more effective stabilizer. The antagonistic effect of ionic strength on the binding of FDP is also observed in the catalytic assay using concentrations of ADP, PEP, FDP, and K^+ which are below K_m (0.5, 0.087, 0.027, and 10.0 mM, respectively) (R. T. Kuczenski and C. H. Suelter, unpublished observation). Increasing the K^+ from 0.01 to 0.23 M in the same assay decreases the observed activity by an order of magnitude, the rate of decrease having a half-life on the order of seconds. Addition of 0.23 M $(CH_3)_4N^+$ has a similar effect, although the decrease in activity is only about half as great as in the case with K^+. Similarly, the observation that K^+ quenches, to a limited extent, the fluorescence of yeast PK, whereas $(CH_3)_4N^+$ does not, again supports a specific cation effect in addition to the effect of ionic strength.

Effect of Substrate. The most marked influence on the binding of FDP to yeast PK is exerted by PEP in conjunction with K^+ (Table I), an effect which might be expected, since kinetically, the K_m's and n_H's for PEP are markedly affected by FDP (Hunsley and Suelter, 1969b). The affinity of the enzyme for FDP in the presence of PEP and K^+ is increased by almost two orders of magnitude over the affinity at the same K^+ concentration in the absence of PEP. On the other hand, PEP alone or PEP in the presence of 0.23 M $(CH_3)_4N^+$ does not decrease the K_D for FDP, suggestive of a specific monovalent cation–PEP–enzyme complex involved in the FDP-induced conversion of the enzyme to its activated conformer. The implication that K^+ might bind at or very near the PEP binding site on the enzyme is consistent with nuclear magnetic resonance data of Kayne and Reuben (1970) using Tl^+ and rabbit muscle pyruvate kinase. Suelter (1970) has recently suggested that certain kinds of reactions are catalyzed by monovalent cation-activated enzymes, implying binding at the active site.

The observation that ADP in the presence of cations also affects the dissociation constant for FDP with yeast PK is surprising. Kinetically, FDP has little or no effect on the K_m for ADP and no heterotropic or homotropic cooperativity has been shown involving ADP, with the Hill slope equal to unity under all conditions (Hunsley and Suelter, 1969b). The addition of 5 mM ADP in the presence of 0.23 M K^+ or $(CH_3)_4N^+$, however, reduces the apparent K_D for FDP, as deter-

mined by the fluorescence titrations, by an order of magnitude. Further, Hill slopes near 2 in the absence of ADP were reduced to unity in its presence, suggesting a significant role for ADP in the determination of conformation of the enzyme, a conclusion not apparent from the kinetic data. Unlike the results obtained for PEP, the enzyme–ADP interaction which is reflected in the apparent K_D for FDP is not mediated by monovalent cations, and occurs in the absence of K^+ and $(CH_3)_4N^+$ consistent with the lack of a specific monovalent cation–ADP–enzyme complex.

Function of K^+. As was noted earlier, an identical V_{max} is obtained for PK from *S. cerevisiae* in the presence or absence of FDP. Both conditions require K^+. However, 0.23 M K^+ even in the presence of Mg^{2+} and PEP does not produce the same fluorescence change in PK as is obtained with FDP. If the fluorescence properties of the enzyme reflect its conformation, and if the spectrum in the presence of FDP reflects an activated conformer, one of two alternatives could be considered. On the one hand, the fluorescence of the enzyme in the presence of K^+, Mg^{2+}, and PEP would reflect a conformation which is intermediate on the path to the kinetically active conformer. In this case ADP is required to complete the transition conformational. Such a possibility cannot be excluded on the basis of kinetic data, since ADP apparently alters the structure of the enzyme in such a manner as to facilitate FDP binding as shown in Table I. On the other hand, if ADP does not provide the driving force to complete the conformational transition, then it would seem necessary to argue the existence of two different active conformers controlled by FDP or K^+. These would be distinguished by their fluorescent properties, and kinetically, by differences in K_m's for PEP. This second alternative is supported by the antagonistic effect of K^+ on the binding of FDP, and further suggests a dual role for K^+: (1) as a required cation for catalysis in the presence and absence of FDP and (2) in producing an apparently unique active conformer in the absence of FDP. Such a dual role is also consistent with the observation (Hunsley and Suelter, 1969b) that Na^+ can substitute for K^+ only in the presence of FDP.

Effect of Temperature. The substantial increase in the apparent K_D for FDP as the temperature is raised from 0 to 30° as determined from fluorescence changes in the presence of Mg^{2+} (Table IB) agrees with the similar increase in the apparent K_D monitored by the FDP-enhanced inactivation of the enzyme in the absence of Mg^{2+} (Kuczenski and Suelter, 1970a, 1971). These observations are consistent with a conformer of yeast PK favored by low temperatures or FDP.

Comparison of the Yeast and Muscle Enzyme. The characteristics of muscle PK are similar in many respects to the yeast enzyme if the latter is compared after addition of the effector FDP. The fluorescence quenching (Suelter, 1967) and increase in sedimentation constant of the muscle enzyme brought about by K^+ and Mg^{2+} (Kayne and Suelter, 1968) requires, for the yeast enzyme, in addition to K^+ and Mg^{2+}, addition of FDP. The yeast enzyme exhibits cooperative kinetics in the absence of FDP and in common with the muscle enzyme, Michaelis–Menten kinetics in the presence of FDP. Lowering the temperature of a solution of the muscle enzyme in the absence of cations results in a conformation change observed by difference spectroscopy (Kayne and Suelter, 1968) which is reflected in the yeast enzyme by a cold inactivation. Addition of FDP in the latter case enhances the inactivation (Kuczenski and Suelter, 1970a).

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