Subunit Structure and Hybrid Formation of Bovine Pyruvate Kinases†

Janet M. Cardenas,* Donald R. Hubbard,‡ and Sonia Anderson

ABSTRACT: After denaturing either type M or L pyruvate kinase by guanidine hydrochloride, urea, or low pH, enzymatic activity and quaternary structure can be recovered by diluting the enzyme into buffer containing β-mercaptoethanol. After denaturation of type M pyruvate kinase by guanidine hydrochloride, the yield and polarization of the intrinsic protein fluorescence, as well as most of the circular dichroism characteristic of the native enzyme, were regained very rapidly, while enzymatic activity was recovered much more slowly. Under the conditions used, about 50% of the original M and 30–50% of the original type L activity were typically recovered. Average half-times for recovery of enzymatic activity were 37 min for type M and 104 min for type L but depended somewhat on the renaturation buffer and on protein concentrations in the renaturation medium. If types M and L pyruvate kinases are renatured together, an approximately random recombination of the two subunits types results in a five-membered hybrid set. We have used this hybridizability to determine the kinetics of reformation of the native tetramer by denaturing each isozyme and beginning its renaturation separately at various times mixing the two isozymes and continuing their renaturation together. These studies indicate that reformation of stable tetramers occurs relatively slowly, qualitatively paralleling the regain of enzymatic activity, and that tetramer formation may be necessary for enzymatic activity. Using a similar technique to test for spontaneous dissociation of the native isozymes in buffer, we find that type L, but not type M, reversibly dissociates into dimers and monomers in buffer solutions. This dissociation is decreased by the presence of the substrate, phosphoenolpyruvate, by Mg^{2+} ions, or by the allosteric effector, fructose bisphosphate.

At least three distinct isozymes of pyruvate kinase differing in their physical and kinetic properties are known to exist in mammals (Susor and Rutter, 1968, 1971; Imamura and Tanaka, 1972; Whittell et al., 1973; Strandholm et al., 1976). Type M, or M_4, the predominant form in skeletal muscle, normally has hyperbolic kinetics with phosphoenolpyruvate and is unaffected by fructose 1,6-bisphosphate. Type L, or L_4, the main isozyme from liver, has sigmoidal kinetics with phosphoenolpyruvate and is activated to give hyperbolic kinetics by fructose 1,6-bisphosphate. Type K, or K_4, is the main form in adult kidney and other internal organs and appears to be the primordial form. It is immunologically cross-reactive with type M but electrophoretically distinguishable from it and has kinetic properties intermediate between those of types M and L.

The isozymes can be reversibly denatured by guanidine hydrochloride and the enzymatic activity then recovered by dilution of the denatured enzyme into buffer containing either dithiothreitol or β-mercaptoethanol (Johnson et al., 1969; Cottam et al., 1969; Cardenas and Dyson, 1973; Cardenas et al., 1975; Strandholm et al., 1976).

When L_4 and M_4 are denatured and renatured together, the result is a five-membered hybrid set consisting of L_4, L_3M, L_2M_2, LM_3, and M_4, the letters and subscripts indicating the apparent subunit composition of each species (Cardenas and Dyson, 1973). The hybrids have been previously separated by isoelectric focusing and characterized (Dyson and Cardenas, 1973). Although low levels of contamination of individual hybrids by other members of the set could occasionally be seen, their tetrameric structure appeared to be quite stable, with no detectable interconversion.

In this paper we report the kinetics of renaturation as determined by monitoring changes in fluorescence and circular dichroism, enzymatic activity, and the availability of enzyme subunits to form hybrids with the other subunit type. We have also found hybridizability to be an extremely sensitive tool for detecting spontaneous dissociation of pyruvate kinase isozymes under nondenaturing conditions.

Experimental Procedure

Guanidine hydrochloride was obtained from Heico Inc., Delaware Water Gap, Pa. Enzyme substrates and other special chemicals were from Sigma Chemical Corp. Cellulose acetate strips and apparatus were obtained from the Gelman Instrument Co. All standard chemicals were reagent grade. Distilled, deionized water was used for making all solutions. Bovine types M and L pyruvate kinases were prepared as described previously (Cardenas et al., 1973; Cardenas and Dyson, 1973).

Enzyme activities were measured by the method of Bücher and Pfleiderer (1955) using an assay medium containing 0.1 M KCl, 0.05 M imidazole hydrochloride, 10 mM $MgCl_2$, 2 mM ADP, 1 mM phosphoenolpyruvate, 0.5 mM fructose 1,6-bisphosphate, and 5 units of lactate dehydrogenase, pH 7.0 at 25 °C.

Both denaturation and renaturation buffers contained 0.05 M Tris[1]-HCl, pH 7.5, 0.1 M KCl, 0.5 M sucrose, 1 mM EDTA, and 50 mM β-mercaptoethanol. Enzymes were centrifuged down from ammonium sulfate suspensions and dissolved in the buffer. Protein denaturation was accomplished by methods similar to those used previously (Johnson et al., 1969; Tobes et al., 1972; Cardenas and Dyson, 1973) as follows: A sufficient volume of 7 M guanidine hydrochloride was added to the enzyme solution at 0 °C to give a final concentration of 3.0–3.5 M. This concentration of guanidine hydrochloride dissociates pyruvate kinase to monomers as deter-

† From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. *Received August 17, 1976.* This work was supported by National Institutes of Health Grants AM-15645 and AM-13192.

‡ Present address: Institute of Paper Chemistry, Appleton, Wisconsin 54911.

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

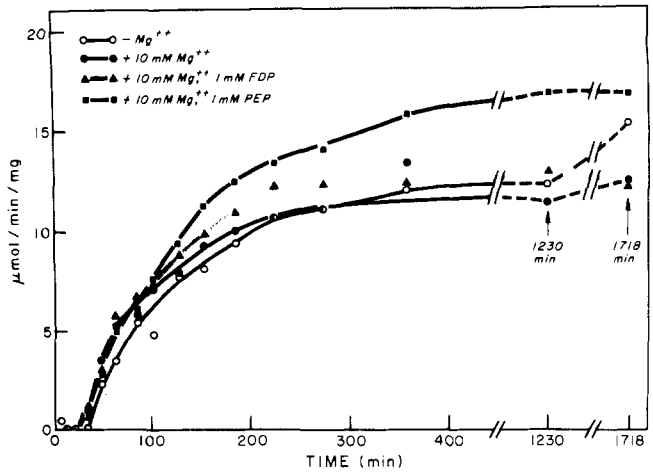

FIGURE 1: Recovery of type L pyruvate kinase activity after denaturation in 3.5 M guanidine hydrochloride, as a function of additives to the renaturation buffer. Denaturation was at 0 °C for 5 min and renaturation was at 15 °C as described in the text. The protein concentration was 16 μg per ml. The control curve (open circles) represents renaturation in 0.05 M Tris-HCl, pH 7.5, 0.1 M KCl, 0.5 M sucrose, 1 mM EDTA, 50 mM β-mercaptoethanol. The other curves were produced in the above buffer to which the following additions were made: (closed circles) 10 mM $MgCl_2$; (closed triangles) 10 mM $MgCl_2$ and 1 mM fructose biphosphate; (closed squares) 10 mM $MgCl_2$ and 1 mM phosphoenolpyruvate.

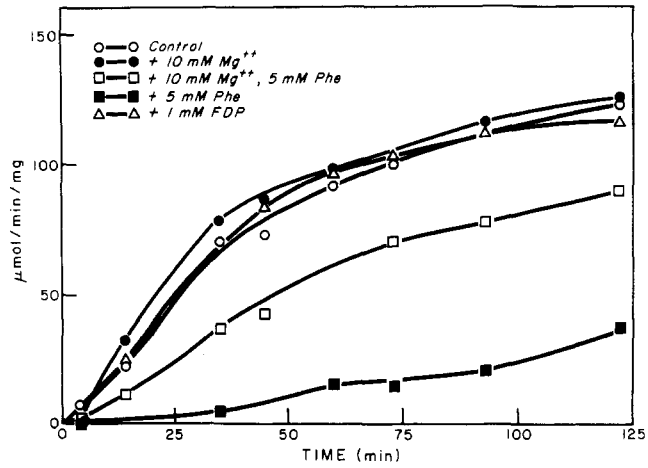

FIGURE 2: Recovery of type M pyruvate kinase activity after denaturation in 3.5 M guanidine hydrochloride, as a function of additives to the renaturation buffer. The protein concentration was 12 μg per ml. Denaturation and renaturation were accomplished as described for Figure 1. The control buffer (open circles) is the same as that described for Figure 1, to which the following additions were made: (closed circles) 10 mM $MgCl_2$; (open squares) 10 mM $MgCl_2$ and 5 mM phenylalanine; (closed squares) 5 mM phenylalanine; (open triangles) 1 mM fructose bisphosphate. Note the different time scales for renaturation of the two isozymes.

mined by sedimentation equilibrium analysis (R. D. Dyson, personal communication) and was shown to destroy all enzymatic activity. After 5 min, an aliquot of the enzyme solution in guanidine hydrochloride was diluted by gentle swirling into at least 25 volumes of the buffer described above in a plastic test tube at 15 °C and the solution was incubated at that temperature for 6–8 h. The renatured enzyme was then dialyzed overnight vs. electrophoresis buffer (0.02 M Tris (pH 7.5 at room temperature), 0.5 M sucrose, 1 mM EDTA, 1 mM fructose 1,6-bisphosphate, 10 mM β-mercaptoethanol), then concentrated by dialyzing for 2–3 h against the same buffer made 2 M in sucrose.

This renaturation procedure resulted in recovery of 50–60% of the original type M pyruvate kinase activity and 30–40% of the original type L activity.

Fluorescence measurements were performed at 15 °C in a Hitachi Perkin-Elmer MPF 2A fluorometer. The emission spectra were recorded using an exciting wavelength of 280 nm. The band-pass of excitation was 10 nm and that of emission was 5 nm. The spectra were corrected for the minor background fluorescence of the solvent and for the wavelength dependence of the detector response. For fluorescence polarization, the excitation and emission wavelengths were 290 and 360 nm, respectively. The band-pass of excitation was 5 nm, while that of emission was 30 nm. The broad emission band-pass embraces a large portion of the emission spectrum, thus enhancing sensitivity, but is sufficient to exclude scattered exciting light. Under these conditions, the fluorescence intensity is almost entirely due to the tryptophan residues.

Circular dichroism spectra were obtained at the same temperature using Jasco J-10 and J-41 recording spectrometers. For the circular dichroism, sucrose was eliminated from the buffer and 1 mM dithiothreitol was substituted for 50 mM β-mercaptoethanol in order to reduce the light absorbance of the solvent. The rate of renaturation of type M pyruvate kinase was approximately the same in this solvent as in the standard renaturation buffer used for most of the experiments.

Electrophoresis was performed on cellulose acetate membranes according to the procedures described by Susor and Rutter (1971) and by Cardenas and Dyson (1973). The electrophoresis buffer contained 0.5 M sucrose, 0.02 M Tris, pH 7.5 at room temperature, 10 mM β-mercaptoethanol, 1 mM EDTA, and 1 mM fructose 1,6-bisphosphate. Electrophoresis was approximately 3 h at 250 V. Electrophoretic patterns were recorded with Kodabromide F-5 paper.

In order to test for subunit dissociation in dilute buffer, solutions containing the native enzyme to be analyzed were incubated at 15 °C for 1 h. The other isozyme was denatured in guanidine hydrochloride and then diluted into renaturation buffer at 0 °C immediately prior to mixing it with the sample of native enzyme. Final guanidine hydrochloride concentrations were 0.05–0.2 M, or lower.

Results

Shown in Figures 1 and 2 are the renaturation curves of types L and M pyruvate kinases at 16 and 12 μg/ml, respectively, as a function of additions to the standard buffer. Note the initial lags, during which time little or no enzymatic activity can be observed. Addition of phosphoenolpyruvate to the renaturation medium appears to increase somewhat the extent of renaturation of type L (Figure 1), but renaturation rates remain fairly constant whether or not 10 mM $MgCl_2$ and/or 1 mM fructose bisphosphate are added. The half-time for recovery of enzymatic activity is approximately 100 min for type L under these conditions. As with type L, the rate of recovery of enzymatic activity for type M pyruvate kinase is approximately the same in the presence or absence of Mg^{2+} or fructose bisphosphate, but the half-time for recovery of enzymatic activity was increased from about 20 min to around 120 min when 5 mM phenylalanine, the allosteric inhibitor, is added to the renaturation medium (see Figure 2).

The half-times for recovery of pyruvate kinase activity as a function of protein concentration were determined at 15 °C in the standard renaturation buffer described above. Half-times for type M were 52 min at 0.004 mg/ml, 46 min at 0.007 mg/ml, 37 min at 0.015 mg/ml, and 43 min at 0.029 mg/ml. Half-times for the recovery of type L pyruvate kinase under the same conditions are: 0.010 mg/ml, 94 min; 0.021 mg/ml,

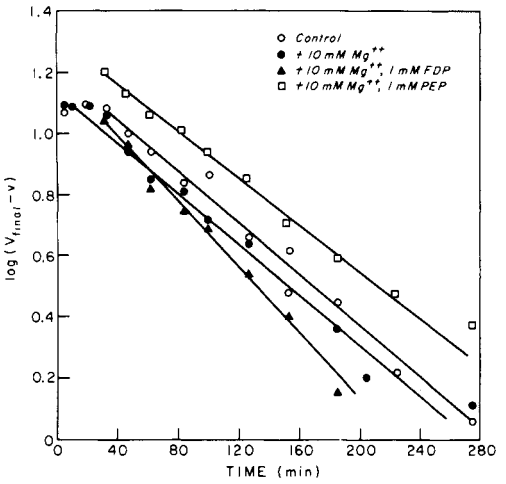

FIGURE 3: First-order plot of the data for activity recovery of type L pyruvate kinase. V_{final} is the final activity recovery, and v is the activity measured at time t. The renaturation buffer contains 0.05 M Tris-HCl, pH 7.5, 0.1 M KCl, 0.5 M sucrose, 1 mM EDTA, 50 mM β-mercaptoethanol, to which the following additions have been made: (open circles) no additions; (closed circles) 10 mM $MgCl_2$; (closed triangles) 10 mM $MgCl_2$ and 1 mM fructose bisphosphate; (squares) 10 mM $MgCl_2$ and 1 mM phosphoenolpyruvate.

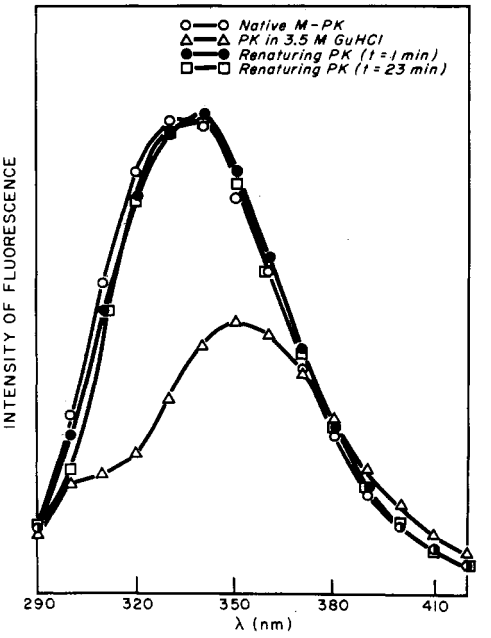

FIGURE 4: Fluorescence spectra of type M pyruvate kinase at 15 °C. All curves were obtained at a protein concentration of 0.03 mg per ml. The fluorescence spectra of native pyruvate kinase and of the renaturing samples were performed in the standard renaturation buffer described for Figure 1. For the spectrum of pyruvate kinase in 3.5 M guanidine hydrochloride, the guanidine hydrochloride and protein were dissolved in the same renaturation buffer. (Open circles) Native pyruvate kinase; (triangles) pyruvate kinase in 3.5 M guanidine hydrochloride; (closed circles and squares) renaturing enzyme 1 and 23 min, respectively, after beginning renaturation.

75 min; 0.042 mg/ml, 74 min. Thus, only a slight concentration dependence can be seen in the concentration range used for either isozyme, but type M tends to renature somewhat faster than does type L.

Shown in Figure 3 are the rates of recovery of enzymatic activity for type L pyruvate kinase, in the presence of different ligands, plotted as a first-order function. Although not shown, similar plots of the recovery of type M pyruvate kinase activity are also linear. The linearity of these plots after the initial lag, combined with the minimal concentration dependence as described above, suggests that the rate-limiting step for recovery of enzymatic activity probably involves refolding of subunit structure, rather than subunit aggregation per se.

Figure 4 contains the fluorescence spectra of native and denatured type M pyruvate kinase. The denaturation in 3.5 M guanidine hydrochloride was accompanied by a decrease in quantum yield and by a shift in the fluorescence emission maximum from 338 to 353 nm. These changes are consistent with increased exposure of the tryptophanyl side chains to solvent (Teale, 1960). The shoulder found near 300 nm in the spectrum of the denatured enzyme suggests that the tyrosine residues contribute to the fluorescence observed in that region. The emission spectrum of tryptophan dissolved in the 3.5 M guanidine hydrochloride solution is nearly identical with that of tryptophan dissolved in water and the quantum yields obtained in the two solutions are within 5% of each other. Thus the differences shown in Figure 4 can not be due to trivial solvent perturbation. Figure 4 also contains spectra of the renaturing enzyme recorded 1 and 23 min after beginning renaturation. The striking observation made in these experiments is the extremely rapid change in the fluorescence of the renaturing enzyme contrasting with the much slower recovery of enzymatic activity.

These experiments were followed up by measurements of the polarization of the protein fluorescence. The polarization measured on excitation at 290 nm is 0.12 for native type M pyruvate kinase and 0.064 for the denatured enzyme. The decrease in polarization accompanying denaturation is consistent with earlier studies on a variety of proteins which showed that denaturation and dissociation result in a uniform decrease in fluorescence polarization throughout the entire range of exciting wavelengths (250–315 nm) (Weber, 1960; Anderson and Weber, 1966). Because of the short fluorescence lifetimes of tyrosine and tryptophan, the decreased polarization reflects increased *local* freedom of rotation of the aromatic side chains rather than dissociation per se. The time course of renaturation showed that, within 15 s of dilution, the polarization had already increased to 0.11 and that, within 3 min of dilution, it had leveled off to 0.12–0.13.

Figure 5 shows the circular dichroism spectra of native type M pyruvate kinase in renaturation buffer and in 3.5 M guanidine hydrochloride at 15 °C. The ellipticity at 220 nm of the enzyme in guanidine hydrochloride was only 14% that of native pyruvate kinase. Within 3 min of beginning renaturation, 60–75% of the original ellipticity at this wavelength had already been recovered (see Figure 6). However, additional increases in the ellipticity occurred over a period of several minutes until 88% of the native level was achieved by 69 min of renaturation. Strong light absorption by guanidine hydrochloride solutions prevented us from determining the circular dichroism at wavelengths below 209 nm. The fluorescence and circular dichroism measurements indicate that a rapid refolding of the polypeptide chain occurs; the slower changes observed in circular dichroism could be due to slower local refolding or could result from subunit interactions.

Since major changes in fluorescence and circular dichroic properties occur very rapidly after beginning renaturation while activity regain is much slower, we wished to determine the kinetics of tetramer formation. Toward this end, we denatured and renatured types L and M pyruvate kinases independently and mixed them together at various times after beginning renaturation in order to test for the presence of hybridizable subunits. The results of this experiment are shown

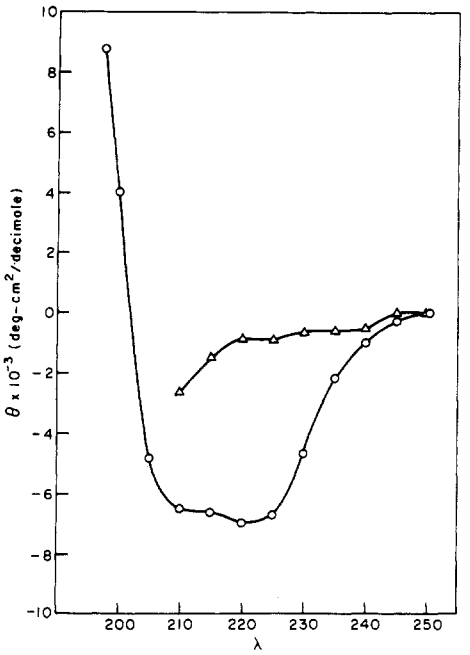

FIGURE 5: Circular dichroism spectra of native type M pyruvate kinase at 0.041 mg per ml in 0.05 M Tris-HCl, pH 7.5, 0.1 M KCl, 0.5 mM dithiothreitol (circles) and denatured pyruvate kinase in 3.5 M guanidine hydrochloride dissolved in the same buffer (triangles). All spectra obtained at 15 °C are corrected for minor contributions made by the solvent.

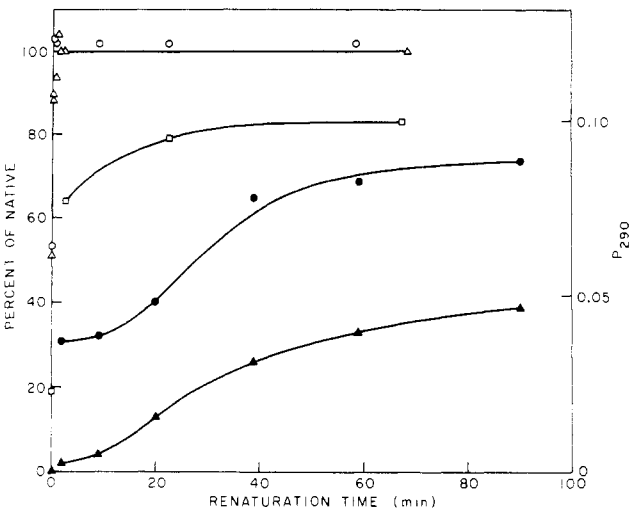

FIGURE 6: Time course for the recovery of native properties during renaturation, followed by five different methods. For comparative purposes the ellipticity, fluorescence intensity, and fluorescence polarization of the enzyme in 3.5 M guanidine hydrochloride are indicated at "zero time". Open triangles: the fluorescence polarization measured using an exciting wavelength of 290 nm and a broad band-pass emission centered at 360 nm. Other conditions are given in legend to Figure 4. Open circles: the fluorescence intensity measured using an exciting wavelength of 280 nm and an emission wavelength of 340 nm. Open squares: the circular dichroism measured at 220 nm. The conditions are the same as those listed under Figure 5 except that 1 mM dithiothreitol was substituted for 50 mM β-mercaptoethanol and that sucrose was eliminated. Closed circles: percent of the total recovered pyruvate kinase activity occurring as M_4 plus L_4. Experimental conditions are as described for Figure 7. Closed triangles: recovery of catalytic activity of type M pyruvate kinase expressed as percent original activity. Renaturation was accomplished using the standard buffer and conditions described for Figure 1.

in Figure 7. A considerable quantity of hybrids occurs when L and M subunits are mixed during the first 20 min after beginning renaturation. By 39 min, hybrids still can form but the parental L_4 and M_4 bands are noticeably heavier, indicating that the majority of either type L or type M subunits, or both, are no longer available for hybridization and presumably have already formed stable tetramers. The skewed patterns toward type L shown in Figure 7 result from the fact that the renaturation was performed with about six times as much type L enzyme as type M because of the lower specific activity and activity recovery of the former. The pattern shown in Figure 7 is probably determined by the renaturation of type M, which tends to renature faster and more completely for, as shown in Figure 8, hybrids are formed at much later times if, at each time point, type L that had been renaturing for the time periods shown is mixed with a sample of type M for which renaturation was begun immediately before mixing. Considerable quantity of hybridizable type L subunit was found for each mixing time, even 215 min after beginning its renaturation, indicating very slow and/or incomplete renaturation. However, decreases in the relative amount of hybrid are seen at the later times of mixing.

A serendipitous finding in the course of this project is the usefulness of hybridizability for detecting reversible subunit dissociation. If we mix native types L and M pyruvate kinases together, no hybrids result. Neither do hybrids occur if denatured type L is mixed with native type M. However, mixing of native type L and denatured type M produces some hybrids, especially L_2M_2 and LM_3, as shown in Figure 9. This indicates spontaneous dissociation of type L but not type M in dilute solution.

We have extended this work to include the effects of some ligands on the stability of the tetrameric structure of L_4 (Figure 9). Sucrose is known to stabilize pyruvate kinase activity (Susor and Rutter, 1968). As shown in Figure 9, greater dissociation of type L pyruvate kinase is seen in the absence of sucrose than in its presence, suggesting that sucrose may stabilize the enzymatic activity of the isozyme by stabilizing its tetrameric structure. Mg^{2+} also appears to stabilize the tetrameric structure, for the hybrids become more prominent if Mg^{2+} is omitted from the medium, even if sucrose is present. The substrate phosphoenolpyruvate and the allosteric activator fructose bisphosphate appear to cause additional stabilization of the tetrameric structure of L_4, at least in the presence of Mg^{2+}, for even smaller quantities of hybrids occur when these substances are added to the incubation medium.

The dissociation of type L does not appear to be caused by the low guanidine hydrochloride concentrations remaining in the renaturation mixture, as the patterns were essentially unchanged by final guanidine hydrochloride concentrations of 0.004–0.2 M. Furthermore, dilution of the denatured type M into renaturation medium prior to its being mixed with type L precluded the occurrence of local high guanidine hydrochloride concentrations. The small amounts of hybrid seen in Figure 9 suggest that the extent of dissociation of type L pyruvate kinase under our conditions is probably only a few percent. This conclusion was confirmed by gel filtration analysis, showing that the amount of dissociation material is so low that it cannot be reliably detected by gel filtration, even in the presence of 1 mM phenylalanine (Cardenas, unpublished results).

Discussion

The goal of this work was to examine the renaturation process of pyruvate kinase by a variety of parameters in order to determine the nature of the events involved in regaining the folded tetramer from unfolded monomers. Presumably, the protein goes through these same steps in vivo in order to achieve the native tetramer from newly synthesized subunits.

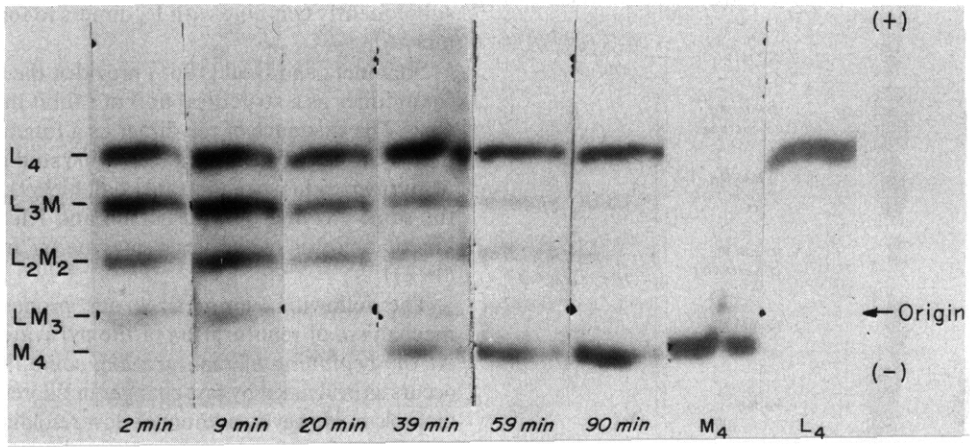

FIGURE 7: Zymograms of pyruvate kinase isozymes and hybrids obtained by mixing renaturing type M and L pyruvate kinases at the times indicated after beginning their respective renaturations at 15 °C. After mixing, the samples were incubated to a total of 6 h, then dialyzed and electrophoresed, and analyzed as described in experimental procedures.

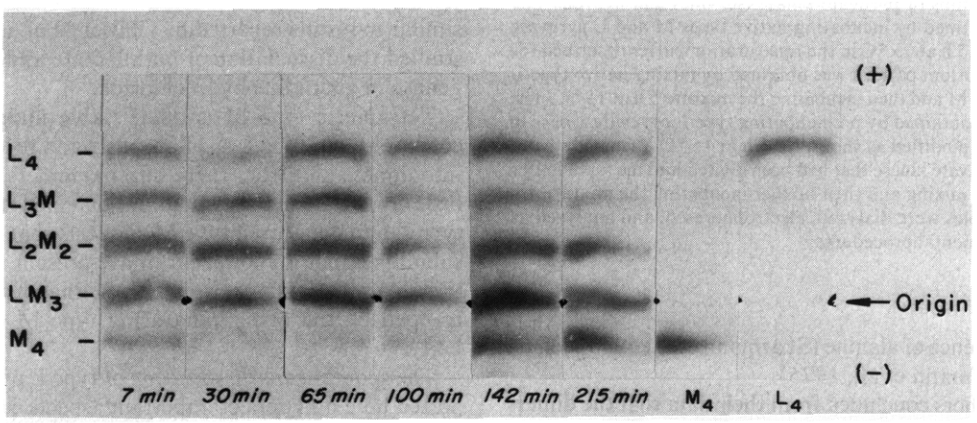

FIGURE 8: Zymograms of pyruvate kinase isozymes and hybrids obtained by mixing type L that had been renaturing in the buffer described for Figure 1 for the time indicated with denatured type M that had been diluted into renaturation medium immediately before mixing. After mixing, samples were incubated to a total of 6 h and then dialyzed, electrophoresed, and analyzed as described in experimental procedures.

Fluorescence spectroscopy provides a very rapid and convenient means of measuring the local environment of fluorescent amino acid residues, particularly of tryptophan and tyrosine, while circular dichroism is useful for a more general overview of folded structure. Hybridizability provides a convenient test for formation of stable tetrameric structure and for detecting subunit dissociation, while recovery of catalytic activity can be compared with the changes in the parameters mentioned above and can serve as a means for monitoring recovery of native structure. Previous studies indicate that active, renatured pyruvate kinase occurs mainly or only as a tetramer (Dyson and Cardenas, 1973).

Shown in Figure 6 is a composite of the changes of several parameters occurring during renaturation. The fluorescence spectrum and polarization characteristic of the native enzyme are recovered almost immediately after beginning renaturation, as is a considerable portion of the circular dichroism spectrum, long before measurable catalytic activity returns. Reformation of stable tetramers and recovery of enzymatic activity are surprisingly slow and closely parallel each other. As demonstrated in Figure 7, at no time during the renaturation is one hybrid appreciably favored over another. It therefore seems likely that, once the prerequisite conformational change occurs, the formation of stable tetramer from monomers is quite rapid.

The activity recovery curves of bovine type M and L pyruvate kinases were qualitatively like those obtained for the rabbit muscle enzyme by Johnson et al. (1969). Also consistent with the earlier work, there was little or no effect of protein concentration on the rate of renaturation for either type L or M, with the half-times for renaturation only slightly longer for the more dilute protein solutions. Our finding of a first-order relationship for kinetics of renaturation during most of the renaturation curve was in agreement with the work of Bornmann et al. (1974) with yeast pyruvate kinase. Therefore, the main rate-limiting step in the renaturation of all the pyruvate kinases studied to date is probably a refolding phenomenon.

Also described in the present paper is the spontaneous and reversible dissociation of bovine type L pyruvate kinase in dilute buffered solutions as detected by hybridizability. Some evidence exists in the literature for the ligand-mediated dissociation of other pyruvate kinases. Ibsen et al. (1971) concluded from data obtained with gel filtration that human erythrocyte pyruvate kinase occurs as monomers, dimers, trimers, and pentamers as well as the normal tetramers. Badwey and Westhead (1975) observed dissociation of the same enzyme when it was stored in the absence of EDTA. Behrisch (1974) found that ATP dissociates the liver enzyme of the arctic ground squirrel into dimers, and the dissociation is reversed by fructose bisphosphate.

The main isozyme of kidney (type K) may also dissociate into half molecules (Ibsen and Trippet, 1972). Subsequently, the pyruvate kinase from Erlich ascites tumor cells, presumably also type K, was found to dissociate completely into half mol-

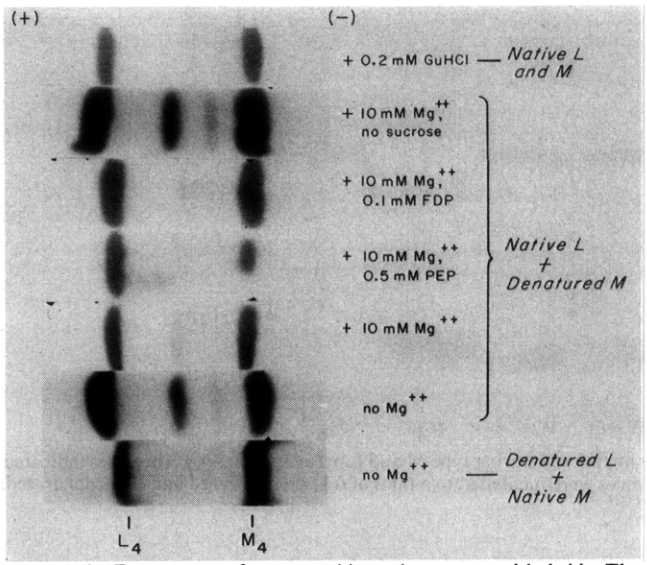

FIGURE 9: Zymograms of pyruvate kinase isozymes and hybrids. The top pattern was obtained by incubating native types M and L pyruvate kinases together for 5 h at 15 °C in the renaturation buffer described for Figure 1, and the bottom pattern was obtained by mixing native type L with denatured type M and then incubating the mixture 5 h at 15 °C. The other patterns were obtained by preincubating type L pyruvate kinase in renaturation buffer modified as shown for 1 h at 15 °C, then adding denatured type M pyruvate kinase that had been diluted into the same buffer immediately before mixing and then further incubating the mixture 4 h at 15 °C. The samples were dialyzed, electrophoresed, and analyzed as described in experimental procedures.

ecules in the presence of alanine (Sparmann et al., 1973; Schulz et al., 1975; Hofmann et al., 1975).

The latter authors concluded from their data that the dimers are catalytically inactive or at least less active than the tetramer. All of the above methods used sucrose density gradient centrifugation or gel filtration to detect changes in molecular weight.

Our hybridization studies with bovine types M and L pyruvate kinases indicate that type M does not dissociate to a detectable extent under mild conditions at the protein concentrations used in this study. Type L does, however, reversibly dissociate into dimers, and some of the dimers appear to reversibly dissociate into monomers, as evidenced by the presence of a band of activity with the electrophoretic mobility of LM_3. The amount of bovine type L dimer appears to be decreased by sucrose, Mg^{2+}, phosphoenolpyruvate, or fructose bisphosphate.

The asymmetry of the patterns of hybrids seen in Figure 9 provides some insight into the mechanism of dissociation of type L pyruvate kinase under mild conditions. The tetramer appears to dissociate into dimers and then to a limited extent into monomers, as indicated below:

$$L_4 \rightleftarrows 2L_2 \rightleftarrows 4L$$

The rationale behind this model is as follows. Between L_4 and M_4, the most prominent band is L_2M_2. The LM_3 band is considerably lighter than L_2M_2 in spite of the preponderance of denatured type M subunits available for hybridization with dissociated type L subunits. A band in the position expected for L_3M is generally not seen; even under optimal conditions, there is much less L_3M than of the other bands. The latter result indicates that significant quantities of trimers probably do not occur under our conditions but result mainly from the combination of types L and M monomers to form dimers that subsequently combine with L_2 dimers to form the L_3M tetramer.

Steinmetz and Deal (1966) provided the first solid evidence for a dimer as a structural unit of rabbit muscle pyruvate kinase. The existence of the dimer as a functional or structural unit is consistent with the dissociation studies with rabbit type M pyruvate kinase of Cottam et al. (1969), the x-ray data on the same enzyme by McPherson and Rich (1972), and the kinetic analysis of the yeast enzyme by Johannes and Hess (1973).

The following summarizes our proposal regarding the mechanism of renaturation of the pyruvate kinase isozymes. At the beginning of renaturation, some fast local refolding occurs as evidenced by fast changes in fluorescence and circular dichroism. However, additional slow refolding is required prior to reformation of stable tetramers, indicated by the low kinetic order for regain of catalytic activity. Once this rate-limiting conformational change occurs, the subunits reassociate rapidly to form active tetramers. The slow rate of recovery of stable tetramers for the pyruvate kinase isozymes described here is similar to results reported by Chilson et al. (1965, 1966), who studied the dissociation of both lactate and malate dehydrogenase in guanidine hydrochloride.

Tetrameric type M is quite stable under normal buffer conditions, but type L spontaneously and reversibly dissociates into dimers and from there into monomers. The presence of little or no L_3M in the zymograms obtained from mixing native type L and denatured type M suggests that trimers of type L subunits either do not occur or are present in very low quantities. Type L monomers, on the other hand, probably often are trapped by the faster renaturing type M subunits to form LM_3.

The spontaneous dissociation of type L pyruvate kinase reported here may reflect important aspects concerning subunit interactions of the allosteric type L pyruvate kinase that must be considered in analyzing the mechanism of allostery in this enzyme. We are continuing our analyses of the renaturation kinetics of pyruvate kinase isozymes in an attempt to more precisely define the mechanisms involved.

Acknowledgments

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Bovine Serum Albumin in Aqueous Guanidine Hydrochloride Solutions. Preferential and Absolute Interactions and Comparison with Other Systems†

Emil Reisler, Yeheskiel Haik, and Henryk Eisenberg*

ABSTRACT: The partial specific volume, $\bar{v}_2^0$, of bovine serum albumin at 25 °C was found to be 0.728 ± 0.001 ml/g in solutions of guanidine hydrochloride (GuHCl), 0.01 M dithioerythritol (DTE), independent of GuHCl concentration (3–6 M). The volume decrease upon denaturation is about 400 ml/mol ($\bar{v}_2^0$ in water at the same temperature was found to be 0.734). From the reduced density increments at constant chemical potential of diffusible solutes, the apparent volumes, ϕ', were found to increase from 0.693 ml/g at 3 M GuHCl to about 0.725 ml/g at 7 M GuHCl. The phenomenological interaction parameter, ξ_3 (grams of GuHCl "bound" per gram of protein), was found to decrease from about 0.2 at 3 M GuHCl to about 0.07 at 6.4 M GuHCl. The phenomenological interaction parameter, ξ_1 (grams of water "bound" per gram of protein), is negative and become less negative with increase in GuHCl concentration. The relation between ξ_3 and ξ_1 and physical binding and exclusion of low-molecular-weight components are discussed in terms of simple model consideration. It is concluded that over the range of GuHCl concentrations studied about 0.2 g of water as well as 0.28 g of GuHCl are bound per gram of protein. This corresponds on the average to 1.3 molecules of water and 0.35 molecule of GuHCl per amino acid residue. Similar results were found by recalculating some previous results for aldolase. These results on proteins in GuHCl solution are in marked contrast to the behavior of DNA at high concentrations of NaCl and CsCl, which is analyzed on the basis of earlier work.

In a previous publication (Reisler and Eisenberg, 1969), we have determined density increments, partial specific volumes, apparent volumes, and interaction parameters of aldolase with low-molecular-weight components in the three component system water–salt–protein. Whereas the primary purpose of that work related to the correct interpretation of the subunit composition of aldolase from equilibrium sedimentation, interesting results with respect to interaction parameters of protein with the low-molecular-weight salt were also derived. Bovine serum albumin was also investigated by us in that work, but in less detail. In the present work, we have extended the measurements on this protein to cover a wide range of concentrations of the denaturing solvent GuHCl.[1] Though bovine serum albumin solutions have been extensively studied in the past, we believe that the new data to be presented below will clarify some basic points relating to the behavior of proteins upon denaturation in GuHCl solutions. The experimental section follows closely the work of Reisler and Eisenberg (1969); the precise description of experiments leading to density increments at constant chemical potential of diffusible solutes and correct partial specific volumes has also been given by Cohen and Eisenberg (1968) in an investigation concerned

† From the Polymer Department, The Weizmann Institute of Science, Rehovot, Israel. *Received June 11, 1976.*

[1] Abbreviations used are: GuHCl, guanidine hydrochloride; DTE, dithioerythritol.