Water-Induced Transitions in the K^+ Requirements for the Activity of Pyruvate Kinase Entrapped in Reverse Micelles†

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ABSTRACT: The activity of pyruvate kinase was studied in reverse micelles formed with cetyltrimethylammonium bromide, *n*-octane, hexanol, and various amounts of water. In systems with 100% water, K^+ is an essential activator of pyruvate kinase [Kachmar, J. F., & Boyer, P. D. (1953) *J. Biol. Chem. 200,* 669–683]; i.e., without and with K^+, the activities observed were 0.07 and 300 μmol/(min·mg), respectively. In the micellar system with 3.6% water (v/v), pyruvate kinase exhibited an activity of about 45 μmol/(min·mg), in the absence of K^+. The k_{cat} was about 450 times larger than that in 100% water without K^+. K_m values for ADP and phosphoenolpyruvate differed, but not markedly from those in 100% water with or without K^+. The kinetics of pyruvate kinase in reverse micelles were not affected by K^+. The activity curve of pyruvate kinase in reverse micelles without K^+ in a pH range of 6.0–8.5 was almost superimposable to that of the enzyme in 100% water with K^+, and it differed drastically from that in 100% water without K^+. The fluorescence emission spectra of pyruvate kinase in 100% water exhibited a blue shift of 3 nm upon the addition of ligands (Mg^{2+}, phosphoenolpyruvate, and K^+) that cause a transition of the enzyme to its active state. Without ligands, the entrapment of pyruvate kinase in reverse micelles with 3.0% water produced a blue shift of nearly 2 nm with respect to that of the enzyme in 100% water without ligands. As water was raised to 7.0% (v/v), the maximal emission shifted to longer wavelengths; these changes paralleled the appearance of the K^+-dependent activity. The plot of the K^+-independent activity versus water concentration in the micellar system was bell-shaped with a maximum at 4% water (v/v). Apparently variations in the amount of water in contact with the enzyme induce structural transitions that reflect the K^+ requirements of the enzyme for carrying out catalysis.

The properties and the catalytic activity of enzymes have been extensively studied in systems that contain low amounts of water and in which the principal component is either an organic solvent (Luisi et al., 1988; Martinek et al., 1986; Klibanov, 1989) or a supercritical fluid (Hammond et al., 1985; Randolph, et al., 1988; Barzana et al., 1989). Enzymes placed in the former conditions acquire properties distinct from those that prevail in conditions with water in excess. For instance, under low water conditions, enzymes acquire a high thermostability (Wheeler & Croteau, 1986; Zaks & Klibanov, 1988; Ayala et al., 1986; Garza-Ramos et al., 1989) and carry out catalysis at temperatures that cause rapid inactivation in 100% water (Zaks & Klibanov, 1984; Garza-Ramos et al., 1990). In addition, in systems formed with organic solvents, enzymes may catalyze reactions that in totally aqueous mixtures are not easily observed; moreover, enzymes in such conditions also exhibit different substrate specificity (Zaks & Klibanov, 1986; Ryu & Dordick, 1989).

As the properties of enzymes are drastically modified when placed under conditions of limiting water, it was considered that the requirements for catalysis that an enzyme exhibits in conventional aqueous systems could be modified when it is transferred to a low water environment. This possibility was explored with pyruvate kinase, since in addition to substrates, catalysis by this enzyme depends on the presence of divalent cations and, of particular importance to this work, on K^+, which is an essential activator of the enzyme (Kachmar & Boyer, 1953).

Pyruvate kinase catalyzes the transfer of the phosphoryl group of phosphoenolpyruvate to ADP, the reaction being largely favored toward the formation of pyruvate and ATP (McQuate & Utter, 1959). Pyruvate kinase requires Mg^{2+} for activity (Mildvan & Cohn, 1965), but Mn^{2+} will also activate the enzyme (Boyer, 1962). Pyruvate kinase also requires K^+ for maximal activity (Boyer, 1962); Rb^+, NH_4^+, and Tl^+ (Kayne, 1971) may partially substitute for K^+. In the presence of Na^+, the enzyme exhibits about 8% of the maximal activity and hardly any activity (0.02%) with tris(hydroxymethyl)aminomethane (Kayne, 1971) or substituted amines (Nowak, 1976).

From NMR data, Mildvan and Cohn (1965) concluded that K^+ affects the conformation of pyruvate kinase only in the presence of PEP[1] and Mn^{2+}. The detailed analysis of the events at the catalytic site studied by NMR techniques indicate that K^+ coordinates the carboxyl group of PEP aligning the phosphoryl group for nucleophilic attack by ADP (Nowak & Mildvan, 1972). Kinetic data with analogs of PEP (Doughtery & Cleland, 1985b) and alternate substrates (Cottam et al., 1968; Doughtery & Cleland, 1985a), as well as the ^3H-trapping experiments of Rose and Kuo (1989), substantiate this model. Although the position of K^+ at the catalytic site of pyruvate kinase has not been directly documented, the available crystallographic data (Stuart et al., 1979; Muirhead et al., 1986, 1987) support the conclusions derived from NMR and kinetic data. Moreover, numerous studies with a wide variety of methodologies (Kayne & Suelter, 1965; Suelter, 1967; Consler & Lee, 1988; Consler et al., 1988) have established

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane.

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that K^+ in conjunction with catalytic site ligands, Mg^{2+} or Mn^{2+}, and PEP induce the formation of the active conformer.

The results of this work show that in the absence of K^+ pyruvate kinase entrapped in reverse micelles exhibits a catalytic activity that is nearly 20% that expressed under optimal conditions in 100% water with K^+. Also as evidenced by its fluorescence spectra, pyruvate kinase in a low water environment without K^+ acquires an active conformation. Thus, fluorescence and activity data suggest that in the absence of K^+ the equilibrium between active and inactive conformations can be controlled by the amount of water in contact with the enzyme.

MATERIALS AND METHODS

Pyruvate kinase from rabbit muscle and heart lactate dehydrogenase were obtained as ammonium sulfate suspensions from Boehringer. The sodium or cyclohexylammonium salts of ADP and PEP, NADH, *n*-octane, hexanol, and cetyltrimethylammonium bromide were from Sigma.

In the assays of pyruvate kinase activity in 100% water, the formation of pyruvate was measured in a coupled system with lactate dehydrogenase and NADH (Büchner et al., 1955). The volume of the reaction was 1 mL. It contained the concentrations of ADP, PEP, $MgCl_2$, and KCl indicated in the Results and Discussion section. Unless otherwise indicated, the buffer was 80 mM Tris-HCl, pH 7.6. In all experiments, the mixture contained 0.24 mM NADH and an amount of lactate dehydrogenase that exhibited an activity at least 10 times higher than that of the measured pyruvate kinase activity. Activity was initiated by introducing pyruvate kinase, and the decreases in absorbance at 340 nm in 1–3 min of reaction time were used for the calculation. Over this period of time, activity was linear.

Reverse micelles were formed in a mixture of 200 mM cetyltrimethylammonium bromide in *n*-octane and hexanol (9:1 v/v) and different amounts of water. The phase diagrams of this system have been described (Laane et al., 1987; Garza-Ramos et al., 1992a). This system has been used for the measurement of several enzyme activities (Hilhorst et al., 1983, 1984; Tyrakowska et al., 1990; Fernández-Velasco et al., 1992), including those that require a trapping enzyme (Laane & Verhaert, 1988; Garza-Ramos et al., 1992b). For assays of enzyme activity in reverse micelles, the reaction mixtures containing the substrates and activators were introduced into the system as water solutions. Micelles were formed by vigorous stirring in vortex. After formation of the micelles, lactate dehydrogenase was introduced followed by the addition of pyruvate kinase to start the reaction. After being vigorously stirred, the mixture was transferred to a spectrophotometer cuvette and the absorbance changes at 340 nm in 1–3 min of reaction time were recorded and used to calculate the activity of pyruvate kinase. The concentrations of ADP, PEP, $MgCl_2$, and KCl are described in the Results and Discussion section. The concentrations indicated refer to those in the water phase of the system. In all experiments, 1 mL of the reverse micelle system contained around 3 μg of lactate dehydrogenase with a specific activity of about 320 μmol/(min·mg) in all water media and 240 nmol of NADH. The activity of lactate dehydrogenase was at least in 10-fold excess over that of pyruvate kinase. With the amount of lactate dehydrogenase used, activity of pyruvate kinase was proportional to concentration in the range of 0.1–0.7 μg of pyruvate kinase/mL of reverse micelle system. Moreover, doubling the concentration of lactate dehydrogenase did not change the activity of pyruvate kinase. In all experiments, pyruvate kinase activity was linear with time until substrate or NADH became limiting.

The formation and the transfer of substrates to the reverse micelles system were carried out at room temperature. The activities in 100% water and in reverse micelles were made at 25 °C.

For the experiments, aliquots of commercial ammonium sulfate suspensions of lactate dehydrogenase and pyruvate kinase were centrifuged. The sediment was dissolved in 100 μL of 10 mM Tris-HCl, pH 7.6, and applied to Sephadex G-50 (fine) filtration–centrifuge columns (Kasahara & Penefsky, 1978). The filtrates were filtered for a second time through similar centrifuge columns. The columns were equilibrated with 10 mM Tris-HCl, pH 7.6. The filtrates obtained after the second filtration were used for measurements of enzyme activity.

The contents of K^+ and NH_4^+ in the chemicals and solvents used in this work were determined by atomic absorption spectroscopy and by the method of Chaney (1962), respectively. The limit of detection for K^+ and NH_4^+ was 10 μM. The procedure for measuring the content of K^+ and NH_4^+ in the organic solvents was as follows: the solvents were vigorously stirred for 60 min with an equal volume of 1 M HCl. After phase separation, the contents of K^+ and NH_4^+ in the water phase were determined. NH_4^+ was also determined in the enzymes after the aforementioned second filtration.

The fluorescence emission spectra of pyruvate kinase in 100% water and in reverse micelles were obtained on an ISS Inc. (Champaign, IL) GREG 200 spectrofluorometer with excitation and emission slits set at 4 nm. Excitation was at 295 nm. The composition of the mixtures is detailed under Results and Discussion, but in all cases the samples contained 75 μg of pyruvate kinase/mL of water mixtures or reverse micelle system. The fluorescence spectra of blanks (no protein) were substracted from those that contained the enzyme. From the latter difference, the spectral center of mass, or average emission wavelength was calculated according to Bismuto et al. (1987) as

$$\lambda_{av} = \frac{\int \lambda I(\lambda)\, d\lambda}{\int I(\lambda)\, d\lambda}$$

where λ and $I(\lambda)$ are wavelength (in nanometers) and fluorescence intensity at a given wavelength, respectively. The standard deviation (SD) was given by

$$SD = \frac{\int \lambda^2 I(\lambda)\, d\lambda}{\int I(\lambda)\, d\lambda}$$

Protein concentrations were determined by measuring the absorbance at 280 nm and using the absorptivity of 0.54 and 1.5 mL mg^{-1} cm^{-1} for pyruvate kinase (Cottam et al., 1969) and lactate dehydrogenase (Pesce & Mc Kay, 1964), respectively.

RESULTS AND DISCUSSION

In confirmation of previous reports (Kayne, 1971), it was observed that in 100% water the inclusion of K^+ in the reaction mixture increased the activity of pyruvate kinase from less than 0.1 to 300 ± 20 μmol/(min·mg). However, pyruvate kinase transferred to reverse micelles formed with 3.6% (v/v) water in the absence of K^+ expressed an activity of approximately 45 μmol/(min·mg). This activity was absolutely dependent on the presence of PEP, ADP, and Mg^{2+} and was

Table I: Kinetics of Pyruvate Kinase in Mixtures with 100% Water and in Reverse Micelles with and without K^+ [a]

substrate	-KCl k_{cat} (s^{-1})	-KCl K_m (mM)	-KCl log (k_{cat}/K_m)	+KCl k_{cat} (s^{-1})	+KCl K_m (mM)	+KCl log (k_{cat}/K_m)
			100% Water			
ADP	0.42 ± 0.03	3.84 ± 0.43	2.0	1457 ± 33	0.53 ± 0.04	6.4
PEP	0.38 ± 0.02	0.28 ± 0.04	3.1	1354 ± 22	0.12 ± 0.01	7.0
			Reverse Micelles (3.6% H_2O)			
ADP	211 ● 18	1.05 ± 0.2	5.3	268 ± 27	1.21 ± 0.26	5.4
PEP	153 ± 7	1.39 ± 0.18	5.0	208 ± 19	2.42 ± 0.28	4.9

[a] In mixtures with 100% water, 1 mL of the reaction media contained 80 mM Tris-HCl, pH 7.6, 7.5 mM $MgCl_2$, 240 nmol of NADH, 3 μg of lactate dehydrogenase, and concentrations of ADP and PEP (cyclohexylammonium salts) that varied between 0.5 mM and 7.5 mM, respectively. Where indicated, KCl was introduced at a concn. of 75 mM. The reaction was started by the addition of pyruvate kinase, either 0.1 μg or 40 μg for the assays with and without K^+, respectively. Where shown, activity was also measured in reverse micelles (see Materials and Methods section) that contained 3.6% water (v/v). One milliliter of reverse micelles contained 80 mM Tris-HCl, pH 7.6, 3 mM $MgCl_2$, ADP and PEP concentrations that varied from 0.5 mM to 4 mM, and 30 mM KCl were shown. These concentrations refer to those in the water phase of the system. In addition, 1 mL of the reverse micelles system contained 240 nmol of NADH and 3 μg of lactate dehydrogenase. The reaction was started by the addition of 0.3 μg of pyruvate kinase. The temperature was 25 °C. Catalytic efficiency is expressed as k_{cat}/K_m. The log values of the ratio are shown. The standard errors from two or three different experiments are shown.

not increased by the inclusion of K^+ up to a concentration of 75 mM, or by NH_4^+ or Tl^+. This K^+-independent activity was proportional to the amount of enzyme in the range of 0.1–0.7 μg/mL of reverse micelle system. Activity was linear up to the time in which substrate concentration for pyruvate kinase, or NADH concentration for lactate dehydrogenase (the trapping enzyme), became limiting.

The contents of K^+ and ammonium in every reagent used for the assay of activity, as well as in cetyltrimethylammonium bromide and the solvents used for the formation of reverse micelles, were below the limits of detection (10 μM). The amount of NH_4^+ in the enzymes recovered after two filtrations (see Materials and Methods) was also below the limits of detection. It was also considered that cetyltrimethylammonium bromide could affect the response of pyruvate kinase to K^+. In experiments in 100% water, it was found that the surfactant in concentrations below and above its critical micellar concentration (0.1–2 mM) did not affect the requirements of the enzyme for K^+. However, it is noted that increasing concentrations of cetyltrimethylammonium inhibited the activity; i.e., 2 mM produced an inhibition of 40%. Of relevance is that, in previous work, Nowak (1976) showed that dimethylammonium, trimethylammonium, and tetramethylammonium chlorides did not activate pyruvate kinase. Additional controls for the experiments of Table I were (a) use of sodium salts instead of cyclohexylammonium salts of ADP and PEP and (b) assay of activity in Tris-HCl, Tris–phosphate, and Tris–acetate buffers. In all conditions, the K^+-independent activity of pyruvate kinase in reverse micelles was essentially the same.

Kinetics of the K^+-Independent Pyruvate Kinase Activity. In 100% water, the kinetics of pyruvate kinase in the presence of K^+ have been extensively studied (McQuate & Utter, 1959). Although the requirements for K^+ on the partial reactions catalyzed by pyruvate kinase have been thoroughly investigated (Tietz & Ochoa, 1958; Cottam et al., 1968; Robinson & Rose, 1972; Kayne, 1974), there are only a few results on the kinetics of the enzyme without K^+. Here, we compared the kinetics

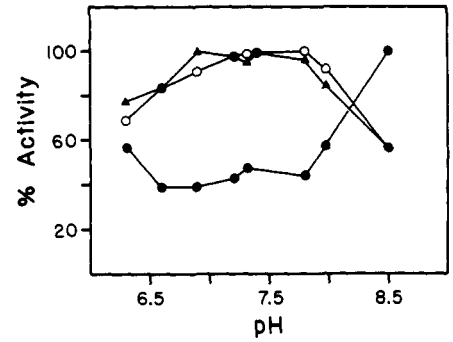

FIGURE 1: Activity of pyruvate kinase in 100% water and in reverse micelles at various pHs. In all water media, activity was measured as in Table I with 7.5 mM ADP and 7.5 mM PEP; (○) indicates percent of activity in mixtures with 100% water that contained 75 mM KCl, whereas (●) illustrates activity without KCl. Activity without K^+ in reverse micelles with 3.6% water (v/v) was measured with 7.5 mM $MgCl_2$, 7.5 mM ADP, and 7.5 mM PEP (▲). The buffer in all experiments was 80 mM Tris–phosphate; the rest of the conditions were as in Table I. The pH indicated refers to that of the reaction mixture of the solution injected to form the micelles. The data were normalized considering the maximal activity detected as 100%; these were 270 μmol/(min·mg) (○), 0.07 μmol/(min·mg) (●) and 44 μmol/(min·mg) (▲).

of pyruvate kinase entrapped in reverse micelles with 3.6% water (v/v) to those in 100% water with and without K^+.

In agreement with the early experiments of Kachmar and Boyer (1953), it was found that in 100% water k_{cat} was several orders of magnitude higher with K^+ than without K^+. This was the most obvious effect of K^+ on the kinetics of the enzyme, albeit, as shown in Table I, K^+ also modified the K_m values for ADP and PEP (Table I). Mostly as a result of the effect of K^+ on k_{cat} values, the catalytic efficiency of the enzyme (log k_{cat}/K_m; in Table I) was about 10^4 times higher in the presence of K^+.

A notable characteristic of the kinetics of pyruvate kinase in reverse micelles (Table I) was that in the absence of K^+ k_{cat} was about 450 times higher than in 100% water without K^+. Pyruvate kinase in reverse micelles exhibited K_m values for ADP three times lower and K_m values for PEP five to six times higher than in 100% water without K^+. The catalytic efficiency of the enzyme was 2–3 orders of magnitude higher in reverse micelles than in 100% water without K^+. It is also of relevance that the addition of K^+ to the reverse micelle system hardly affected the kinetics of the enzyme (Table I).

As noted, the results with pyruvate kinase entrapped in reverse micelles described in Table I were obtained from experiments carried out with the cyclohexylammonium salts of ADP and PEP, but similar results were obtained with the sodium salts (not shown).

pH Activity Profiles of Pyruvate Kinase in 100% Water Mixtures and in Reverse Micelles. The data described on the characteristics of the activity of pyruvate kinase in the interior of reverse micelles without K^+ appeared to indicate that in a low water environment the enzyme acquired a catalytically competent conformation that could resemble that attained in 100% water with K^+. In fact, data on the activity of pyruvate kinase as a function of pH in 100% water and in reverse micelles with 3.6% water (v/v) (Figure 1) supported this idea. In 100% water with K^+, the activity curve was bell-shaped with a plateau between pH 7.0 and 7.8, which agrees with the profile reported by Gregory and Ainsworth (1981). In the absence of K^+, the pH activity profile was drastically different; i.e., maximal activity was at pH 8.5. In reverse micelles that did not contain K^+, the pH activity curve was almost superimposable with that in 100% water with K^+.

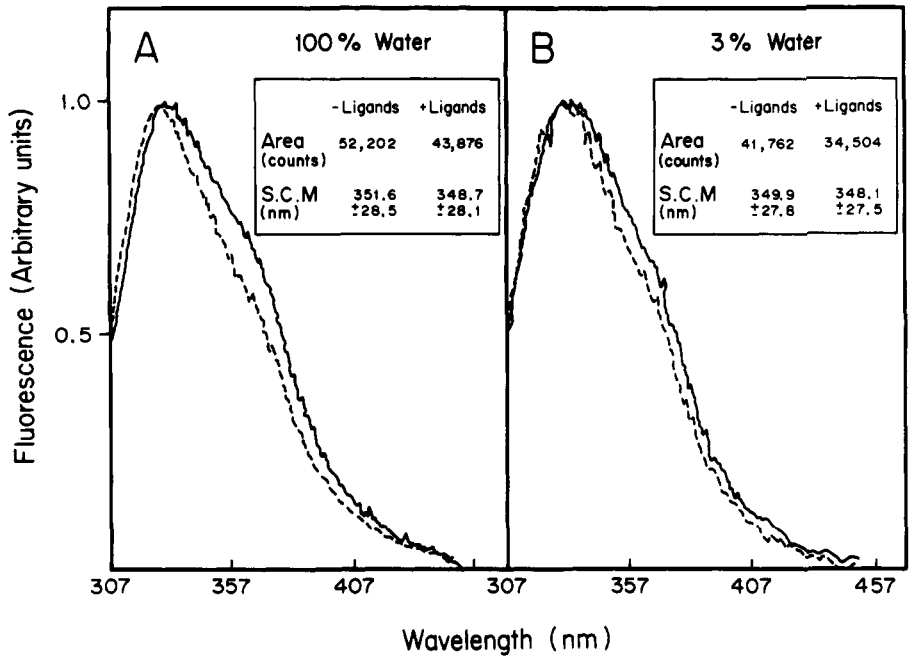

FIGURE 2: Fluorescence emission spectra of pyruvate kinase in all water media and in reverse micelles with and without ligands. The spectra were recorded at an excitation wavelength of 295 nm. The spectra in 100% aqueous media (A) were obtained from mixtures that contained pyruvate kinase at a concentration of 75 μg/mL in 80 mM Tris-HCl, pH 7.6 (continuous line); the trace illustrated by the dashed line also contained 7.5 mM PEP, 3 mM $MgCl_2$, and 30 mM KCl. Panel B shows the spectra in reverse micelles with 3.0% water (v/v) that contained 75 μg of pyruvate kinase/mL of reverse micelles. The continuous and the dashed lines correspond to spectra of micelles that contained 80 mM Tris-HCl, pH 7.6, without and with ligands, respectively. The inset shows the area of the curve throughout the whole emission spectra in counts, and the spectral center of mass (SCM) was calculated as described under Materials and Methods. The spectra shown were obtained after substracting the spectra of the blanks which had identical compositions from the experimental, except for the enzyme. The spectra were normalized to maximal emission = 1.

Fluorescence of Pyruvate Kinase in Reverse Micelles and in All Aqueous Media. The NMR studies by Nowak and Mildvan (1972) indicate that in the presence of Mn^{2+} there is an interdependence in the binding of PEP and K^+ at the catalytic site. These observations together with kinetic (Reuben & Kayne, 1971) and crystallographic (Muirhead et al., 1986, 1987) data indicate that in the presence of Mg^{2+} or Mn^{2+}, K^+ coordinates to the carboxyl group of PEP. The ligand-induced arrangements of the catalytic site produce the catalytically active conformation.

The active and inactive conformations of pyruvate kinase can be visualized by differences in ultraviolet and fluorescence spectra. These have been attributed to perturbations of tryptophan residues (Suelter & Melander, 1963; Kayne & Suelter, 1965; Suelter, 1967). In pyruvate kinase there is a tryptophan in a region identified as domain B. In the inactive conformation, this region is partially exposed to the solvent (Muirhead, 1986; Consler et al., 1988). The other two tryptophan residues lie within domain C of the protein (Muirhead, 1986). Regarding fluorescence of pyruvate kinase in 100% water, Suelter (1967) showed that the binding of Mn^{2+} and K^+ to the enzyme produces a blue shift of 2–3 nm of its polarization spectra. By using small-angle neutron scattering, Consler et al. (1988) found that the binding of PEP produces an additional conformational change of the enzyme–metal complex.

We recorded the emission fluorescence spectra of pyruvate kinase in reverse micelles and compared them to those obtained in 100% water with and without the ligands (K^+, Mg^{2+}, and PEP) that produce the active conformation. In accordance with reported data (Suelter, 1967), the emission spectrum of pyruvate kinase in mixtures in 100% water that contained ligands was 3 nm lower than in their absence (Figure 2A). The fluorescence spectra of pyruvate kinase entrapped in reverse micelles (Figure 2B) with 3.0% water (v/v) differed from those in 100% water in several respects. The area of the emission spectra of pyruvate kinase was 20% smaller than in 100% aqueous media; (see insets in Figure 2), and its spectral center of mass (see Materials and Methods section) was about 1 nm lower than in 100% water without ligands. Also, it was observed that in reverse micelles the ligand-induced shift in the peak of maximal fluorescence was less marked than in 100% water (Figure 2B). On the other hand, in the presence of ligands, in both, reverse micelles and 100% water, the spectral centers of mass were strikingly similar. Thus, in the overall data of Figure 2 it is apparent that in the absence of ligands pyruvate kinase in reverse micelles exhibited a fluorescence emission that approached that observed in 100% water with ligands.

Effect of Water on the Fluorescence and Activity of Pyruvate Kinase Entrapped in Reverse Micelles. The spectral center of mass of pyruvate kinase entrapped in reverse micelles without K^+ was determined at various water concentrations. As the amount of water was increased, the spectral center of mass progressively shifted to longer wavelengths (Figure 3). These shifts could be due to structural arrangements of the protein or to changes in the environment of tryptophan residues on placement in reverse micelles with different water contents. Therefore, it was explored if the fluorescence of free tryptophan transferred to reverse micelles was affected by the amount of water. The intensity of tryptophan fluorescence in reverse micelles was 20% less than that observed in 100% aqueous media (not shown). A similar decrease in fluorescence intensity of pyruvate kinase entrapped in reverse micelles was observed (Figure 2). In addition, there was a blue shift in the spectral center of mass of tryptophan, i.e., 365 nm in 100% water and 359 nm in reverse micelles. This probably indicates a relatively less polar environment of the interior of the reverse micelles. However, in contrast to pyruvate kinase, the spectral center of mass of tryptophan was not affected by the amount

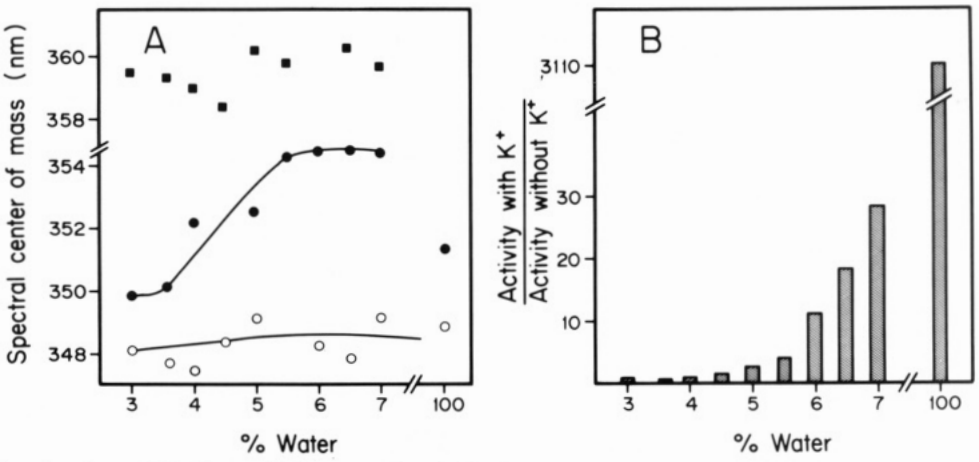

FIGURE 3: Spectral center of mass (A) of pyruvate kinase and tryptophan in reverse micelles with different water concentrations and (B) activity ratios of pyruvate kinase in reverse micelles with and without K^+. In (A), the experimental conditions were as in Figure 2 except that the amount of water in the reverse micelle system was as indicated. (●) and (○) show the spectral center of mass obtained from emission spectra obtained from reverse micelles without and with ligands, respectively. (■) shows the spectral center of mass of 10 μM tryptophan transferred to reverse micelles with the indicated water concentrations. (B) shows the ratio of activities with and without 75 mM KCl in reverse micelles that had the indicated water concentration. Activities were measured as in Table I with 7.5 mM ADP and 7.5 mM PEP.

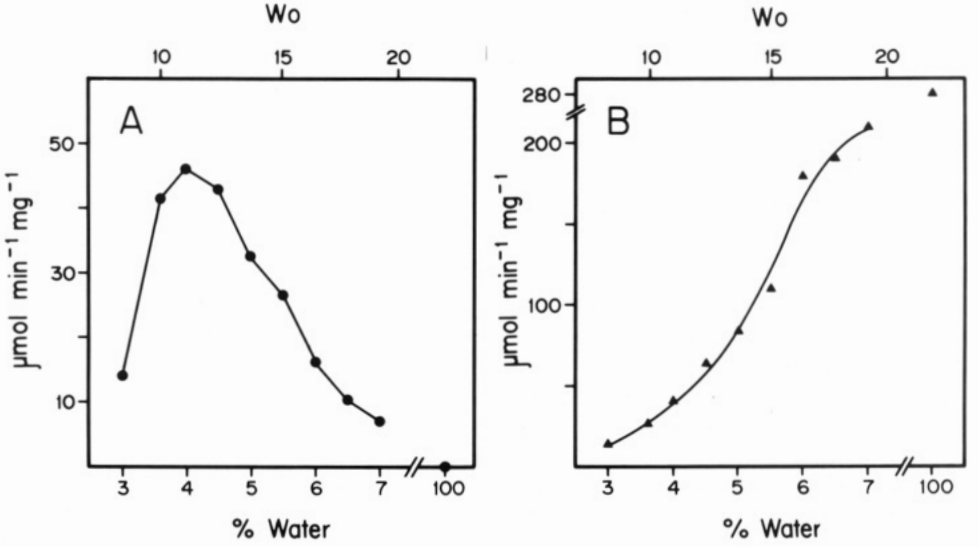

FIGURE 4: Activity of pyruvate kinase in reverse micelles without (A) and with (B) 75 mM KCl at various water concentrations. The experimental conditions were as in Table I with 7.5 mM ADP, 7.5 mM PEP, and the indicated water concentrations.

of water (Figure 3). Therefore, it is very likely that the distinct fluorescence pattern of pyruvate kinase at various water concentrations reflects different conformations of the protein that affect the environment of one, or all, of the tryptophan residues.

Fluorescence data were also collected with pyruvate kinase entrapped in reverse micelles that contained the ligands (Mg^{2+}, PEP, and K^+) that produce the active conformation. Under these conditions, the differences in the spectral center of mass at various water concentrations were rather small (Figure 3A). Thus, the overall data of Figure 3A indicate that in reverse micelles, the conformation that pyruvate kinase acquires depends on two factors: the presence of ligands and the amount of water in contact with the enzyme. In addition, it would appear that without ligands the enzyme has a structure that is susceptible to modulation by water, whereas in the presence of ligands, the enzyme is locked in a conformation that is unaffected by water concentration.

The characteristic patterns of fluorescence emission of the enzyme with water concentration in the absence of ligands suggested that the amount of water in contact with pyruvate kinase could be central in the equilibrium between the catalytically active and inactive conformers. Hence, it was explored if the fluorescence characteristics of pyruvate kinase at various water concentrations were accompanied by distinct K^+ requirements in catalysis. It was found that as the water content of the micellar system was gradually increased, there was a progressive rise in the ratio of activities with and without K^+ (Figure 3B). In fact, the ratio of activities with and without K^+ probably indicates an equilibrium between active and inactive conformations.

Effect of Water on the Rate of Catalysis by Pyruvate Kinase. It has been described that the activity of numerous enzymes entrapped in reverse micelles, including the type of micelles employed here (Laane et al., 1987), depends on the amount of water (Hilhorst et al., 1983, 1984; Laane et al., 1987; Martinek et al., 1986; Luisi et al., 1988; Tyrakowska et al., 1990, Fernández-Velasco et al., 1992; Garza-Ramos et al., 1992b). The data of Figure 4 show how the amount of water in contact with pyruvate kinase affects its activity in the presence and absence of K^+. The plot of the K^+-independent activity versus the amount of water (Figure 4A) was bell-shaped with a maximum at about 4% water (ratio of water to surfactant molecules, Wo, = 11). In contrast, the activity

with K^+ increased with the amount of water (Figure 4B). This pattern most likely reflected the water requirements for catalysis that have been documented for many enzymes.

At low amounts of water, conformational mobility of enzymes is severely restricted due to hindrances in solvent–protein interactions. As water is progressively raised, enzyme flexibility increases and activity becomes higher (Careri et al., 1980; Finney & Poole, 1984; Garza-Ramos et al., 1992a). Thus, regarding the data of Figure 4, it would appear that below 4% water, activity is independent of K^+ but restricted due to impairments in solvent–protein interactions [for review, see Garza-Ramos et al. (1992c)]. As water is raised above 4%, and the enzyme acquires a higher flexibility, the K^+-independent activity decreases simultaneously with the appearance of the K^+-dependent activity (Figures 3B and 4). Therefore, it would appear that the amount of water in contact with pyruvate kinase is central in the prevalence of enzyme conformations with distinct catalytic and fluorescence properties.

At the moment, the intimate molecular mechanisms that induce in pyruvate kinase the ability to function in the absence of K^+ are not known. Indeed, due to the nature of the experimental system employed, it is possible that the amount of water, as well as interactions of the protein with the micellar wall, accounts for the described behavior and structural features of pyruvate kinase. In this respect, it is relevant that Desfosses et al. (1991) suggested that the blue shift of 17 nm in fluorescence emission produced by entrapping human serum albumin in reverse micelles could be due to alterations of protein structure induced by its attachment to the micellar interphase. Strambini and Gonelli (1988) reached similar conclusions from phosphorescence data on tryptophan of liver alcohol dehydrogenase entrapped in reverse micelles. Regarding this possibility, the data of this work show that at different amounts of water distinct fluorescence patterns of pyruvate kinase occur in parallel to the expression of the K^+-independent activity (Figures 3 and 4). If under identical conditions ligands of the enzyme are included, these changes do not take place. Therefore, unless water only affects the interaction of the protein with the micellar wall when ligands are absent, the results would suggest that the amount of water in contact with the enzyme controls the structural and catalytic characteristics of pyruvate kinase.

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