Analysis of the Renaturation Kinetics of Bovine Muscle Pyruvate Kinase†

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ABSTRACT: Bovine type M pyruvate kinase can be reversibly denatured by solutions of guanidine-HCl. Subsequent dilution of the enzyme into buffer containing 2-mercaptoethanol or dithiothreitol results in recovery of enzymatic activity with half-times that vary from 185 min at 0 °C to 4 min at 45 °C. In the temperature range 0–25 °C, 90% of the enzymatic activity is recovered. Above about 32 °C, the recovery drops off sharply, with a yield of only 13% at 45 °C. Removal of inactive nonspecific aggregates and denatured monomer by gel filtration yields an enzyme with the same specific activity as the starting material. At enzyme concentrations below 3 μg/mL at 16 °C or below 25 μg/mL at 7.8 °C, the reactivation kinetics show a concentration dependence. At higher concentrations of protein and at temperatures of 16 °C or higher, no protein concentration dependence is seen, and the rate of reactivation is described by two first-order relaxations. The rate constants have apparent activation energies of 10.6 and 11.9 kcal/mol. Combining the results presented here with earlier work from this laboratory [Cardenas, J. M., & Dyson, R. D. (1973) *J. Biol. Chem. 248*, 6938–6944; Cardenas, J. M., Hubbard, D. R., & Anderson, S. (1977) *Biochemistry 16*, 191–197] leads to the conclusion that a rapid, major folding produces two species which undergo transconformational steps. This is followed by subunit association which yields the native tetramer.

Protein folding occurs spontaneously and is determined by information contained in the specific amino acid sequence and its aqueous environment (Wetlaufer & Ristow, 1973). The enzyme pyruvate kinase (EC 2.7.1.40) is composed of four identical polypeptide chains. Thus, reconstitution from its unfolded monomers must involve both folding and reassociation processes.

Previous work has demonstrated the reversible denaturation of pyruvate kinase. Such reversible denaturation was first reported by Cottam et al. in 1969. These workers were able to recover 35–50% of the original enzymatic activity after denaturation of rabbit muscle pyruvate kinase in 5 M urea by subsequently removing the urea by gel filtration or dialysis. Independently, Johnson et al. (1969) were able to recover up to 70% of the original enzymatic activity by using careful dilution as the method for removing the denaturant, and they further defined some of the conditions required for optimal renaturation. The latter workers found that native and renatured enzymes had the same sedimentation coefficients, K_m values, and heat stability profiles.

In later studies on the renaturation of pyruvate kinase from yeast, Bornmann et al. (1974) found the presence of L-valine to be necessary for the renaturation of this enzyme. In the presence of L-valine, recovery of enzymatic activity followed pseudo-first-order kinetics, and 50% of the original enzymatic activity could be recovered. Bornmann et al. proposed that L-valine serves as a nucleation center for the folding of the monomer.

Previous work with bovine muscle pyruvate kinase resulted in 50–60% recovery of activity at 15 °C after denaturation in 3.5 M guanidine-HCl (Cardenas et al., 1977). The half-time of recovery of activity at this temperature was 37 min and was not affected by concentrations of guanidine-HCl in the range of 0.05–0.2 M. In the protein concentration range 4.0–29 μg/mL, little or no effect of protein concentration on the half-time of recovery at 15 °C was seen. Fluorescence properties were fully recovered within 1 min of dilution of the guanidine-HCl, indicating rapid general folding of the polypeptide chains, but recovery of stable quaternary structure, as demonstrated by the hybridizability of type M subunits with those from the type L isozyme, was slow and roughly paralleled the regain of enzymatic activity.

In the present paper, we describe the effect of temperature and of a wide range of protein concentrations on the renaturation of bovine muscle pyruvate kinase. From these studies, a much clearer picture of the renaturation mechanism of muscle pyruvate kinase has emerged. At 16 °C and at protein concentrations of 3.0–25.6 μg/mL, there is no apparent protein concentration dependence on the renaturation rate, and the reactivation can be described by two first-order relaxations having apparent activation energies of 10.6 and 11.9 kcal/mol. When the renaturation temperature is lowered to 7.8 °C, a definite concentration dependence can be seen for the protein concentration range of 1–25 μg/mL.

Experimental Procedures

Substrates and lactate dehydrogenase were obtained from Sigma Chemical Co. Guanidine hydrochloride was obtained from Pierce Chemical Co. Sucrose and ammonium sulfate were of special enzyme grade from Schwarz/Mann. Common chemicals were of standard reagent grade. Distilled, deionized, or double-distilled water was used to make all solutions. Bovine type M pyruvate kinase was prepared as described by Cardenas et al. (1973).

Enzyme that had been stored as an ammonium sulfate precipitate was centrifuged down and dissolved in 0.05 M Tris-HCl, pH 7.5, and 0.1 M 2-mercaptoethanol to give a protein concentration 52 times greater than the protein concentration to be desired during renaturation. Protein concentrations were determined by the absorbance at 280 nm by using $E_{280}^{0.1\%} = 0.55$. Pyruvate kinase activity was assayed by the method of Bücher & Pfleiderer (1955) in 1.0 mL of a medium containing 0.05 M imidazole-HCl, pH 7.0, 0.1 M KCl, 10 mM $MgCl_2$, 2.0 mM ADP, 1.0 mM phosphoenolpyruvate, 0.16 mM NADH, and 5.5 units of lactate dehydrogenase. The assay temperature was the same as the

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corresponding reactivation temperature with the following exceptions. (1) For the dependence of renaturation on enzyme concentration at 7.8 °C, enzyme assays were performed at 15 °C. (2) For other renaturations involving temperatures of less than 12.5 °C, enzyme assays were performed at 12.5 °C. The denaturation–renaturation procedure is basically the same as described by Cardenas & Dyson (1973) for making hybrids of bovine type M and type L pyruvate kinases. When either type M or type L pyruvate kinase was renatured alone under these conditions, these workers found that the kinetic properties of the renatured enzyme were the same as those of the native, undenatured form, albeit they achieved only a 60% activity yield. For denaturation, an aliquot of the enzyme solution prepared and analyzed as described above was mixed with an equal volume of 8 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol and incubated at 0 °C for 15 min. Reactivation was begun by diluting, with gentle swirling, an aliquot of enzyme in guanidine hydrochloride into 25 volumes of 0.05 M Tris-HCl, pH 7.5, 0.5 M sucrose, 0.1 M KCl, 5 mM $MgCl_2$, and 5 mM dithiothreitol at the desired temperature. Aliquots were removed at appropriate time intervals and assayed for pyruvate kinase activity as described above.

Parallel controls consisted of enzyme that was not exposed to denaturing conditions but was treated identically in all other respects. Sufficient solid guanidine-HCl was added to the control enzyme buffer so that control and renaturing enzymes were exposed to the same concentration of guanidine-HCl (0.15 M).

Gel filtration at 4 °C was employed to detect protein aggregates formed during renaturation. A total of 2.4 mg of enzyme was subjected to the denaturation–renaturation procedure and was concentrated by ammonium sulfate precipitation. The precipitate was collected by centrifugation, dissolved in 0.05 M potassium phosphate, pH 7.0, 0.1 M KCl, and 10 mM 2-mercaptoethanol, and applied to a column (1.5 × 38 cm) of Bio-Gel A-1.5m, 100–200 mesh, that had previously been equilibrated with the same buffer. Gravity flow from a Marriot flask was used to set the flow rate at 8.8 mL/h, and 0.88-mL fractions were collected. Fractions were assayed for pyruvate kinase activity as described above, and protein concentrations were determined from the absorbance at 280 nm.

Results

Shown in Figure 1A are the reactivation curves of type M pyruvate kinase at temperatures from 0 to 20 °C. Figure 1B shows the corresponding reactivation curves for temperatures from 35 to 45 °C. For each data point, enzyme assays were performed on both the renaturing enzyme and a control solution of the same protein concentration (25 μg/mL) that had not been denatured. Each data point therefore represents a comparison of enzyme activity concentrations in renaturing and control samples. Control enzyme samples typically retained 85–100% of their original activity throughout the course of the experiment for all temperatures (see Table I for data from a typical set of experiments). At all temperatures, the recovery of activity as a function of time is sigmoidal. This indicates that activity recovery is probably not dependent on a single first- or second-order rate-limiting step and therefore agrees with the earlier work of Tobes et al. (1972) with yeast pyruvate kinase and with the studies of Cardenas et al. (1977).

L-Valine is necessary for (yeast) or enhances (Neurospora) the renaturation of some pyruvate kinases (Bornmann et al., 1974; O'Brien & Kapoor, 1980). However, L-valine had no effect on the renaturation of bovine muscle pyruvate kinase (Porter & Cardenas, 1980).

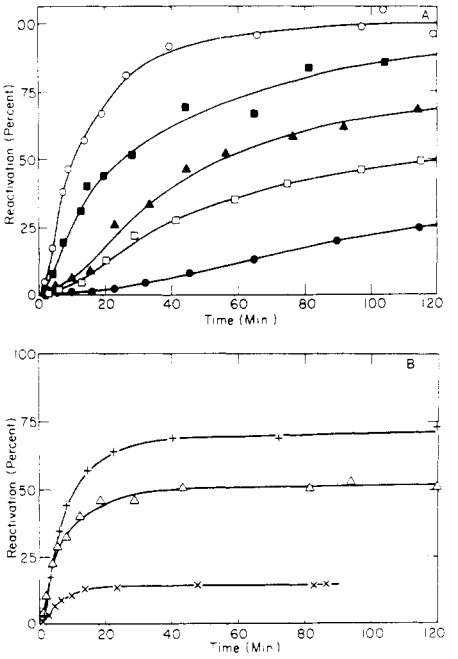

FIGURE 1: Reactivation as percent of control of type M pyruvate kinase after denaturation in 4 M guanidine-HCl. The protein concentration was 25.6 μg/mL. For renaturation at 0, 5, and 10 °C, enzymatic activities were determined at 12.5 °C. In each case, no reactivation in the assay cuvette was observed as evidenced by a linear change in absorbance with time. No enzymatic activity could be detected in enzyme samples taken directly from solutions of 4 M guanidine-HCl. Experimental procedures are described in the text. (A) 0 (●), 5 (□), 10 (▲), 15 (■), and 20 °C (○). (B) 35 (+), 40 (△), and 45 °C (×).

Table I: Half-Times and Percent Recovery for the Enzymatic Activity of Bovine Type M Pyruvate Kinase as a Function of Temperature[a]

t (°C)	half-time (min)	% recovery	control enzyme act., final % compared to initial value
0	185 ± 7.0	89 ± 3	88 ± 3
7.0	64.0 ± 4.0	93 ± 4	80 ± 3
11.2	29.3 ± 4.0	90 ± 6	90 ± 2
16.0	17.8 ± 0.60	89 ± 3	86 ± 3
20.3	10.5 ± 0.59	91 ± 3	85 ± 3
25.3	6.31 ± 0.20	88 ± 3	96 ± 4
30.3	5.85 ± 0.13	87 ± 3	94 ± 4
35.1	4.67 ± 0.29	73 ± 3	95 ± 4
40.0	5.12 ± 0.72	52 ± 3	100 ± 3
45.2	4.33 ± 0.32	13 ± 2	100 ± 3

[a] Experimental procedures are given in the text.

Half-times for recovery of enzymatic activity decreased with increasing renaturation temperature throughout the temperature range studied and varied from 185 min at 0 °C to 4 min at 45 °C. Decreases in half-times of renaturation as a function of increasing renaturation temperature were much less marked above 30 °C (see Table I).

Final activity yields were determined by assaying renaturing and control enzymes at least 24 h after beginning renaturation for the higher temperatures and up to 3 days after beginning renaturation for the lower temperatures. The results are presented in Table I. Final activity yields were constant at about 90% of control values for samples that were renatured between 0 and 25 °C. In the temperature range 25–45 °C, the percent recovery decreased with increasing renaturation temperature. Thus, while 91% of the activity, based on control samples of enzyme, was recovered when renaturation occurred at 20 °C, only 73% of the activity could be recovered when

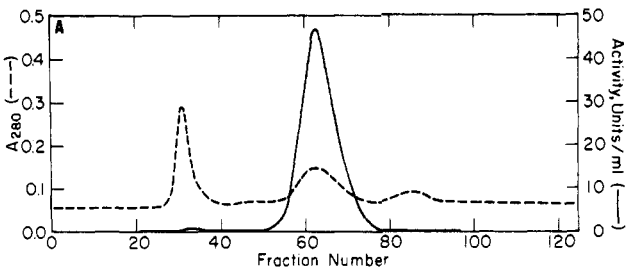

FIGURE 2: Gel filtration of enzyme renatured at 25 °C. Absolute absorbance at 280 nm (---) is uncorrected for buffer absorbance. Pyruvate kinase activity is expressed as units/mL (—). Procedures are described in the text.

renaturation occurred at 35 °C, and a final activity yield of only 13% was found when renaturation took place at 45 °C.

Kayne & Suelter (1968) found that an Arrhenius plot of catalytic activity of rabbit muscle pyruvate kinase consisted of two effectively linear segments of different slopes (larger slope at lower temperature) connected by a curved region. We have found the same type of Arrhenius plot with bovine muscle pyruvate kinase (unpublished results). The plot is essentially linear at temperatures below about 31 °C. There is, therefore, no kinetic evidence to suggest a critical conformational change in the temperature range 0–31 °C, although such a change may occur at 31 °C or above. It is interesting to note that the break in the Arrhenius curve for enzymatic activity occurs at about the same temperature where the recovery of enzymatic activity after renaturation begins to decrease.

We were interested in determining whether decreased recovery of enzymatic activity at the higher renaturation temperatures was due to the formation of enzyme of lower specific activity or whether some competing process, such as protein aggregation, might simply reduce the amount of enzyme available for renaturation. We therefore employed gel filtration in order to determine whether protein aggregates could be observed after renaturing the enzyme at any of the temperatures. Shown in Figure 2 is a gel filtration pattern of enzyme that had been renatured at 25 °C. The OD_{280}-absorbing peak that preceded the peak of enzymatic activity probably represents aggregated protein while the peak trailing the activity peak probably represents denatured protein subunits. The specific activity of the active enzyme approximated the value for native enzyme, thus indicating that decreased yields of enzyme activity are caused by protein aggregation or by failure of protein subunits to reassociate to form a stable tetramer. These processes become more prevalent when renaturation is performed at higher temperatures. Renaturation at 45 °C produced aggregates so large that they did not pass through the column but could be seen at the top of the column. A somewhat larger quantity of inactive, monomeric protein was obtained, but the active enzyme had a specific activity that was similar to that of native pyruvate kinase.

In order to determine whether aggregated or otherwise denatured protein affected the renaturation process of other enzyme subunits, we performed a renaturation experiment at 45 °C for the purpose of producing large amounts of denatured protein and then reequilibrated this solution of mainly aggregated protein to 11.5 °C. A second sample of protein in guanidine-HCl was added to the solution containing the aggregates and was allowed to renature at the lower temperature. The renaturation of the second aliquot of protein, added at 11.5 °C, was unaffected by the presence of aggregated protein from the renaturation attempt at 45 °C. Renaturation controls demonstrated that enzyme activity recovered during the 11.5 °C incubation was due to the protein sample that was added

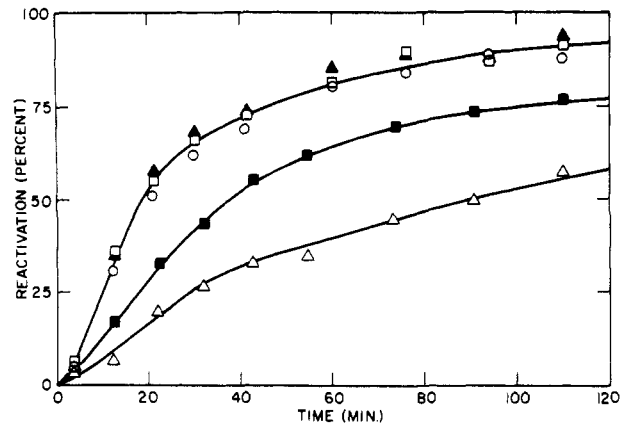

FIGURE 3: Reactivation at 16 °C in the presence of 1 mg/mL bovine serum albumin. Enzyme concentrations (μg/mL) are 25.6 (□), 12.8 (▲), 6.4 (○), 2.5 (■), and 1.0 (△). Each data point is the ratio of the percent reactivation at time t to the percent final reactivation.

at that temperature and not due to additional activity regained from the sample that had been previously incubated at 45 °C.

We also studied the effect of protein concentration on the reactivation of pyruvate kinase. The renaturation kinetics at 16 °C over the concentration range 6.4–25.6 μg/mL show no dependence on protein concentration (results not shown). Within experimental error, the percent final reactivation was approximately the same for each protein concentration (about 90%). Similar results were obtained for renaturation at 55 μg/mL protein and 16 °C (results not shown). Renaturation at 110 μg/mL resulted in only a 54% final reactivation, probably due to increased nonspecific aggregation. At protein concentrations of 3.2 μg/mL or below, renaturation occurred with considerably reduced final yields (58%). Teipel & Koshland (1971) have also observed reduced yields at low protein concentrations. Low concentrations of aldolase (Chan et al., 1973) and of phosphofructokinase (Parr & Hammes, 1976) have been stabilized by the addition of bovine serum albumin. We found that the addition of bovine serum albumin (1.0 mg/mL) to the pyruvate kinase renaturation mixture eliminated the problem of reduced final activity yields at the lower concentrations of enzyme.

Other than increasing the final recovery of enzymatic activity at the lower concentrations of pyruvate kinase, bovine serum albumin had no significant effect on the renaturation kinetics of pyruvate kinase at any of the temperatures or concentrations of enzyme tested. Cook & Koshland (1969) have shown that the renaturation of a given enzyme is generally independent of the renaturation of other added purified enzymes. Thus, we feel that bovine serum albumin in our renaturation medium functions in a relatively nonspecific manner, neutralizing factors such as vessel wall effects that tend to decrease the amount of enzyme available for renaturation.

Figure 3 shows the renaturation kinetics in the presence of 1 mg/mL bovine serum albumin at 16 °C. No concentration dependence was seen in the enzyme concentration range 6.4–25.6 μg/mL, and the renaturation results were similar to those obtained in the absence of bovine serum albumin. However, the renaturation rate was slower at enzyme concentrations of 2.5 μg/mL or less than it was at higher concentrations of enzyme (see Figure 3). In the presence of bovine serum albumin, the final recovery of enzymatic activity was approximately 90% of all enzyme concentrations tested.

Figure 4 shows the reactivation curves for various concentrations of enzyme at 7.8 °C in the presence of 1 mg/mL bovine serum albumin. Here we see a concentration depen-

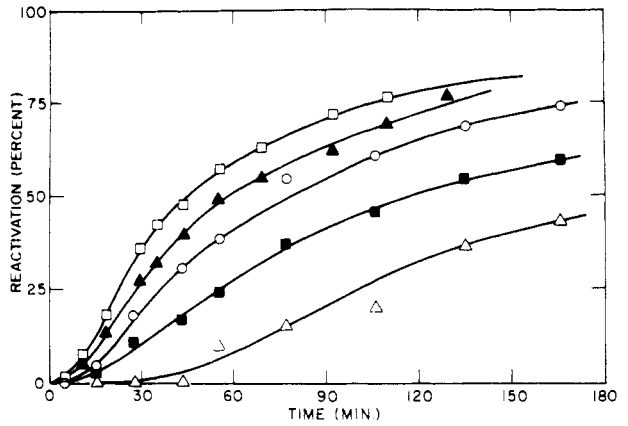

FIGURE 4: Reactivation at 7.8 °C in the presence of 1 mg/mL bovine serum albumin. Enzyme concentrations (μg/mL) are 25.6 (□), 12.8 (▲), 6.0 (○), 2.5 (■), and 1.0 (△). Each data point is the ratio of the percent reactivation at time t to percent final reactivation. Enzymatic activities were measured at 15 °C.

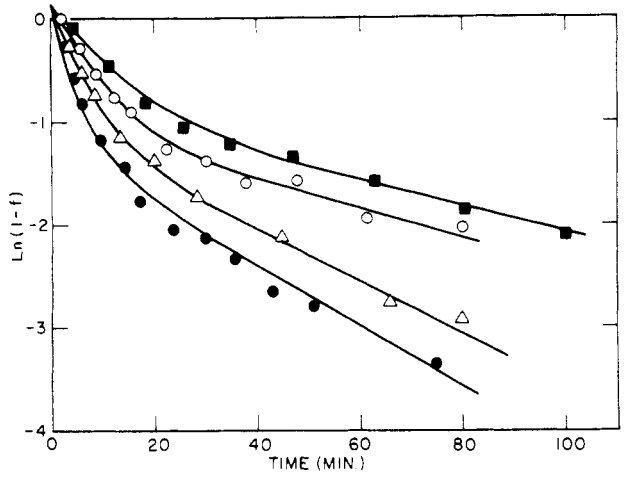

FIGURE 5: First-order plot of reactivation data at 25.6 μg/mL for the following temperatures (°C): 16.0 (■), 20.3 (○), 25.3 (△), and 30.3 (●). The solid lines were drawn based on eq 1 by using the parameters given in Table II.

dence in the range 6.0–25.6 μg/mL as well as at lower concentrations. Thus, manipulation of enzyme concentration and/or renaturation temperature can produce conditions in which the association processes involved in the assembly of active pyruvate kinase are slow enough that they contribute to the rate of recovery of enzymatic activity. This is shown by the enzyme concentration effects at low renaturation temperatures and/or concentrations. By raising the renaturation temperature and/or enzyme concentration, the concentration effects on enzyme renaturation rates are eliminated. Thus, by choosing the appropriate temperature and enzyme concentrations for renaturation, we can examine the transconformational processes involved in the reassembly of pyruvate kinase.

Figure 5 shows a first-order plot of reactivation data points for temperatures between 16 and 30.3 °C. The solid lines were calculated as described below. The enzyme concentration in each case was 25.6 μg/mL. No concentration dependence on the renaturation kinetics could be seen for protein concentrations in this range and for the temperatures used. The reactivation rates clearly do not follow simple first-order kinetics, but neither do they follow second-order kinetics (results not shown). However, the data can be satisfactorily represented as the sum of two exponentials according to

$$1 - v/V_f = ae^{-\lambda_1 t} + be^{-\lambda_2 t} \quad (1)$$

Table II: Values of the Parameters in Equation 1 Determined for Temperatures of 16–30.3 °C[a]

t (°C)	a	$\lambda_1 \times 10^3$ (s^{-1})	b	$\lambda_2 \times 10^4$ (s^{-1})
16	0.662 ± 0.032	1.65 ± 0.30	0.487 ± 0.040	2.08 ± 0.38
20.3	0.766 ± 0.044	2.22 ± 0.02	0.380 ± 0.024	2.40 ± 0.45
25.3	0.800 ± 0.050	3.00 ± 0.42	0.342 ± 0.057	4.13 ± 1.08
30.3	0.850 ± 0.089	3.88 ± 0.55	0.317 ± 0.105	5.03 ± 1.70

[a] Each value is the mean ± the standard deviation of three determinations.

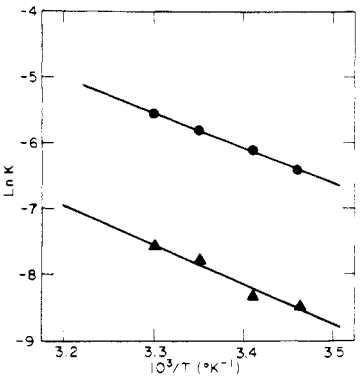

FIGURE 6: Arrhenius plot for the rate constants λ_1 (●) and λ_2 (▲).

where a and b are constants, v is the enzyme activity at time t, V_f is the final recovery of enzymatic activity, and λ_1 and λ_2 are the macroscopic rate constants.

The data were analyzed by the method of Ray & Koshland (1961) and yielded the values given in Table II. The value for a increases with increasing temperature while b decreases. The sum $a + b$ is essentially constant and equal to 1.14. The solid lines of Figure 5 were calculated from eq 1 by using the parameters given in Table II. As seen in Figure 5, the calculated lines provide a good fit for the actual experimental data points.

Figure 6 is an Arrhenius plot of the rate constants λ_1 and λ_2. The data were highly linear and yield apparent activation energies of 10.6 and 11.9 kcal/mol, respectively, for λ_1 and λ_2.

We have also found the reactivation kinetics of pyruvate kinase at 16 °C and 25.6 μg/mL to be identical (a) whether denaturation is performed in 4.0 or 6.0 M guanidine-HCl, (b) whether 2.5 or 5.0 mM dithiothreitol or 0.1 M mercaptoethanol is used in the denaturation buffer, (c) at any dithiothreitol concentration in the renaturation buffer from 5 mM up to at least 15 mM, (d) whether the enzyme remained in the denaturation medium 8, 15, or 30 min prior to beginning renaturation, (e) regardless of whether or not the solutions were flushed with N_2 prior to use, and (f) whether enzyme denaturation is performed in a solution containing Tris-HCl and 2-mercaptoethanol or in renaturation buffer (see Experimental Procedures).

Discussion

Earlier work from this laboratory (Cardenas et al., 1977) showed that the fluorescence properties and most of the circular dichroism characteristics of bovine muscle pyruvate kinase are recovered within 1 min after beginning renaturation. Furthermore, recent results are consistent with the formation of an L-phenylalanine binding site early during the renaturation process (Porter & Cardenas, 1980). However, Cardenas et al. (1977) found by hybridization studies that the formation of stable tetramers was much slower and approximately

paralleled the rate of recovery of enzymatic activity. The results cited above indicate that major folding of the peptide chain occurs on a much shorter time scale than does the regaining of either native tetrameric structure of enzymatic activity. Thus, the results reported in this paper describe transconformational and association processes which occur after the major refolding of the polypeptide chain.

Evaluated in this paper are the effects of temperature and protein concentration on the renaturation of bovine muscle pyruvate kinase. Activity recoveries are essentially constant at about 90% for temperatures from 0 to 25 °C. Above 32 °C, recoveries drop off sharply and appear to be the result of nonspecific aggregation and formation of apparently incorrectly folded but stable monomer. Decreasing the protein concentration at the higher renaturation temperatures did not result in an increase in recovery of enzymatic activity.

At 16 °C, the reactivation shows no dependence on enzyme concentration in the range 6.4–25.6 μg/mL, but at lower concentrations there is a noticeable dependence. If renaturation occurs at 7.8 °C, the concentration dependence can be seen at protein concentrations up to 25.6 μg/mL. If this change in concentration dependence with temperature is the result only of a temperature effect on rate constants, the activation energy for the association process is much larger than the activation energy for the limiting first-order relaxations. In previously published work with porcine muscle lactate dehydrogenase, Rudolph et al. (1977) obtained an apparent energy of activation of 58 kcal/mol for the association reaction. We note that this energy of activation for the association of lactate dehydrogenase subunits is considerably greater than the energies of activation (10.6 and 11.9 kcal/mol, respectively) for the two first-order relaxations of pyruvate kinase. A concentration dependence based solely on diffusion rates is unlikely since diffusion-limited reactions have low activation energies (about 2–3 kcal/mol; Gardiner, 1969).

Another possibility is that lower temperature could destabilize interactions involved in maintaining quaternary structure. However, equilibrium centrifugation of native bovine muscle pyruvate kinase at 4 °C in the analytical centrifuge yielded only one species of molecular weight 230000 (Cardenas et al., 1973). Hybridization experiments by Cardenas et al. (1977) performed at 15 °C and at enzyme concentrations of approximately 20 μg/mL did not reveal any reversible subunit dissociation. Furthermore, we find the specific activity of control enzyme samples to be independent of protein concentration at the low temperatures used in this work. Thus, no evidence has been found for a destabilization of subunit interactions by low temperature.

Since pyruvate kinase is composed of four subunits, renaturation will involve both folding and association processes. Under appropriate conditions of temperature and enzyme concentration, subunit association is rapid enough that it is not rate limiting, and the reactivation kinetics are satisfactorily represented by the sum of two exponentials (see eq 1). Under these conditions, the results are consistent with the presence of two different populations at zero time that may renature either (a) via a single pathway

$$A \rightarrow B \rightarrow N$$

where $[B] \neq 0$ at $t = 0$ or (b) via parallel pathways

$$A \rightarrow N \leftarrow B$$

The data rule out reactivation via a single pathway in which the initial concentration of B is zero. Since the reactivation conditions strongly favor the native enzyme (shown by the high percent final activity recovery), the rate-limiting steps during renaturation are expected to be irreversible.

A short induction period occurs during renaturation, as reflected by the lag in activity recovery seen in Figures 1 and 4 and by the failure of the lines in Figure 5 to converge to ln $(1 - f) = 0$. This lag is seen under conditions of temperature and enzyme concentration high enough that no concentration dependence is observed for the overall renaturation process. However, the lag is more pronounced at the lower renaturation temperatures and enzyme concentrations where a concentration dependence occurs. The lag could not result from an inaccurate assessment of time zero for initiation of enzyme renaturation, as mixing of solutions is complete within 5–10 s and the lag lasts for several minutes. Part of the induction period could result from the fast-folding process described above. Under conditions where we see a concentration dependence, part of the lag is no doubt due to the kinetic contribution of the association process. However, the fact that the lag is so long and is seen even under conditions where no protein concentration dependence is evident suggests that the renaturation process could involve additional kinetically significant steps that we have not yet described.

The results of previous work (Cardenas & Dyson, 1973; Cardenas et al., 1977) with the formation of hybrids from muscle and liver pyruvate kinases make it unlikely that significant amounts of dimer are formed early during renaturation. Since species A and B are produced very early, the limiting transconformational processes described in this paper probably precede the association processes involved in the renaturation. On this basis, we expect that renaturation begins with a major refolding [as seen with fluorescence and circular dichroism studies; Cardenas et al. (1977)] which produces two partially folded species. These species undergo transconformational steps followed by association processes that produce the native tetramer.

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Gene Expression in Normal and Neoplastic Mammary Tissue†

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ABSTRACT: The techniques of molecular hybridization and cell-free translation have been utilized to analyze total cellular poly(A^+) RNA populations from hormone-dependent, 7,12-dimethylbenz[*a*]anthracene (DMBA) induced mammary adenocarcinomas and from normal 14-day midpregnant mammary glands in Sprague-Dawley rats. These studies were designed to elucidate any marked changes in gene expression that might occur in these primary hormone-dependent mammary tumors, resulting in a deviation from normal hormone-regulated growth and differentiation. Homologous and heterologous hybridizations of poly(A^+) RNAs from DMBA-induced mammary tumors and normal mammary glands to complementary DNAs (cDNAs) revealed no detectable qualitative differences between the two RNA populations. However, there were significant quantitative differences in the abundancies of certain poly(A^+) RNA sequences. Specifically, the milk protein sequences were reduced 100-fold in the tumor when compared to the normal midpregnant mammary gland. Saturation hybridization of the poly(A^+) RNAs to ^{3}H-labeled single copy DNA revealed that both RNA populations annealed to 7.5% of the single copy haploid genome, as did mixtures of the two RNA populations, which again indicated that the great majority of the poly(A^+) RNA sequences were shared between the two tissues. Analysis of cell-free translation products by two-dimensional gel electrophoresis indicated quantitative and possibly qualitative differences in the proteins specified by the two mRNA populations. These data suggest that large alterations in gene expression are not required for the altered function found in the neoplastic tissue and that differences in relative abundancies of specific RNAs may provide a mechanism by which the concentration of specific proteins and ultimately the expression of the transformed phenotype may be regulated.

The same hormones that are important regulators of growth and differentiated function in the normal mammary gland are also necessary for the growth of hormone-dependent mammary adenocarcinomas. Administration of the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA)[1] to female rats results in the induction of mammary adenocarcinomas. Approximately 80–90% of these tumors are hormone dependent, as demonstrated by endocrine ablation and hormone replacement (Huggins & Bergenstal, 1952). Hormonally responsive DMBA-induced mammary tumors may exhibit differentiated functions that are characteristic of the normal mammary gland. Previous studies in our laboratory utilizing a specific cDNA hybridization probe revealed that casein mRNA was present in 70% of the DMBA-induced mammary tumors that were assayed. However, these casein mRNA levels were only 1–10% of those observed in 8-day lactating rat mammary tissue (Rosen & Socher, 1977).

The purpose of this study was to utilize the techniques of molecular hybridization and cell-free translation to compare the total poly(A^+) RNA populations in the DMBA-induced mammary tumor and the normal mammary gland. This approach was based on the rationale that changes in the patterns of RNA synthesis or processing lead to qualitative as well as quantitative alterations in the pool of mRNA sequences translated into proteins that ultimately modulate cellular function and phenotype. Molecular hybridization employing both cDNA and unique sequence DNA has been used to measure the number of poly(A^+) RNA sequences present in the cells in question, while cell-free translation allowed a comparison of the proteins that are specified by the mRNA populations in the tumor and the normal mammary gland.

Several previous studies have employed molecular hybridization to compare poly(A^+) RNA populations in different tissues (Hastie & Bishop, 1976; Chikaraishi et al., 1978), during different stages of differentiation (Paterson & Bishop, 1977; Affara et al., 1977) and, more importantly for this study, in normal and transformed cells (Getz et al., 1977; Kuo et al., 1976; Williams et al., 1977; Moyzias et al., 1976). However, to date, the comparisons of poly(A^+) RNA populations between normal and transformed cells have been done in cultured cells or in transplantable tumors. Since neoplastic progression is known to be a characteristic of both transformed cell lines and transplantable tumors, it is likely that the pattern of gene expression in these cells may be different from that of the

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[1] Abbreviations used: DMBA, 7,12-dimethylbenz[*a*]anthracene; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Na_2EDTA, disodium ethylenediaminetetraacetic acid; cDNA, complementary DNA; RNA_{mp} and RNA_t, poly(A^+) RNA from 14-day midpregnant mammary glands and DMBA-induced mammary tumors, respectively; $cDNA_{mp}$ and $cDNA_t$, complementary DNAs synthesized by using poly(A^+) RNA from 14-day midpregnant mammary gland and DMBA-induced mammary tumors as templates; C_0t, DNA concentration in moles of nucleotide per liter × time in seconds; R_0t, RNA concentration in moles of nucleotide per liter × time in seconds.

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