The Temperature-Dependent Conformational Transitions of Pyruvate Kinase*

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ABSTRACT: Rabbit muscle pyruvate kinase has been shown to undergo protein conformational changes following the addition of the catalytically required monovalent or divalent cations and upon changing the temperature of a solution of the enzyme. The conformational changes produced by cations are observed by the production of ultraviolet difference spectra (characteristic of the solvent perturbation of tryptophan), changes in optical rotatory dispersion parameters, and the sedimentation velocity of the enzyme. Solvent perturbation studies and reaction of the protein tryptophyl residues with *N*-bromosuccinimide or 2-hydroxy-5-nitrobenzyl bromide have failed to show differences in the degree of exposure of this amino acid residue to the external environment.

The temperature-dependent conformational change studied under varying solution conditions follows the behavior expected for an equilibrium between two forms of the enzyme. Decreasing pH, increasing ionic strength, ^2H_2O substitution for H_2O, and binding of activating cations favor the low-temperature form of the enzyme. The high-temperature form is favored by the addition of protein structure disrupting agents such as urea and Triton. The catalytic activity of the enzyme exhibits an anomalous Arrhenius plot with a curvature from a high energy of activation at low temperatures to a low energy of activation at high temperatures. The temperature about which this curvature occurs depends on the solution pH, ^2H_2O substitution, and ionic strength. The transition temperatures observed by the curvature in the Arrhenius plots parallel but are not identical with those observed by ultraviolet difference spectrophotometry. Taken together, the results suggest that the low-temperature form and the cation-activated form of the enzyme are more compact than the high-temperature form.

Previous papers in this series (Suelter and Melander, 1963; Kayne and Suelter, 1965; Suelter *et al.*, 1966; Suelter, 1967) have demonstrated that rabbit muscle pyruvate kinase (EC 2.7.1.40) undergoes conformational changes under the following conditions: (1) upon the addition of either the required monovalent or divalent cations, (2) upon the addition of the substrates PEP[1] or pyruvic acid and, (3) upon changing the temperature of a solution of the enzyme. These conformational changes were demonstrated and followed by changes in the protein ultraviolet difference spectrum and fluorescence emission and polarization. Previous work (Kayne and Suelter, 1965) had also suggested that a curvature in the Arrhenius plot of the enzyme catalysis may be related to the observed conformational change.

This communication will describe the effects of ionic strength, pH, cation interaction, and other solvent environments on the temperature-dependent transitions as observed by difference spectroscopy, Arrhenius plots, sedimentation velocity, and optical rotatory dispersion.

Experimental Section

The enzyme used in these studies was isolated from frozen rabbit muscle (Pel-Freeze Biologicals, Rogers, Ark.) by a modification of the method of Tietz and Ochoa (1958) as described previously (Kayne and Suelter, 1965). The enzyme preparations had specific activities in the range of 160–200 μmoles of product/min mg of protein at 25°. Reagent or analytical reagent grade inorganic and organic chemicals were obtained from commercial sources. Cacodylic acid (dimethylarsinic acid), Tris (Trizma base), 2-hydroxy-5-nitrobenzyl bromide (ϕ-Br), PEP (tricyclohexylammonium salt), and ADP (sodium salt) were obtained from Sigma. Imidazole from the same source was recrystallized from a mixture of chloroform and petroleum ether (bp 30–60°). Urea was recrystallized twice from 95% ethanol and fresh solutions were made prior to use. $(CH_3)_4NCl$ and $(CH_3)_4NOH$ were products of Eastman. *N*-Bromosuccinimide was obtained from Matheson Coleman and Bell and recrystallized from benzene. Sodium dodecyl sulfate was from the same source and Triton X-100 was a product of Rohm & Haas. Deuterium oxide was obtained from a number of commercial sources and in all cases was greater than 99.6 mole % ^2H_2O. Dimethyl

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: NBS, *N*-bromosuccinimide; ϕ-Br, 2-hydroxy-5-nitrobenzyl bromide; PEP, phosphoenolpyruvate.

sulfoxide was shaken with $NaHCO_3$ and redistilled *in vacuo*.

A Cary Model 15 spectrophotometer was used for recording the ultraviolet difference spectra. Temperatures in spectrophotometer cells and reaction vessels were measured with the use of glass-probe thermistors obtained from Sargent and Fenwal Electronics Inc. The readability and accuracy achieved under optimal conditions was usually better than $\pm 0.1°$ (for details, see Kayne, 1966).

A Radiometer TTT-1/SBR2/SBU1/TTA31 automatic recording titrator was used for routine pH measurements, pH-Stat determinations of enzymatic activity and for the determination of titration curves.

Optical rotatory dispersion measurements were carried out on a Durrum-Jasco ORD/UV/CD-5 spectropolarimeter and circular dichroism recorder. Maximum absorbance of the protein solutions was kept below 1.0. The instrument was adjusted to zero rotation with the cell compartment empty and solvent base lines were usually recorded at the beginning of each series of runs.

Sedimentation velocity and diffusion measurements were made in a Beckman-Spinco Model E analytical ultracentrifuge equipped with the schlieren optical system and the rotor temperature indicating and control unit. Two enzyme samples were run simultaneously in the analytical ultracentrifuge. One solution was 0.10 M $(CH_3)_4NCl$ while the other was 0.10 M KCl plus 0.005 M $MnCl_2$. Both were in 0.05 M $(CH_3)_4N$–cacodylate (pH 7.0 or 7.8) and each had identical protein concentrations. The rotor was maintained at 20.0° during the run and average $s_{20,w}$ values were calculated. A partial specific volume for the protein of 0.747 cm^3/g at 20° was used for the calculations.[2] Densities of the respective solvents at 20° were measured with the use of hydrometers. Diffusion coefficients, $D_{20,w}$, were only determined at pH 7.0, but otherwise under the same conditions and extrapolated to zero protein concentration.

The pH-Stat assay for pyruvate kinase activity was used as described previously (Kayne and Suelter, 1965) except that 0.008 M $MgCl_2$ was used in the reaction mixture. This procedure also allowed the assays to be conducted in ^2H_2O solutions with only slight modifications. First, the reaction mixture was lyophilized, dissolved in 2 ml of ^2H_2O allowed to stand at room temperature for 30 min, lyophilized again, and diluted to its final volume with ^2H_2O. The titrant used in this case was ^2HCl at a concentration of about 0.002 M, prepared by diluting concentrated HCl with ^2H_2O and then standardizing. The p^{2}H of the solution was determined from the relationship p^{2}H = pH meter reading plus 0.40 (at 100% ^2H_2O) for the glass electrode (Glascoe and Long, 1960).

The activation energy for the catalytic reaction was also determined by using the pH-Stat assay. The reaction vessels were maintained at the desired temperature and the pH meter was standardized with buffer using the temperature corrections supplied with the buffer. At low temperatures it seemed best to keep the absolute rates low because of a slower electrode response and the increased overshoot of the individual titration steps.

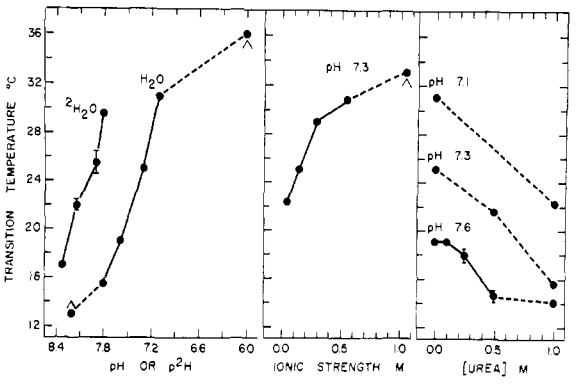

FIGURE 1: Transition temperatures for nonactivated pyruvate kinase under varying conditions. Left section: dependence on pH and p^{2}H with enzyme in 0.1 M $(CH_3)_4NCl$ and 0.05 M $(CH_3)_4N$–cacodylate at the indicated pH. Center section: dependence on ionic strength with enzyme in 0.05 M $(CH_3)_4N$–cacodylate (pH 7.3) and varying $(CH_3)_4NCl$ concentrations. Right section: dependence on urea concentration with enzyme in 0.1 M $(CH_3)_4NCl$ and 0.05 M $(CH_3)_4$–cacodylate at the indicated pH.

The temperature-perturbed protein difference spectra were obtained as previously described (Kayne and Suelter, 1965). The experiments with the enzyme in ^2H_2O solution were accomplished by lyophilization of the appropriate enzyme solution and subsequent dissolution in ^2H_2O. Best results were usually obtained when the enzyme was lyophilized in the appropriate salt solution with the buffer solution in ^2H_2O being added later. In these cases, the enzyme was still not completely solubilized in the ^2H_2O solution and centrifugation was necessary before the spectra were recorded. The specific activity of the enzyme was not significantly altered by this treatment.

In all cases where the temperature was varied, cacodylic acid buffer ($(CH_3)_4N$–cacodylate) was used, since its acid dissociation constant is very insensitive to temperature changes. Protein concentrations used in these experiments were 1.5–3.0 mg/ml. The molar difference absorptivity (maximum) for these spectral changes is about 4500 (Kayne and Suelter, 1965).

Results

The temperature-dependent conformational transitions of muscle pyruvate kinase previously reported (Kayne and Suelter, 1965, Figure 3) have been examined under a variety of conditions. These data are shown in Figure 1. The left portion of the figure shows that decreasing pH markedly increases the transition temperature of the enzyme. The same increase is seen in ^2H_2O. The transition temperature in ^2H_2O, however, should be corrected for the lower degree of ionization of enzyme-dissociable groups in ^2H_2O. This was done by assuming that the protonation of imidazole residues are primarily responsible for the pH effects. The pK of imidazole determined in ^2H_2O had a value of 7.25 which

[2] This was based on a value of $\bar{v} = 0.740$ cm^3/g at 10° (Warner, 1958) corrected to 20° as described by Taylor and Lowry (1956). M. A. Steinmetz and W. C. Deal (unpublished data) have calculated a value of 0.741 cm^3/g from amino acid analysis.

TABLE I: Transition Temperatures of Pyruvate Kinase in the Presence of Various Cations Determined by Difference Spectroscopy.[a]

Cation (M)	Transition Temp (°C)
$(CH_3)_4N^+$ (0.1), pH 7.6	19.0
$(CH_3)_4N^+$ (0.1) + 0.02% Triton X-100, pH 7.6	17.5
$(CH_3)_4N^+$ (0.1) + Ca^{2+} (0.001), pH 7.6	25.5
$(CH_3)_4N^+$ (0.1)	15.5
$(CH_3)_4N^+$ (0.1) + Mn^{2+} (5×10^{-6})	17.4
$(CH_3)_4N^+$ (0.1) + Mn^{2+} (4.5×10^{-5})	22.2
$(CH_3)_4N^+$ (0.1) + Mn^{2+} (1×10^{-4})	25.9
$(CH_3)_4N^+$ (0.1) + Mn^{2+} (0.005)	26.7
$(CH_3)_4N^+$ (0.1) + Ca^{2+} (0.001)	17.3
$(CH_3)_4N^+$ (0.1) + Ca^{2+} (0.001) + EDTA (0.002)	14.9
K^+ (0.1)	22.4
Na^+ (0.1)	21.0
Li^+ (0.1)	22.4
K^+ (0.1) + Mn^{2+} (0.005)	32.4
K^+ (0.1) + Mn^{2+} (0.005) + urea (1.0)	29.4
Li^+ (0.1) + Mn^{2+} (0.005)	32.4
K^+ (0.1) + Ca^{2+} (0.001)	26.6

[a] All solutions in 0.05 M $(CH_3)_4N$–cacodylate (pH 7.8) except where noted. The transition temperature was determined from plots as described in text and as previously shown in Figure 4 of Kayne and Suelter (1965).

TABLE II: Characteristics of Arrhenius Plot for Pyruvate Kinase under Varying Conditions.[a]

Solution	E_{act} (kcal/mole) High-Temp Form	E_{act} (kcal/mole) Low-Temp Form	Arrhenius "Break" Temp (°C)
pH 8.0	11		Not observed
pH 7.8	11	20	14
pH 7.6	14	18	16
pH 7.4	9	14	20–22
pH 7.6 (μ = 0.55)[b]	6	13	21
p^{2}H 7.8–7.9 (^2H_2O)	6	15	22.5

[a] Concentrations of substrates and activating cations were the same as those used for standard pH-Stat assay described in text. [b] Additional 0.45 M $(CH_3)_4NCl$ with usual 0.10 M KCl.

is *ca.* 0.3 higher than the value in H_2O (Edsall and Wyman, 1958). Such a change in enzyme protonation would still allow for an increase of around 5° in the transition temperature of pyruvate kinase in ^2H_2O.

As shown in the center section of Figure 1, the transition temperature increases with increasing ionic strength at a constant pH. The right-hand side of the figure shows a significant decrease in the transition temperature as the urea concentration is increased at three different pH values. In this case a constant ionic strength of 0.1 M was employed using $(CH_3)_4NCl$. The ΔH for all transitions observed under the previously described conditions ranged between 35 and 40 kcal/mole.

Additional transition temperature changes are presented in Table I. Triton X-100, a nonionic detergent, causes a lowering of the transition temperature as does urea. Ca^{2+}, a competitive inhibitor of the enzyme with respect to the divalent cation, produces a slight increase in the transition temperature both at pH 7.6 and 7.8. Addition of EDTA to the same solution reverses this effect and also shows that small differences in transition temperature are readily detectable. Also illustrated here are the differences observed at pH 7.8 in the presence of the activating K^+ and Mn^{2+}, the weak activator Na^+, the nonactivating and inhibiting cations, Li^+ and Ca^{2+}, and the effect of urea on the fully activated enzyme.

Urea tends to disrupt protein structure (Tanford, 1964) and in this case favors the presence of the high-temperature form. Although the effect is not as great as that of pH, it is considered significant especially since low urea concentrations were utilized to avoid any dissociation phenomena (Steinmetz and Deal, 1966). Triton X-100, another structure disrupting agent, shows the same effect as urea on the transition temperature.

The binding of cations to the enzyme always results in an increase in the transition temperature relative to the enzyme in $(CH_3)_4N^+$, *i.e.*, the low-temperature form of the enzyme is stabilized. Solutions of K^+, Na^+, and Li^+ all of which are known to bind (Suelter *et al.*, 1966), but not necessarily activate, show approximately the same transition temperature, an increase of *ca.* 6° over the value in $(CH_3)_4N^+$. When the enzyme is nearly saturated with Mn^{2+} alone, the increase is *ca.* 11° while near saturation with Ca^{2+} only resulted in a slight increase in the transition temperature. It is significant that optimal catalytic levels of both the monovalent and divalent cations show a further increase in the transition temperature, to some 17° over that with only $(CH_3)_4N^+$. This occurs as well when a nonactivating monovalent cation, Li^+, is used but not with the nonactivating divalent cation (Ca^{2+}). This synergistic effect is consistent with a previous observation (Suelter *et al.*, 1966) in which the monovalent or divalent activating cations were found to mutually influence the affinities for each other. The effect of urea on the fully activated enzyme is similar to that observed with nonactivated enzyme.

The temperature-dependent conformational transition observed by difference spectroscopy is apparently also reflected in the catalysis by the observation of nonlinear Arrhenius plots. A curvature (so-called "break") in the Arrhenius plot for pyruvate kinase under the standard assay conditions was previously described (Kayne and Suelter, 1965). Arrhenius plots have now been obtained under other assay conditions of which Figure 2

TABLE III: Moffitt Parameters for Pyruvate Kinase Solutions.[a]

Enzyme Soln (M)	$b_0°$	$a_0°$
$(CH_3)_4NCl$ (0.1)	−102	−182
KCl (0.1) + $MnCl_2$ (0.005)	−112	−190
KCl + $MnCl_2$ + PEP (0.001)	−115	−188
KCl + $MnCl_2$ + ADP (0.001)	−102	−228
$(CH_3)_4NCl$ + ADP (0.001)	−106	−167
$(CH_3)_4NCl$ + 0.1% sodium dodecyl sulfate	−110	−278
KCl + $MnCl_2$ + urea (1.5)	−119	−132
$(CH_3)_4NCl$, 29.6° ($(CH_3)_4N$-cacodylate (0.05))	−111	−170
$(CH_3)_4NCl$, 9.8° ($(CH_3)_4N$-cacodylate (0.05))	−117	−149

[a] All solutions in 0.05 M Tris (pH 7.8), temperature 23° except as noted.

is an example showing the data obtained in ^2H_2O. The solid line in Figure 2 is a theoretical curve calculated as described previously (Kayne and Suelter, 1965). The observed initial velocities were maximal in both the high- and low-temperature ranges. This was checked by doubling the substrate concentration during measurements at low temperatures.

In this case, the energies of activation in the high- and low-temperature ranges are 6 and 15 kcal/mole, respectively, with the curvature centered near 22.5°, an increase of some 6° over that observed under standard conditions in H_2O. Parenthetically, it should be stated that a considerable deuterium isotope effect was observed in these measurements; $k_{H_2O}/k_{^2H_2O} \cong 5$. Characteristics of Arrhenius plots obtained under other assay conditions are given in Table II. The "break temperature" is the temperature at the apparent intersection of the linear portions of the plot; approximately the center of curvature.

Preliminary determinations of the sedimentation coefficient of the enzyme indicated small differences in the values between the activated and nonactivated forms of the enzyme (Kayne and Suelter, 1965). However, since these differences were essentially within experimental error, a more extensive examination of the sedimentation properties was made as a function of protein concentration at two different pH values. These results are presented in Figure 3. The lines are calculated for a least-square fit of the data. The $s_{20,w}$ at infinite dilution for enzyme in the presence and absence of activating cations at pH 7.0 are 8.93 and 8.65 S, a difference of 0.28 S, a value outside the range of experimental error (*ca.* 1.5%). The same difference is observed at pH 7.8. The differences between the values observed at pH 7.0 and 7.8 are just within the range of experimental error. However, the values at lower pH are consistently higher. The sedimentation velocities were not measured as a function of temperature, since the uncertainties in the

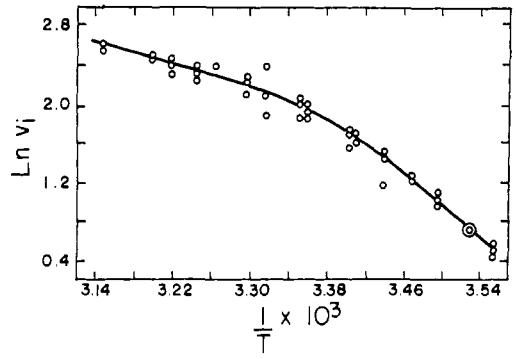

FIGURE 2: Arrhenius plot for pyruvate kinase in ^2H_2O. v_i is the initial velocity at p^{2}H 7.85.

estimated value for the partial specific volumes at other temperatures would probably not allow a firm conclusion regarding the data.

The molecular weights calculated for the enzyme either in the presence or absence of activating cations from the sedimentation and diffusion constants obtained at infinite dilution were identical within experimental error.

The optical rotatory parameters determined for various solutions of the enzyme are presented in Table III. The observed rotations at the various wavelengths (310–245 mμ) were converted into specific rotations and plotted as described by Moffitt and Yang (1956). Refractive index corrections were not deemed necessary since only in the case of urea solutions did the refractive index of the solution vary considerably. The data were found to fit linear Moffitt plots using a λ_0 of 220 mμ. The specific rotation at the Cotton effect maximum ($[\alpha]_{233}$ −5450°) and the calculated 29% α-helix compared favorably with those values reported by Jirgensons (1965) ($[\alpha]_{233}$ −5600° and 32% helix). The b_0 values were determined from the slope of the Moffitt–Yang plots as drawn by eye. While sufficient samples were not obtained for a statistical analysis of the small differences seen in the b_0 values, it is clear that the conformational

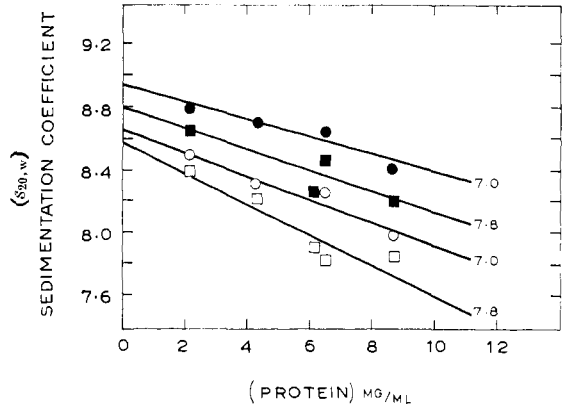

FIGURE 3: Sedimentation velocity of pyruvate kinase in the presence and absence of activating cations. Filled circles: enzyme in 0.1 M KCl + 0.005 M $MnCl_2$ and 0.05 M $(CH_3)_4$-cacodylate at the indicated pH. Open circles: enzyme in 0.1 M $(CH_3)_4NCl$ and 0.05 M $(CH_3)_4N$-cacodylate at the indicated pH. Lines are calculated by the method of least squares.

changes observed with pyruvate kinase are not reflected by large changes in optical rotation.

Independent attempts were also made to determine the number of tryptophyl residues in the protein exposed to the solvent environment and whether or not any change in this number occurred during the process of cation activation. The solvent perturbation technique of Williams *et al.* (1965), reaction of the exposed tryptophans with NBS in 0.1 M Tris-HCl (pH 7.8) (Spande *et al.*, 1966), and reaction with ϕ-Br using the method of Horton and Koshland (1956) were attempted. The data obtained were not conclusive. Results from the first two methods showed no differences in the number of exposed tryptophans while, in the latter case, 4.6 $\pm$ 0.8 moles of ϕ-Br reacted with the nonactivated enzyme and 3.3 $\pm$ 0.3 moles reacted with the activated enzyme. Since a blue shift in the protein tryptophan absorption is observed, one would normally expect an increase and not a decrease in the number of exposed tryptophans following cation activation. Furthermore, it is unlikely that the latter result reflects gross structural changes since the resulting protein had, in both cases, a specific activity of *ca.* 120 μmoles/min mg of protein.

Discussion

These results and those of the previous studies (Kayne and Suelter, 1965; Suelter, 1967) suggest that the differences observed in the spectrum of pyruvate kinase are brought about by an environmental perturbation of tryptophyl residues. The blue (bathochromic) shift observed upon addition of cations or substrates or a lowering of the temperature may be explained in a variety of ways. In one case, the chromophore may be shifted from a region of the protein whose solvent properties are more similar to a nonpolar, low dielectric solvent into a relatively more polar or aqueous environment. However, the spectral effects may also be produced by the breaking of intramolecular hydrogen bonds between the indole chromophore and a nearby acceptor, for example, the carbonyl group of the peptide backbone (C. H. Suelter and G. Weber, unpublished observations; Yanari and Bovey, 1960) or by other so-called "internal changes" (Kronman *et al.*, 1965). A direct interaction of the chromophore with a charged protein group or the cationic cofactor is considered unlikely in this case since similar spectral shifts were produced by simply lowering the temperature. Furthermore, charge perturbations of the indole chromophore do not produce an appreciable spectral shift (Donovan *et al.*, 1961).

To delineate the origin of the spectral perturbation, attempts were made to determine differences in the number of exposed tryptophyl residues between the two conformations of the enzyme. The results have indicated that the solvent perturbation techniques (with sucrose or dimethyl sulfoxide) in themselves give rise to conformational changes in the protein which either mimic or mask those changes which were under investigation. Chemical modification of the protein by oxidation with NBS or reaction with 2-OH-5-NO_2-benzyl bromide resulted in the uncertainty of whether or not the conformation of the protein was the same after reaction with the first equivalent of reagent and whether this affected the reaction with subsequent amounts of reagent. Thus, available evidence based on seemingly well-established procedures is not conclusive. Either no appreciable differences in the number of tryptophyl residues exposed to the external medium exist between the activated and nonactivated forms of the enzyme or else the exposed tryptophyl residues are located in crevices not allowing interaction with perturbants other than H_2O. Kronman and Holmes (1965) reported no increase in the number of exposed tryptophans of α-lactalbumin following the blue shift of the tryptophyl spectrum of α-lactalbumin as observed during a conformational change when the pH was lowered from 6 to 1.7. Similar perturbations may, in fact, be responsible for the blue shift observed with pyruvate kinase.

The changes in the transition temperature with varying solution conditions show that the forces maintaining these conformations are sensitive to external influences (Figure 1). The results obtained upon varying the pH and p^{2}H show that protonation of the enzyme favors the presence of the low-temperature form, *i.e.*, higher temperatures are required to affect the transition into the high-temperature form. This same effect is observed when the ionic strength is increased.

Substitution of ^2H_2O for H_2O also favors the low-temperature form. This effect may be explained in a number of ways. First, the actual substitution of readily exchangeable protons in the protein could lead to an increased strength of internal hydrogen bonding (Némethy and Scheraga, 1964). Second, the structure of ^2H_2O is somewhat more ordered than H_2O at the same temperature and thus, transitions in these structures will occur at higher temperatures (Némethy and Scheraga, 1964). Finally, hydrophobic bonds between protiated amino acid side chains are stronger in ^2H_2O than in H_2O (Kresheck *et al.*, 1965).

The transition temperatures observed by difference spectroscopy were previously correlated with the temperatures of the transitions as obtained from the curved Arrhenius plots for the enzyme (Kayne and Suelter, 1965). In fact, the ΔH observed for the temperature-dependent conformational change was used for the calculation of the theoretical Arrhenius plot. The studies presented here have shown that the initial assumption may be wrong, that is, the temperatures of the conformational transition and Arrhenius plot "break" were not the same under identical conditions. The original coincidence was due to the slightly different pH values used in the first conformational change study and in the normal assay. The Arrhenius plots for pyruvate kinase have now been determined under varying conditions similar to those studied for the temperature transition. Table IV compares data from the Results section showing the relationship of the Arrhenius plot "breaks" under the same conditions. The large shift in transition temperature with deuterium substitution previously discussed is paralleled by a corresponding shift in the "break" of the Arrhenius plot.

The degree of curvature in the Arrhenius plot is related to the ΔH for the equilibrium between the two forms of the enzyme. Massey has recently discussed a

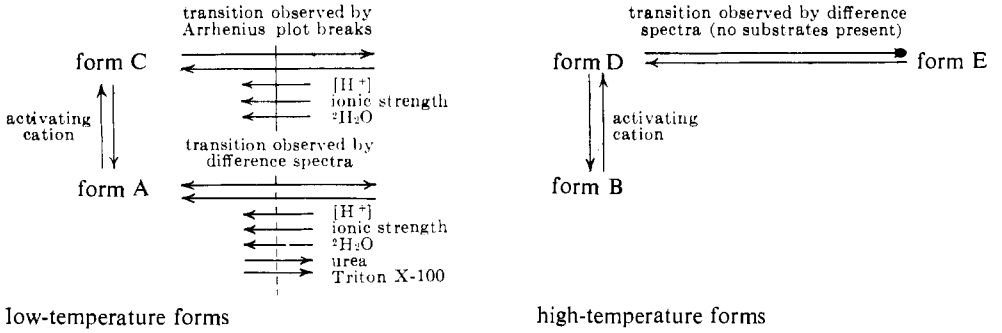

FIGURE 4: Hypothetical scheme depicting the effect of increasing concentrations of modifying conditions on the conformational transitions as observed by difference spectroscopy and breaks in Arrhenius plots.

similar effect in D-amino acid oxidase (Massey *et al.*, 1966). The theoretical Arrhenius plots presented there show a sharp break if ΔH's (conformational) of the order of 100 kcal/mole are used. At the present time, pyruvate kinase seems to be the only enzyme for which a curvature of the Arrhenius plot is documented rather than a relatively sharp break as found to date in other systems with anomalous Arrhenius plots (for a listing, see Massay *et al.*, 1966).

The temperature-dependent transitions found in the pyruvate kinase system are discussed in terms of a diagram shown in Figure 4. The lower portion portrays an equilibrium between two forms of the nonactivated enzyme as previously discussed (Kayne and Suelter, 1965), one predominant at low temperatures and the other at higher temperatures. Increasing hydrogen ion concentrations, ionic strength, ^2H_2O, urea, and Triton are shown under this as they were observed to affect the supposed equilibrium.

In relating the conformational transitions observed by difference spectroscopy to the transitions as observed by the Arrhenius plot as portrayed in the upper part of Figure 4, we must realize that the latter can be observed only under conditions where both monovalent and divalent activating cations and substrates are present. It should be noted that the difference spectra produced by temperature or cation perturbation are not exactly identical when all substrates are present. The two transitions as observed by difference spectroscopy and Arrhenius plots are affected in a similar manner by a variety of conditions of pH, ionic strength, and ^2H_2O, yet there always appears to be a 1.5–3° differential under identical conditions. Furthermore, the cation-activated enzyme (0.1 M K^+ plus 5×10^{-3} M Mn^{2+}) has a transition temperature at 32° with no apparent curvature in the Arrhenius plot at this temperature. This is portrayed in the upper right-hand corner of Figure 4. Presumably, the high-temperature form under these conditions still contains bound cation since the enzyme does exhibit catalytic activity. In addition, the difference spectra produced by temperature or cation perturbation are not identical.

While a firm conclusion cannot be made, the data suggest two distinct transitions, one which is observed by difference spectroscopy and may or may not affect catalysis and one observed by a curvature in the Arrhenius plot, which presumably affects the catalytic parameters. The suggestion that these two transitions are related is admittedly based on circumstantial evidence, specifically, the very similar manner in which their equilibria are affected by changes in the external environment. Additional data suggest that there may be distinct processes or steps in the over-all conformational equilibria of the protein.[3]

While not shown in Figure 4, a more compact protein structure at the lower temperatures or when activating cations are added is supported by three independent lines of evidence. First, the consistently higher sedimen-

TABLE IV: Relationship between the Temperature of the Arrhenius Plot "Breaks" and the Transition Temperature of the Conformational Change Observed by Difference Spectroscopy.

Solution	Arrhenius "Break" Temp (°C)[a]	Transition Temp[b] (estimated) (°C)
pH 8.0	Not observed[c]	(13–14)
pH 7.8	14	15.5
pH 7.6	16	19
pH 7.4	20–22	(23)
pH 7.6 (μ = 0.55)	21	(25)
p^{2}H 7.8–7.9 (^2H_2O)	22.5	25.5–29.5

[a] From data in Table II. [b] From data in Figure 1. [c] Actual "break" may occur but at too low a temperature for accurate observation.

[3] In preliminary kinetic (relaxation) studies on the conformational change, at least two relaxation times are seen for the process observed by difference spectroscopy. This can be an indication for at least three states of the protein, and it is possible that the relaxation times represent distinct steps in the over-all conformational change process. This further suggests that additional related steps may be responsible for the transition observed in the Arrhenius plot curvature.

tation velocity, with no change in the s/D molecular weight in the presence of activating cations or at the lower pH, indicate that this form of the enzyme is more compact (less asymmetric). Although the differences between pH 7.0 and 7.8 under either condition are very small they are consistent and agree with the model in that decreasing pH favors the low-temperature form of the enzyme.

Second, urea and Triton, protein structure disrupting agents, effect a decrease in the difference spectra transition temperature, that is, they favor the high-temperature form of the enzyme (Table I and Figure 1). Thus the protein becomes less compact as the temperature is raised, which is what one would normally expect. In contrast, in the presence of activating cations, the transition temperature is increased to 32° to effect, as previously concluded, a more compact protein structure. This is also consistent with the marked stabilization toward heat denaturation brought about by these cations (F. J. Kayne and C. H. Suelter unpublished observations; Wilson *et al.*, 1967).

Third, support for a more compact protein molecule at low temperatures or in the presence of activating cations is given by studies on the polarization of the enzyme fluorescence (Suelter, 1967). A marked increase in fluorescence polarization was noted in both cases indicating that the emitting tryptophyl residues are more constrained, *i.e.*, they experience a more compact environment. Similar results have been reported for yeast enolase by Brewer and Weber (1966).

The preceeding results and discussion confirm previous conclusions that pyruvate kinase undergoes conformational transitions with changes in temperature or following addition of activating cations (Kayne and Suelter, 1965; Mildvan and Cohn 1966). These can be directly observed by a number of physical methods which all suggest that the changes involved are relatively small in magnitude. The individual observations are adequately explained on the basis of a temperature-dependent conformational equilibrium between two forms of the enzyme (Kayne and Suelter, 1965). Although muscle pyruvate kinase does not exhibit the sigmoidal kinetics characteristic of an allosteric enzyme, a cooperative interaction does exist in the binding of monovalent or divalent cations when measured in the absence of each other (Suelter *et al.*, 1966). In the related enzyme, pyruvate kinase from yeast, a cooperative interaction is seen in the allosteric activation of the enzyme by K^+ *or* fructose 1,6-diphosphate (Hess *et al.*, 1966; Hess and Haeckel, 1967; Hunsley and Suelter, 1967).

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