Effects of Temperature, Substrate, and Activating Cations on the Conformations of Pyruvate Kinase in Aqueous Solutions

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The binding of cationic cofactors and substrate to the enzyme pyruvate kinase results in a pronounced ultraviolet difference spectrum. A similar effect is also observed upon variation of the temperature of a sample when compared with a reference at 25°. This change is completely reversible and the thermodynamic parameters for it have been determined. Corresponding with this temperature effect, the Arrhenius plot for the activity of the enzyme exhibits a curvature from an E_{act} of 13.5 kcal./mole above 30° to 18.0 kcal./mole below 0°. An equilibrium between protein conformers is invoked to explain these observations.

It has recently been shown that the interaction of some monovalent and/or divalent cations with the enzyme pyruvate kinase (EC 2.7.1.40) leads to a pronounced protein ultraviolet difference spectrum.[1,2] This difference spectrum is qualitatively similar to others observed in the cases of denaturation, solvent perturbation, substrate, and coenzyme binding with various proteins.[3-8] These spectra are usually explained by assuming changes in the solvating environments of the absorbing aromatic amino acid residues.[9] However, such difference spectra may also be caused by charge interactions.[10] This paper describes experiments which indicate that, in the case of pyruvate kinase, these difference spectra probably reflect changes in the solvating environment of protein chromophores which, in turn, reflect changes in the conformation of the protein.

Experimental

The pyruvate kinase used in these studies was prepared by the method of Tietz and Ochoa[11] from frozen rabbit muscle (Pel-Freeze Biologicals, Rogers, Ark.). The protein precipitated by $(NH_4)_2SO_4$ between 40 and 60% saturation was desalted by elution from a 1.7 × 17 cm. column of Sephadex G-25 at 0° and stored at 4°. The Sephadex was previously equilibrated with 0.005 *M* Tris·HCl, pH 8.6 [tris(hydroxymethyl)-aminomethane, Sigma], and this buffer was used for the elution. Deionized distilled water was used in all procedures. "Special Enzyme Grade" $(NH_4)_2SO_4$ (Mann Research Laboratories) or $(NH_4)_2SO_4$ recrystallized from 0.001 *M* EDTA (ethylenediaminetetraacetic acid disodium salt, Eastman) was used. Dialysis tubing (Visking) was boiled for 15 min. in two changes of 0.001 *M* EDTA before use.

The enzyme preparations had specific activities in the range of 120–180 μmoles of substrate/min./mg. of protein at 25°. The standard reaction mixture for assay contained: 0.1 *M* KCl, 0.0016 *M* $MgCl_2$, 0.002 *M* adenosine-5′-diphosphate sodium salt (ADP, Pabst), and 0.001 *M* phospho(enol)pyruvic acid tricyclohexylamine salt (PEP, Sigma), adjusted to a pH of *ca.* 7.6 with tetramethylammonium hydroxide. One milliliter of this was placed in the jacketed electrode vessel of the Radiometer TTT-1/SBR2/SBU1/TTA31 automatic recording titrator and the pH was adjusted to 7.50 by the addition of 1.85×10^{-3} *M* HCl, 0.1 *M* in KCl. The reaction was started by the addition of 5–20 μl. of 0.1 *M* KCl containing enzyme at 0.1–0.2 mg./ml. and the initial rate (1–3 min.) of HCl addition to maintain pH 7.50 was recorded.

Beckman buffers were used to standardize the pH meter. Assay temperatures were maintained to within ±0.05° with a circulating water bath and corrected for in the instrument by the manual temperature compensator. Temperature was measured by immersing a glass probe thermistor (Sargent) into the solution in the reaction vessel. Duplicate or triplicate assays were always performed. Protein concentrations were routinely determined from the absorbance in 0.1 *M* KCl at 280 mμ, using a molar extinction coefficient of 1.28×10^5 based on a molecular weight of 237,000[12] for the enzyme. From both the titration with N-bromosuccinimide according to Patchornik, *et al.*,[13] and the spectrophotometric method of Bencze and Schmid[14] 12–14 tryptophan residues were estimated per molecule of enzyme. The sedimentation coefficient of the enzyme was measured in a Spinco Model E ultracentrifuge at 59,980 r.p.m. with the rotor maintained at 21°. Optical rotation of the enzyme was measured in a Zeiss precision photoelectric polarimeter.

The difference spectra were recorded with a Cary Model 15 spectrophotometer using 1-cm. silica cells of 2.0 ml. capacity (Pyrocell). For the solvent and substrate perturbation spectra, the double-tandem cell method of Herskovits and Laskowski[15] was used to

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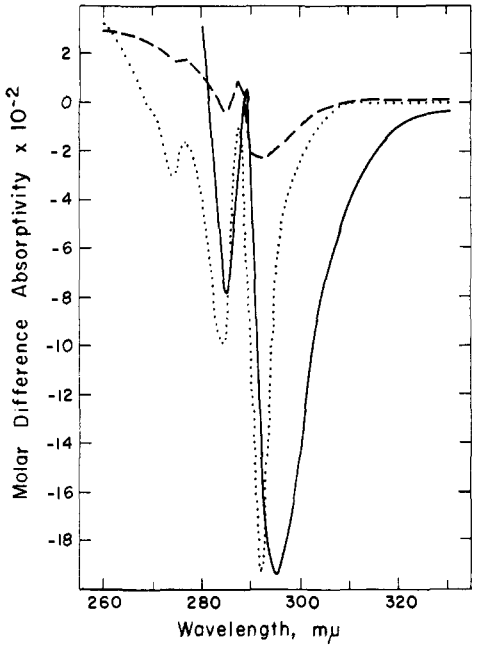

Figure 1. Ultraviolet difference spectra: ———, pyruvate kinase in 0.1 *M* KCl + 0.001 *M* $MgCl_2$ *vs.* pyruvate kinase in 0.103 *M* TMACl, calculated for saturation with Mg^{2+}; . . . ., L-tryptophan in H_2O *vs.* L-tryptophan in 94% dioxane-water; -------, L-tryptophan at pH 6 *vs.* L-tryptophan at pH 1.

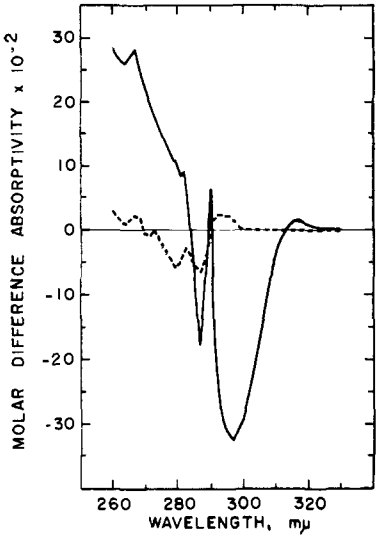

Figure 2. Ultraviolet difference spectra: ———, nonactivated (0.1 *M* TMACl) pyruvate kinase in both sample and reference positions, with PEP in sample position, calculated for saturation with PEP; ---------, activated (0.1 *M* KCl + 0.001 *M* $MnCl_2$, corrected for saturation with Mn^{2+}) pyruvate kinase in both sample and reference positions, saturated with PEP in sample position.

cross blank any absorption due to solvent or substrate. The double-tandem cells were manufactured by Pyrocell, Inc., New York, N. Y., and consisted of two 1-cm. silica cells fused together with a single silica window between the two halves. The path length was thus 1 cm. per compartment. Cell matching and spectrophotometer balance were checked by recording air *vs.* air spectra between 350 and 220 mμ. For the studies of the effect of temperature on the spectrum, thermostated cell holders supplied by Cary were mounted on plastic blocks in both the sample and reference compartments. Separate circulating baths controlled each cell to within ±0.05°. Temperature was measured in this case by immersing the thermistor probe into the solution in the cuvette.

For all enzyme difference spectra, the ionic strength and pH of both sample and reference solutions were identical. Ionic strength was maintained by the use of nonactivating tetramethylammonium (TMA) chloride in the cases where cation activation was not desired. Protein concentrations between 2.1 and 2.5 mg./ml. (*ca.* 10^{-5} *M*) were used. The buffer used for all enzyme difference spectra at 25° was 0.05 *M* Tris·HCl, pH 7.8. When the temperature of the enzyme solution was varied, 0.05 *M* TMA cacodylate buffer, pH 7.8, was substituted.

Spectral grade *p*-dioxane (Matheson Coleman and Bell), ethanol ("Gold Shield," Commercial Solvents Corp.), and ethylene glycol (Fisher) were used without further purification for the solvent-perturbed difference spectra. The tandem cell method corrects for absorption by any impurities in these solvents. No effects attributable to peroxides were noted in solutions of tryptophan in dioxane. Amino acids and their derivatives, used without further purification, were: L-tryptophan, L-tyrosine, L-phenylalanine, N-acetyl-L-tyrosine (all Calbiochem), and N-acetyl-L-tryptophanamide (New England Nuclear-Yeda).

Results

Figure 1 illustrates the difference spectra due to solvent and charge perturbation of tryptophan and the difference spectrum of pyruvate kinase in the presence *vs.* absence of activating cations. The pyruvate kinase difference spectrum is calculated for saturation with Mg^{2+} and the molar absorptivities are based on two active sites per molecule.[16] This is compared with the difference spectrum occurring between L-tryptophan in H_2O and L-tryptophan in a 94% dioxane–H_2O mixture and that between L-tryptophan at pH 6 and L-tryptophan at pH 1 as found by Hermans, *et al.*[10]

Binding of one of the substrates, PEP, to either the activated (K^+ + Mn^{2+}) or nonactivated (TMA^+) enzyme, results in the difference spectra shown in Figure 2. The activated enzyme was saturated with PEP and the spectrum of the nonactivated enzyme has been corrected for saturation with PEP. The difference spectrum brought about by the interaction of PEP with pyruvate kinase in the presence of activating cations is typical of tyrosine, while in the case of nonactivated enzyme the spectrum indicates a perturbation of tryptophan. Titration of activated enzyme with PEP and measurement of the difference in absorption at 287 mμ yields a dissociation constant of 1.5×10^{-5} *M*, which is in excellent agreement with the value of 2×10^{-5} *M* obtained by Mildvan and Cohn[17] by the use of n.m.r. techniques. Titration of nonactivated enzyme with PEP and measurement of the difference in absorption at 295 mμ gives a dissociation constant of 3×10^{-4} *M*.

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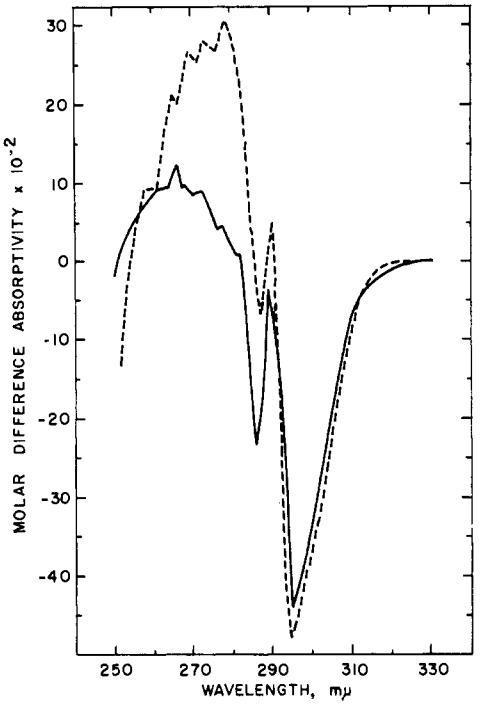

Figure 3. Ultraviolet difference spectra: -------, nonactivated (0.1 *M* TMACl) pyruvate kinase with sample position at low temperature relative to reference, corrected to theoretical maximum; ———, pyruvate kinase in 0.1 *M* KCl + 0.001 *M* $MnCl_2$ *vs.* pyruvate kinase in 0.103 *M* TMACl, calculated for saturation with Mn^{2+}.

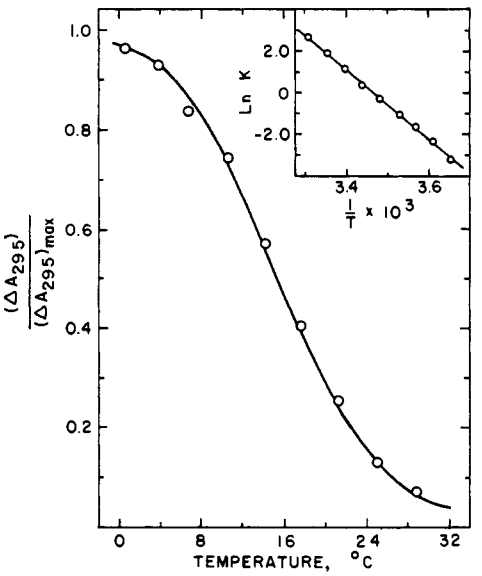

Figure 4. Temperature-dependent difference absorbance of pyruvate kinase at 295 mμ: nonactivated (0.1 *M* TMACl) pyruvate kinase in both sample and reference positions, reference position maintained at 25° and sample position varied from 0.5 to 28.9°. The solid line is a theoretical curve calculated from data presented in insert.

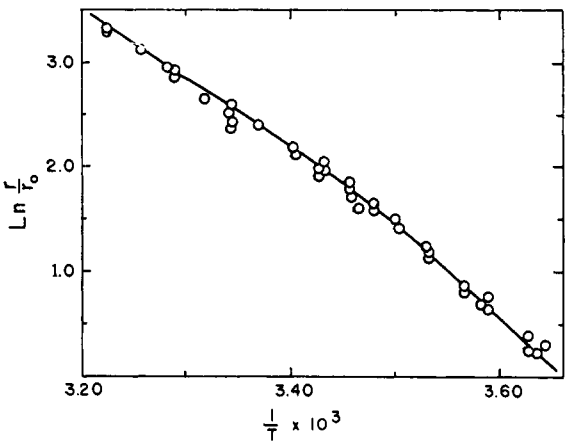

Figure 5. Arrhenius plot for pyruvate kinase: r is the initial velocity of the forward reaction at pH 7.50 and r_0 is the initial velocity at 0° (extrapolated). The line is calculated as described in text.

When nonactivated enzyme is cooled in the sample compartment of the spectrophotometer and measured against nonactivated enzyme in the reference compartment at 25°, the difference spectrum shown by the dashed line in Figure 3 is obtained. This has been corrected to illustrate the maximum difference that would occur with a sample temperature $< -10°$ and a reference temperature $>40°$. Comparison with the cation-activated difference spectrum at 25° indicates a similar perturbation of tryptophan, and, indeed, this is probably the same tryptophan, since upon lowering the temperature of the activated enzyme only minor changes in the difference spectrum are observed with practically no difference at 295 mμ.

The change in optical density at 295 mμ is presented in Figure 4 as a function of temperature. The solid line is calculated from the slope of the van't Hoff plot (insert). The maximum change in absorption at 295 mμ [$(\Delta A_{295})_{max}$] was estimated in order to obtain the equilibrium constant at each temperature noted in the insert. The phenomenon indicated is completely reversible within experimental error. The thermodynamic parameters calculated from this data are: $\Delta H° = 33.1$ kcal./mole, $\Delta S° = 107$ cal./deg./mole, and $\Delta F° = -1.1$ kcal./mole.

Figure 5 is the Arrhenius plot for the reaction catalyzed by pyruvate kinase (ADP + PEP $\rightleftharpoons$ ATP + pyruvate). The curved line has been calculated from Arrhenius equations for two forms of the enzyme existing in an equilibrium as indicated by Figure 4. From this equilibrium, the values for α, the fraction of enzyme in the form predominant at low temperature, and $(1 - \alpha)$, the fraction of the form predominant at higher temperatures, were calculated. The equation used was

$$k_{calcd} = \alpha k_1 e^{(E^1_{act}/R)(1/T_1 - 1/T)} + (1 - \alpha) k_2 e^{(E^2_{act}/R)(1/T_2 - 1/T)}$$

where k_1 is the rate constant at temperature T_1 of the conformer with energy of activation E^1_{act}. This was normalized so that the rate at 0° is equal to 1. The E_{act} was estimated from the data to be 13.5 kcal./mole ($\Delta H^* = 12.9$ kcal./mole) above 30° and 18.0 kcal./mole ($\Delta H^* = 17.4$ kcal./mole) below 0°, since the Arrhenius plot is linear at these extremes. The rates are initial velocities at optimum pH (7.5) and at saturating levels of substrate and are maximal in both the high and low temperature ranges of the Arrhenius plot.

The sedimentation velocity of pyruvate kinase in 0.1 *M* KCl and 0.001 *M* $MnCl_2$ was 9.33 S while that in

0.103 M TMACl was 9.22 S. Both solutions were 0.05 M in Tris·HCl, pH 7.8. Specific rotations [α] of the enzyme were identical for an activated (0.1 M KCl + 0.0017 M $MnCl_2$) and a nonactivated (0.1 M TMACl) sample in the above buffer at the wave lengths 578, 546, 436, 405, and 365 mμ; $[\alpha]_{365}$ $-1920°$ at 25°.

Discussion

The difference spectra produced by interaction of substrate or cationic cofactors with pyruvate kinase or by changes in temperature are due, no doubt, to changes in the solvating environments of protein chromophores. While there may be some effect on the spectrum due to changes in the electrostatic environments of the chromophores, these effects must be small compared with the solvent perturbation. For example, if the total difference spectrum characteristic of tryptophan is due to a charge perturbation caused by cation binding, then, based on the molar difference absorptivity for the charge perturbation noted in Figure 1 and the estimation of 12–14 tryptophans per molecule of pyruvate kinase, it would be necessary to invoke a perturbation of every tryptophan in the molecule.

The difference spectrum of the cation- or temperature-perturbed enzyme arises from a blue shift in the spectrum of the enzyme, while a red shift in the spectrum occurs when tryptophan is measured in dioxane relative to tryptophan in water. Thus the difference spectrum of the enzyme is due to a change in the solvating environment of certain protein chromophores which are in a nonaqueous environment at 30° or in the absence of cations, and in an aqueous environment at 0° or in the presence of cations. This interpretation is substantiated by the fluorescent properties of the enzyme[18] studied under similar conditions.

The preliminary evidence from optical rotation and sedimentation velocity measurements indicates that the changes in conformation are small relative to the entire molecule. If the thermodynamic parameters calculated from the temperature-induced difference spectrum are mainly characteristic of the interaction energies involved, they would be consistent with the hydrophobic bonding of 5–30 apolar side-chain residues per molecule.[19-21] This may also represent a minor change in a protein of some 2500 amino acid residues. Since the hydrophobic interactions are weaker at lower temperatures,[19] the amino acid sequence containing the perturbed chromophores would be solvated by water at the lower temperatures. This is consistent with the blue shift in the spectrum of the protein. The perturbation due to cations must, therefore, be indirect. That is, after the cation binds, the hydrophobic forces responsible for maintaining the tryptophyl residues in a nonaqueous environment are counterbalanced by some other force, so that they are now in an aqueous environment.

It has been suggested that the curvature in an Arrhenius plot for an enzyme-catalyzed reaction is due to a temperature effect on the rates of the individual steps in the reaction as discussed by Stearn[22] and by Kistiakowsky and Lumry.[23] In the case of pyruvate kinase, however, the effect is probably due to an equilibrium between at least two different conformations of the enzyme. This is supported by the theoretical Arrhenius plot shown in Figure 5, which was calculated by assuming two conformations of the enzyme, which are present in equal concentrations at 15.5°. It should be noted that, in this case, the temperature-dependent transition is gradual in that the effect occurs over a span of some 30°. A sharper curve in the Arrhenius plot may result when the structural modification occurs over a shorter temperature range. This may be the case for the muscle proteins myosin[24] and fumarate hydratase,[25] where a sharper break in the Arrhenius plot is observed, also near 16°. This particular temperature may be significant in determining the balance of the various forces which maintain the conformation of muscle proteins.

As noted, the binding of substrate to the enzyme produces a change in the environment of certain tryptophyl residues. If these residues are already perturbed by either the temperature or the cation effect, additional perturbation of the tryptophyl residues is not observed. Yet PEP binds to the cation-perturbed enzyme (Figure 2) and the enzyme is catalytically active at the lower temperatures. Therefore, the tryptophyl residues do not appear to be associated directly with the enzyme catalytic site but are perturbed indirectly as a result of the change in the protein conformation. Thus it can be argued that the enzyme exists as an equilibrium among protein conformers and the substrate (or cofactor) preferentially binds one conformer, shifting the equilibrium to form a different population of conformers as saturating levels of substrate or cofactor are reached. These results, while consistent with the "induced fit" hypothesis proposed by Koshland,[26] indicate the possibility of another mechanism by which the active conformations can be stabilized.

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