Metabolic Control and Structure of Glycolytic Enzymes. VIII. Reversal of the Dissociation of Rabbit Muscle Pyruvate Kinase into Unfolded Subunits*

George S. Johnson,† Marlene Steinmetz Kayne,‡ and William C. Deal, Jr.

ABSTRACT: The dissociation of tetrameric rabbit muscle pyruvate kinase (mol wt 237,000) into unfolded subunits (mol wt 57,000) in 6 M guanidine hydrochloride has been shown to be reversible. A systematic study of the factors affecting the reversal of the dissociation led to conditions where up to 70% of the initial catalytic activity was regained. The reversal procedure (Deal, W. C., Jr. (1969), *Biochemistry* (in press)) consisted of two phases: (1) a 100-fold dilution of guanidine hydrochloride dissociated enzyme (0°) into a reversal solvent at 0° and (2) incubation of the resulting solution at a higher temperature, usually 16°. Conditions for optimum reversal of dissociation were (1) pH 8; (2) protein concentration, 0.04 mg/ml; (3) ionic strength, 0.3; (4) reducing agent, 0.06 M β-mercaptoethanol; and (5) temperature, 0° dilution, followed by 6 hr at 16°. The half-time for activity recovery was approximately 45 min at both 0.02 and 0.09 mg per ml enzyme concentration.

Two metabolites, insulin and phosphate ion, were found to greatly influence the reversal of dissociation. Insulin decreased the activity recovery upon reversal, in contrast to what would be expected for an inducer of the enzyme. Phosphate ion yielded activity recovery at 36°; negligible activity was recovered at that temperature in its absence. The reversal of dissociation was not affected significantly by the addition of a number of metabolites including adenosine triphosphate, adenosine diphosphate, 5′-adenosine monophosphate, 3′,5′-adenosine monophosphate, lactate, fructose diphosphate, and nicotinamide–adenine dinucleotide. The reassociated enzyme had the same K_m, heat stability, and sedimentation coefficient as the native enzyme.

We have previously shown (Deal, 1967, 1969; Deal and Constantinides, 1967) that, contrary to the widely accepted view, some proteins apparently require[1] metabolites for *in vitro* reassembly from their unfolded subunits. The experimental evidence was that for *in vitro* reversal of dissociation of yeast glyceraldehyde 3-phosphate dehydrogenase, NAD was required. It was suggested that the *in vitro* requirement for NAD may reflect a similar *in vivo* requirement for NAD for proper folding of nascent polypeptide chains as they are being synthesized, thereby providing for control of enzyme synthesis. The possibility was raised that similar effects might occur with other enzymes.

As part of an analysis of the metabolic control and structure of rabbit muscle pyruvate kinase (Steinmetz and Deal, 1966; G. S. Johnson and W. C. Deal, Jr., in preparation), it was of interest to determine whether rabbit muscle pyruvate kinase could be reassembled *in vitro* from its unfolded subunits (Steinmetz and Deal, 1966; Kayne, 1966), and if so, whether metabolites were required for, or had any effect upon, the process.

Pyruvate kinase has often been postulated to be a control point in glycolysis and the activities of pyruvate kinases from various sources are known to be affected by various compounds (Hess *et al.*, 1966; Milman and Yurowitskii, 1967; Tanaka *et al.*, 1967; Taylor and Bailey, 1967; Maeba and Sanwal, 1968). Also, the synthesis of pyruvate kinase from various sources is known to be affected by various conditions. Rat liver pyruvate kinase, but not kidney cortex pyruvate kinase, decreases upon starvation or low carbohydrate diets and increases with high carbohydrate diets (Krebs and Eggleston, 1965). This effect may be explained by the observation that insulin induces pyruvate kinase synthesis in alloxan diabetic rats (Weber *et al.*, 1965, 1966). This suggested that the synthesis of pyruvate kinase might be regulated as an important step in metabolic control, and that metabolites or related substances might be the agents which would accomplish such regulation.

Pyruvate kinase has been well characterized, and its properties have been discussed in an excellent review by Boyer (1962). Morawiecki (1960) reported that pyruvate kinase dissociated into subunits of mol wt 150,000 in 6 M urea and concluded that the enzyme consisted of at least two polypeptide chains. Our previous work (Steinmetz and Deal, 1966) and that of others (Cottam *et al.*, 1969) have shown that tetrameric rabbit muscle pyruvate kinase (mol wt 237,000) is completely dissociated into unfolded subunits (mol wt 57,000) by high concentrations of urea (4 M or greater) or guanidine. Optical rotatory dispersion analysis indicated that the urea denaturation,

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[1] This means that in the absence of the metabolite, the rate of correct folding is extremely slow. The metabolite may, or may not, be absolutely essential for correctly formed enzyme.

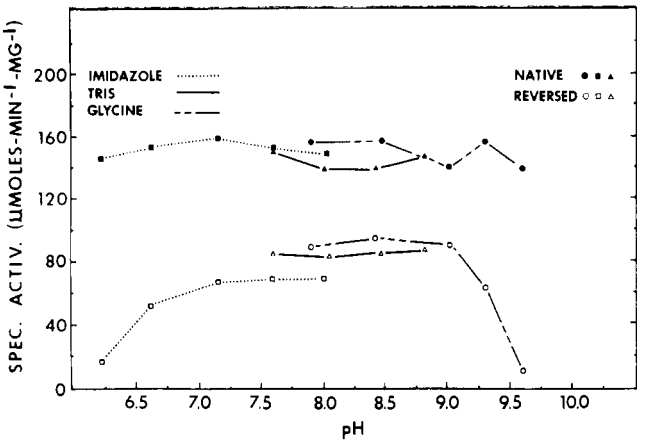

FIGURE 1: Effect of pH on the reversal of pyruvate kinase. The enzyme was dissociated in a guanidine hydrochloride solution. Then reversal was accomplished by dilution of the samples into the reversal solvent at 0°, followed by incubation at 16°. The pH of the reversal solvent was varied from pH 6.2 to 9.6 using imidazole, Tris, and glycine buffers. The optimal reversal conditions (see Methods) were used except that the 0.05 M buffer species was varied. All samples were assayed at pH 7.5 after an 18-hr incubation at 16°.

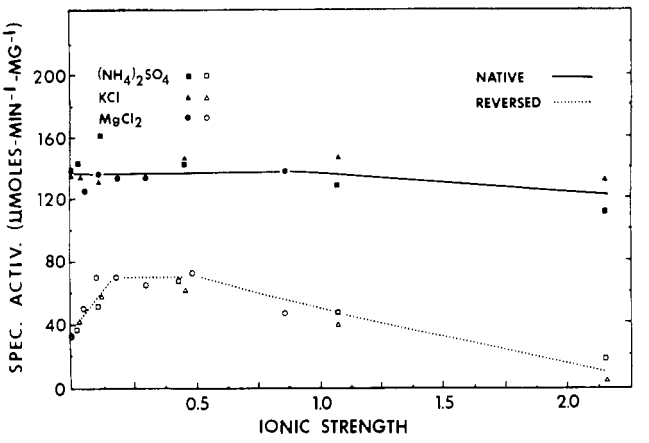

FIGURE 2: Effect of ionic strength on the reversal of pyruvate kinase. The optimal reversal conditions were used except for the variations in salt species and concentration. The concentration of the added salts in the reversal solvent was varied so that the ionic strength ranged from 0 to 2.15. The reversed enzyme was assayed after a 6-hr incubation at 16°. The contribution of the buffer, approximately 0.025, is not included in the values of ionic strength. Each experimental point represents the average of two results with two test samples.

and presumably the guanidine denaturation, resulted in essentially complete unfolding of the polypeptide chains as well (Kayne, 1966).

Although Morawiecki (1960), using a dialysis procedure, was able to obtain activity recovery of pyruvate kinase exposed to 2.5 M urea—which completely inactivates (Morawiecki, 1960), but only partially dissociates it (Steinmetz and Deal, 1966)—he concluded that the dissociation in 5 M urea was irreversible, since only 5% of the activity could be regained.

This paper describes a systematic analysis of factors affecting reversal to determine the optimal conditions necessary for the renaturation of rabbit muscle pyruvate kinase. Up to 70% of the native activity was regained upon careful dilution of the denatured enzyme into the proper renaturation solvent. Independently, Cottam *et al.* (1969) obtained 35–50% activity recovery upon removal of the denaturing agent by dialysis or by gel filtration.

Two metabolites were found to influence the reversal of dissociation in a specific way. Insulin inhibited the reversal, in contrast to what would be expected for an inducer of the enzyme. The presence of phosphate ion yielded significant activity recovery at 36°; none was obtained in its absence at that temperature.

Results

Preliminary experiments using the reversal[2] solvents and procedures (Deal, 1967, 1969) for glyceraldehyde 3-phosphate dehydrogenase immediately gave significant recovery of pyruvate kinase activity upon appropriate dilution of the guanidine–enzyme solution into the reversal solvent. A systematic study of variables influencing the reversal was then conducted, using a successive approximation technique. All results reported are for experiments where all variables except that under study were at their optimal values, unless indicated otherwise.

Effect of pH and Buffer Species. Using imidazole, Tris, and glycine buffers (0.05 M), the pH of the reversal solvent was varied from pH 6.2 to 9.6. The range for optimum reversal was found to lie between pH 7.0 and 9.0 (Figure 1). Except for imidazole, all the buffers tested, including Tris, glycine, bicine, tricine (Good *et al.*, 1966), bicarbonate, and phosphate, gave essentially identical recoveries of activity upon reversal at 16° (but see the phosphate effect at 37°). However, at a given pH, the recoveries appeared to be lower in imidazole. To eliminate the possibility that impurities in the imidazole might have been interferring with the reversal, the experiments were repeated using imidazole recrystallized from absolute ethanol; however, this did not improve the recovery.

Effect of Salt Species and Ionic Strength. An ionic strength optimum of 0.2–0.5 was found for ammonium sulfate, potassium chloride, and magnesium chloride (Figure 2). Similar results were obtained with sodium chloride. The cations of all these salts have been shown to bind pyruvate kinase (Kayne and Suelter, 1965; Suelter *et al.*, 1966). Tetramethylammonium chloride, whose cation does not bind, gave similar effects. These results suggested that the salt requirement was solely an ionic strength effect and not salt specific.

Effect of Temperature and Time of Incubation. To determine the optimal time and temperature of incubation for reversal, reversal samples were first diluted at 0° (the first stage of reversal) and then exposed to various temperatures ranging from 0 to 37°. The samples were assayed at the indicated times (Figure 3). The results indicated that 12–16° was the temperature range for optimal reversal. They also showed that the activities of the reversed enzyme samples and those of the controls were constant for 24 hr (additional data not shown indicated complete stability in this system for up to 42 hr). Maximal reversal was obtained in 2–3 hr at 21°, but

[2] The words "reversal" and "renaturation" stand for "reversal of dissociation or unfolding or inactivation."

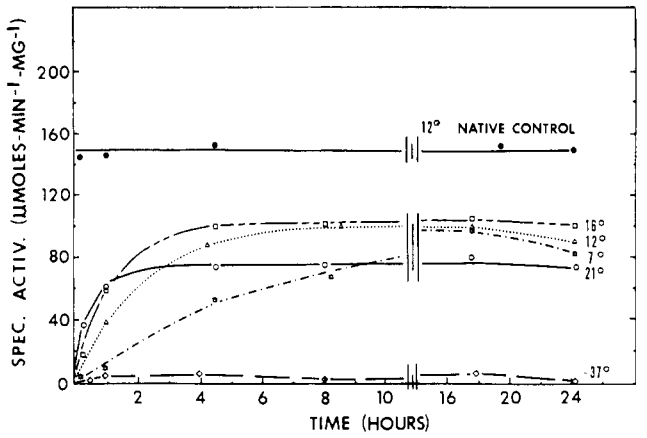

FIGURE 3: Effect of temperature and length of incubation on the reversal of pyruvate kinase. The optimal reversal conditions were used. The dissociation enzyme was diluted into the reversal solution at approximately 2°. The various enzyme samples were then immediately taken to the indicated temperatures and assayed at the indicated times.

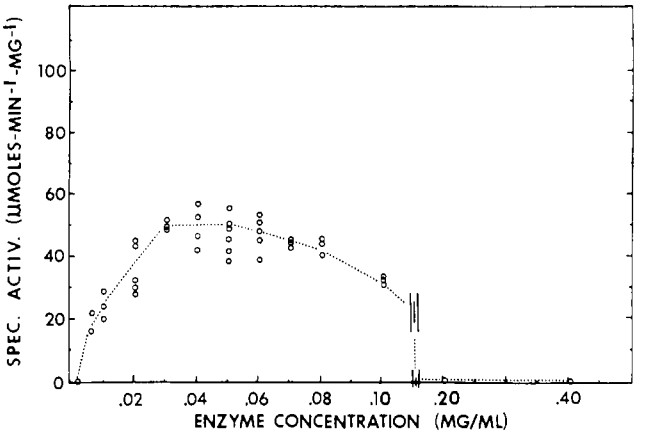

FIGURE 4: Effect of enzyme concentration on the reversal of pyruvate kinase. (See text for special dissociation and reversal procedures.) The enzyme concentration in the reversal solvent was varied from 0.002 to 0.4 mg per ml. The reversal solution also contained 0.1 mM ADP, 0.1 M Tris-HCl (pH 8.75), 0.165 M ammonium sulfate, 0.05 M β-mercaptoethanol, and 0.001 M EDTA. The data shown are the results of several experiments.

about 5 hr of exposure was needed for the 12 and 16° samples to attain maximal recovery. Essentially no reversal occurred in the samples at 0° (not shown).

The Phosphate Effect. The failure to obtain significant reversal under these conditions at 37° was surprising, since this is the temperature at which the chains must fold *in vivo*. Since a visible precipitate was observed at the 0.04 mg/ml concentration at 37°, it was thought that a lower enzyme concentration might lead to less aggregation and a greater recovery of activity. However, even with concentrations of 0.02 and 0.009 mg per ml in the renaturation solution at 37°, no activity was recovered. Nor did a 30-min preincubation at 0° provide any increase in recovery.

In an effort to obtain recovery at 37° in Tris buffer, four other reversal conditions were used: (1) the optimal conditions (see Methods), with the omission of the 0.4 M KCl; (2) the conditions described in the legend for Figure 4; (3) the conditions in 2 minus the EDTA, and (4) the conditions in 2 minus the ADP. However, none gave significant activity recovery.

Other buffers were used and reversal was finally obtained at 36° with phosphate, but not with bicarbonate or imidazole buffers. Maximum recovery occurred in the range of 0.04–0.1 M phosphate. An unusual characteristic of the phosphate–36° system was the dependence of activity recovery upon time of preincubation at 0° (see Methods). Preincubation times of 30 sec and 90 min at 0° yielded activity recoveries of 10 and 30%, respectively. Denatured enzyme diluted at 36° directly, instead of at 0°, precipitated. In contrast, denatured enzyme diluted at 16° directly yielded 30% activity recovery with no precipitation. Also, a 5-min preincubation at 0° was adequate to yield maximal recovery at 16°.

Effect of Protein Concentration. Since some phase or phases of the reversal process involved the successive association of the subunits into the dimeric and then tetrameric species, it was expected that the concentration of enzyme in the reversal mixture might be important for activity recovery. To test the effect of enzyme concentration on the reversal, stock enzyme solution was diluted with the dissociation solvent, the final composition[8] of which was 3.5 M guanidine hydrochloride, 0.06 M β-mercaptoethanol, 0.02 M Tris-HCl buffer (pH 8.0), 0.07 M KCl, 0.001 M EDTA, plus residual 0.01 M imidazole buffer (Kayne, 1966). After a 1-hr incubation in the dissociation solvent, varying aliquots of the enzyme solution were diluted appropriately with the dissociation solvent to yield various protein concentrations. Samples from these solutions were then diluted 100-fold to yield reversal samples containing various enzyme concentrations ranging from 0.002 to 0.4 mg per ml. Native enzyme controls were not run for this particular series because of the extensive amounts of enzyme required. The data obtained (Figure 4) indicated that enzyme concentrations in the narrow range of 0.03–0.08 mg/ml were optimal. One technical problem preventing analysis of a wider range was that the enzyme precipitated at protein concentrations greater than 0.2 mg/ml in the reversal solution.

Effect of Sucrose. The presence of sucrose in the reversal solution increased the percent recovery at higher, but not at lower, enzyme concentrations. Reversal solutions with 10% sucrose gave 45% recovery of activity at 0.12 mg/ml of enzyme and 11% recovery at 0.2 mg/ml; under otherwise identical conditions, samples without sucrose yielded 15 and 0% recovery, respectively. However, at a protein concentration of 0.4 mg/ml, no reversal was obtained, even in the presence of 10% sucrose and a precipitate formed during the incubation. In contrast to the enhancement by sucrose of reversal recovery at higher protein concentrations, there was no effect of either 10 or 20% sucrose on the reversal recovery using a protein concentration of 0.04 mg/ml, the optimal enzyme concentration (Figure 4).

[8] The enzyme was stored in aqueous solution (0.02 M imidazole buffer) at a concentration of 20–80 mg/ml. The 3.5 M guanidine was a compromise to allow the highest possible protein concentration in the dissociation solution with a sufficient guanidine concentration to ensure complete dissociation and unfolding of the enzyme. Since guanidine usually seems to accomplish the same result as urea at roughly one-half the concentration, 3.5 M guanidine hydrochloride seemed adequate to ensure dissociation and unfolding in this experiment (Steinmetz and Deal, 1966).

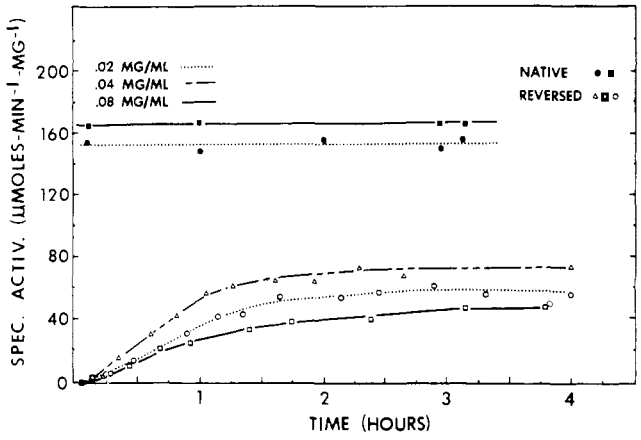

FIGURE 5: Effect of enzyme concentration on the half-time of reversal of pyruvate kinase. The optimum reversal conditions were used except for the omission of the 0.4 M KCl. Samples of the dissociated enzyme were diluted into the reversal solution to the final enzyme concentration indicated, at approximately 2°. These diluted enzyme solutions were then placed into a 16° temperature bath and the enzyme was assayed at 25° at the appropriate times. The time at which the samples were brought to 16° was designated zero time. All the assays were linear, indicating that no change in activity occurred during the assays.

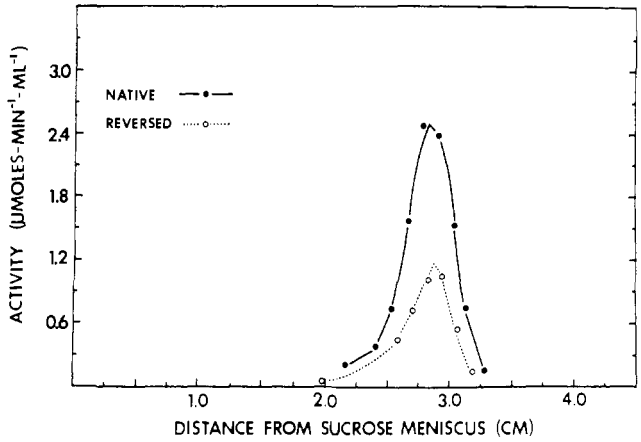

FIGURE 7: Sucrose density gradient centrifugation pattern of native and reversed pyruvate kinase. The optimum reversal conditions were used except for the omission of 0.4 M KCl and the substitution of 0.1 M bicine for 0.05 M Tris-HCl. Approximately 0.01 mg of the native or renatured enzyme was layered on the sucrose gradient, and centrifuged for 18 hr at 40,000 rpm at 2°. The sucrose solutions also contained 0.02 M imidazole-HCl (pH 7.15), 0.15 M KCl, and 0.001 M EDTA.

Half-Times for Reversal. Since native pyruvate kinase consists of tetramers and also apparently exists as dimers or monomers (3.6S species) under certain conditions (Steinmetz and Deal, 1966), it was of interest to see whether association had to occur in order for activity to be regained. The concentration dependence of the half-times of activity recovery at 16° was studied to provide information on this question. The half-time for recovery, determined experimentally as the time at which 50% of the maximum activity was recovered, was found to be 50 min for both the 0.09- and 0.02-mg per ml samples; the half-time was 44 min for the 0.04-mg/ml samples (Figure 5). These differences were presumably not significant, since the two extremes of concentration had the same half-times. Although these results were not conclusive, they did suggest that at these concentrations a first-order process, a folding step, was rate limiting for the recovery of activity.

The half-time for reversal at 16° was surprisingly long. However, the half-time for reversal at 36° in phosphate buffer and 0.04 mg/ml was determined to be only 4–5 min. This is nearer to the rate of folding expected *in vivo*.

Effect of Reducing Agent Species and Concentration. Since the reversal experiments were conducted under aerobic conditions, the possibility existed that random formation of disulfide bonds might be hindering the specific refolding of the enzyme. To test this possibility, a reversal study was performed with various concentrations of the reducing agent β-mercaptoethanol ranging from 0 to 0.44 M. Maximal reversal was obtained with mercaptoethanol concentrations in the range of 0.05–0.15 M (Figure 6). Negligible recovery was obtained from the sample reversed without β-mercaptoethanol.

Other reducing agents were also tested. Reversal solutions containing 0.06 M dithiothreitol or 3-mercapto-1,2-propanediol gave results similar to those with 0.06 M β-mercaptoethanol. Glutathione, which is expected to exist in fairly high concentrations *in vivo*, also gave good recovery of activity. However, in the glutathione system both the control enzyme and the reversed enzyme were unstable during incubation, losing approximately 50% of their original activity within 10 hr.

Effect of Added Metabolites. Previous work in this laboratory had shown that the refolding of yeast glyceraldehyde 3-phosphate dehydrogenase was aided by the metabolite NAD (Deal, 1967, 1969; Deal and Constantinides, 1967). To test for a similar type of effect on the reversal of pyruvate kinase, reversal solutions 5 mM in ATP, ADP, 3′,5′-AMP, phosphoenolpyruvate, or lactic acid were tested. All the metabolites were tested in the presence of 0.1 M $MgCl_2$. Also, 3′,5′-AMP, ADP, and ATP were tested in 0.4 M KCl. Occasionally ADP and ATP in 0.4 M KCl appeared to give about 5–10% enhancement of activity recovery over that in their absence, but

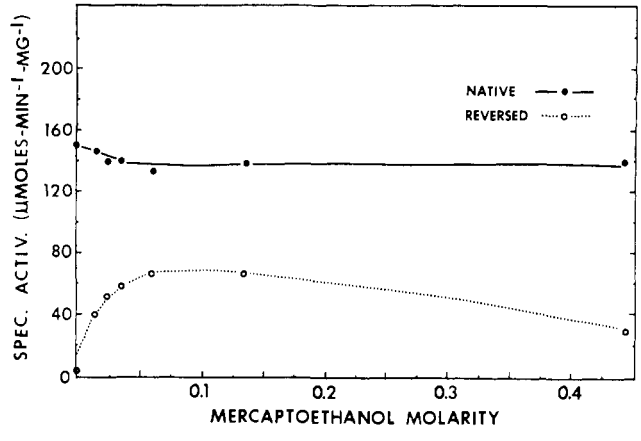

FIGURE 6: Effect of β-mercaptoethanol on the reversal of pyruvate kinase. The optimum reversal solution was used except for the omission of 0.4 M KCl. The concentration of β-mercaptoethanol in the reversal solution was varied from 0 to 0.45 M. The renatured enzyme was assayed after a 6-hr incubation at 16°. Each point represents the average of results with two test samples.

TABLE 1: Effect of Metabolites on the Reversal in 0.2 M KCl.[a]

Conditions	% of Ref Act.[b,c] 16°	Conditions	% of Ref Act.[b] 16°
0.05 M Tris, pH 8.0	100	0.05 M K_2HPO_4, pH 8.0	107
0.05 M Tris, pH 8.0		0.05 M K_2HPO_4, pH 8.0	
+ 2.5 mM ADP	100	+5 mM ADP	98
+ 2.5 mM ATP	111		
+ 5 mM 5'-AMP	86	+5 mM ADP	
+ 5 mM 3',5'-AMP	86	+5 mM AMP	106
+ 2.5 mM FDP	90	+5 mM ADP	
+ 2.5 mM FDP		+5 mM AMP	
+ 5 mM 3',5'-AMP	91	+5 mM α-D-glucose	111
+ 0.73 mM Phosphoenol-pyruvate	90	+5 mM ADP	
		+5 mM α-D-glucose	100
+ 2.5 mM β-NAD	90		
+ 5 mM α-D-glucose	100		
+ 5 mM glucose 6-phosphate	90		
+ 2.5 mM ADP			
+ 10 mM $MgCl_2$	106		

[a] The reversed enzyme was assayed after incubation for 5–7 hr at 16° in the reversal solution which, in addition to the buffer contained 0.2 M KCl and 0.06 M β-mercaptoethanol. [b] The reference activity is that obtained after reversal for 5–7 hr at 16° in a reversal solution which contained 0.05 M Tris (pH 8.0), 0.2 M KCl, and 0.06 M β-mercaptoethanol, and no metabolite. A *reversal* sample is the reference. [c] No activity was recovered at 37° under any of these conditions.

this was not observed in 0.1 M $MgCl_2$. None of the other metabolites significantly aided the reversal under these conditions.

It seemed possible that in this system ionic strength might have an effect superimposed on metabolite effects, as it had in the reversal system of yeast glyceraldehyde 3-phosphate dehydrogenase. The requirement of that enzyme for NAD was essentially absolute at low ionic strength (0.15 M KCl) and this requirement was virtually abolished when the ionic strength was raised to 0.8 (Deal, 1969). However, reduction of the KCl concentration in the reversal mixture from 0.4 to 0.2 M still did not yield any pronounced metabolite effects at 16°, using the previously mentioned metabolites and some additional metabolites (Table I).

Since phosphate had previously been found to uniquely aid recovery at 36°, it also seemed possible that it might aid the recovery at 16° and perhaps produce a synergistic enhancement of reversal with other metabolites. However, it only slightly aided the reversal at 16° and there was no significant effect of added metabolites in the presence of phosphate (Table I).

Insulin, an inducer of rat liver pyruvate kinase (Weber *et al.*, 1965), showed a pronounced interference with reversal at 16° and an even more pronounced interference at 37° (Table II). Insulin had no effect on the native enzyme. Ribonuclease and bovine serum albumin at the same concentration (0.2–0.01 mg/ml) had no significant effect on the reversal recovery of pyruvate kinase, suggesting that the effect was specific for insulin and not a general protein effect.

Comparison of Native and Reversed Enzyme. The native and reversed enzymes were found to have identical characteristics as shown by measurements of sedimentation coefficients (Figure 7), K_m values for ADP and K^+, and heat-stability profiles at 50°.

Discussion

The systematic analysis of variables influencing the refolding and reassembly of guanidine-dissociated and unfolded rabbit muscle pyruvate kinase into its active native tetrameric structure led to conditions where substantial activity recovery (up to 70%) was obtained. In contrast Morawiecki (1960) had earlier reported the dissociation in high concentrations of urea to be essentially irreversible, since he had obtained less than 5% activity recovery. Also in limited studies Cottam *et al.* (1969) obtained 35–50% recovery of activity upon removal of the denaturing agent by dialysis or gel filtration. Thus, the yeast glyceraldehyde 3-phosphate dehydrogenase reversal conditions and procedures (Deal, 1967, 1969), which served as the starting point for this study, have been found to give substantial recovery of activity for rabbit muscle pyruvate kinase, as they have for a number of other enzymes (see Deal, 1969).

As might be expected, the general optimum requirements for reversal of rabbit muscle pyruvate kinase are similar to those of these other enzymes. For the yeast glyceraldehyde 3-phosphate dehydrogenase reversal system and for pyruvate kinase in particular, two key factors in obtaining significant reversal were: (1) the removal of the denaturing agent by dilution of the guanidine–enzyme (0°) into a reversal solvent at 0° and then bringing the sample to higher temperatures (12–35°) to produce the refolding–reassociation reactions and (2) the use of low protein concentrations, about 0.05 mg/ml, for

TABLE II: Effect of Insulin on the Reversal.[a]

Conditions	% of Ref Act.[b,c] 16°	Conditions	% of Ref Act.[b] 16°	% of Ref Act.[b] 37°
0.05 M Tris, pH 8.0	100	0.05 M K_2HPO_4, pH 8.0	107	36
0.05 M Tris, pH 8.0		0.05 M K_2HPO_4, pH 8.0		
+ 0.2 mg of bovine serum albumin	107	+ 0.2 mg/ml of insulin	34	6
+ 0.2 mg/ml of ribonuclease	90	+ 0.08 mg/ml of insulin	59	7
+ 0.2 mg/ml of insulin	32	+ 0.2 mg/ml of insulin		
+ 0.08 mg/ml of insulin	64	+ 2.5 mM FDP	27	<3
+ 0.01 mg/ml of insulin	92	+ 0.2 mg/ml of insulin		
+ 0.2 mg/ml of insulin		+ 5 mM α-D-glucose		
+ 0.5 mM 3′,5′-AMP	40	+ 5 mM ADP	30	<3

[a] The reversed enzyme was assayed after incubation for 5–7 hr at 16° in the reversal solution which, in addition to the buffer, contained 0.2 M KCl and 0.06 M β-mercaptoethanol. [b] The reference activity is that obtained after reversal for 5–7 hr at 16° in a reversal solution which contained 0.05 M Tris (pH 8.0), 0.2 M KCl, and 0.06 M β-mercaptoethanol, and no metabolite. A *reversal* sample is the reference. [c] No activity was recovered at 37° under any of these conditions.

the reversal process. Both of these factors seemed to operate to avoid nonspecific aggregations which would have caused the protein to precipitate, or at least, to have attained rather irreversibly an aggregated state.

Possible Effects of Metabolites. When this research was begun, the possibility seemed good that metabolites might affect the folding and synthesis of pyruvate kinase and/or its activity. Because of the possible direct relationship presumed (Deal, 1969) between folding control by metabolites *in vitro* and regulation of protein synthesis by inducers *in vivo*, it was natural to consider as prime candidates for "folding control" those compounds thought likely to be inducers (or repressors) of pyruvate kinase. In this connection the report by Weber and coworkers (Weber *et al.*, 1965, 1966) that insulin is an inducer of rat liver pyruvate kinase was of special interest. This raised the possibility that insulin, or some product of insulin action, might control the folding of pyruvate kinase. Unfortunately, the primary products of insulin action have not yet been elucidated. Therefore, within this group, the only feasible test was that of the ability of insulin to directly enhance reversal.

This study showed that under the conditions used, insulin did affect the reassembly of pyruvate kinase, albeit in a negative way rather than the positive way expected. It also showed that the binding was specific for insulin; neither bovine serum albumin nor ribonuclease had any effect on the reversal recovery. Furthermore, the insulin effect was not a nonspecific inhibition of enzyme activity, since the activity of the native enzyme was not significantly affected by insulin under identical conditions. Since insulin does interact with the unfolded polypeptide chains of pyruvate kinase, and since other variables may superimpose their effects onto those of insulin *in vivo*, the possibility cannot be ruled out that the insulin interaction might favor assembly *in vivo*, rather than impede it.

It also seemed reasonable that the metabolites expected to influence enzyme activity might be expected to be prime candidates for affecting the rates of synthesis of the enzyme. A number of compounds have been shown to affect the activity of muscle pyruvate kinase. The ion K^+ was reported to be required for the enzyme to be in the proper conformation for activity (Melchior, 1965). The binding of substrates and activating cations was reported to have resulted in a change in protein conformation (Kayne and Suelter, 1965). Other studies showed an interaction of the Mg^{2+}–ADP complex with the enzyme (Melchior, 1965). Weber *et al.* (1967) showed that acetyl-CoA was an inhibitor of both liver and rat muscle pyruvate kinase.

Also, a number of compounds have been shown to affect the activity of the pyruvate kinase enzymes obtained from various sources other than muscle. Pyruvate kinase from *Escherichia coli* was activated by both AMP and FDP (Maeba and Sanwal, 1968) and the enzyme from developing loach embryos was activated by 3′,5′-AMP and FDP (Milman and Yurowitskii, 1967). The pyruvate kinases from yeast (Hess *et al.*, 1966) and liver (Taylor and Bailey, 1967) were strongly activated by FDP. Although there has been no evidence that pyruvate kinase from rabbit muscle was activated by FDP, Taylor and Bailey (1967) have suggested that *in vivo* it might have possessed this characteristic and lost it during isolation and purification of the enzyme.

Of the metabolites and ions tested in this study, only phosphate and insulin affected the *in vitro* folding and assembly of pyruvate kinase from its unfolded subunits. Phosphate seemed to be absolutely required for this process at 36°, although not at 16°. Since this is near the temperature at which the *in vivo* folding of rabbit muscle pyruvate kinase occurs, this may be a very significant requirement.

In this regard, it is of interest that the rabbit muscle pyruvate kinase apparently can exist in two temperature-dependent states (Kayne and Suelter, 1968). Since the midpoint of the transition from one state to the other is in the range of 16–22°, it is possible and even likely that the low-temperature form, which does not require phosphate for folding, may not

occur *in vivo*. That is, possibly only the high-temperature form occurs *in vivo* and it may require phosphate for folding. However, an alternative possibility is that phosphate keeps the enzyme in the low-temperature form where it reverses easily. Also, the possibility cannot be overlooked that *in vivo* other effects might operate in conjunction with the phosphate effect. These subjects are receiving further study.

In general, the muscle is expected to have a considerably different set of metabolic priorities and types of controls than liver or yeast because it does not have the ability to carry out gluconeogenesis, nor does it have, in significant amounts, many of the other metabolic pathways (such as the pentose phosphate cycle) which liver and yeast possess. It will thus be of much interest to compare these results for rabbit muscle pyruvate kinase with pyruvate kinases from other sources.

Materials and Methods

Enzyme Preparation and Assay. Rabbit muscle pyruvate kinase (EC 2.7.1.40) was isolated from frozen rabbit (Pel-Freez Biologicals, Inc.) using the modifications (Steinmetz and Deal, 1966) of the method of Tietz and Ochoa (1958). The enzyme was assayed at 25° by coupling the pyruvate kinase reaction to the lactic dehydrogenase reaction and following the decrease in absorbancy at 340 mμ (Kubowitz and Ott, 1944) using a Beckman DU spectrophotometer attached to a Gilford multiple-sample absorbance recorder. Enzyme concentrations were determined by measuring the optical density at 280 mμ and using the extinction coefficient of $E_{280\ m\mu}$ 0.54 ml/g cm (Bucher and Pfleiderer, 1955). The specific activity of the native enzyme was found to be 140–160 μmoles of NADH consumed per min per mg of protein. The enzyme concentration in the assay was 0.2 μg/ml and the reaction was monitored for 1 min.

Reagents. Reagent grade chemicals were obtained from the following commercial sources: Tris (Trizma base), phosphoenolpyruvate (tricyclohexylamine salt), glucose 6-phosphate (disodium salt), bovine serum albumin, bovine pancreas ribonuclease, AMP (sodium salt), bovine pancreas insulin, FDP (sodium salt), 3′,5′-AMP (sodium salt), and lactic acid from Sigma (St. Louis); ATP (sodium salt), ADP (sodium salt), β-NADH (disodium salt), and β-NAD^+ (disodium salt) from P-L Biochemicals (Milwaukee); tricine and bicine (Good *et al.*, 1966) and dithiothreitol from Calbiochem; imidazole and tetramethylammonium chloride from Eastman (Rochester, N. Y.); glycine from General Biochemicals (Chagrin Falls, Ohio); 3-mercapto-1,2-propanediol from Aldrich Chemical Co. (Milwaukee); β-mercaptoethanol from Matheson Scientific (Elk Grove Village, Ill.); α-D-glucose from Pfanstiehl Laboratories, Inc. (Waukegan, Ill.); and beef heart lactic dehydrogenase from Worthington (Freehold, N. J.).

Urea (Baker Analytical reagent) was recrystallized from absolute ethanol and allowed to dry at 50° to remove residual ethanol. Guanidine-HCl was prepared (Anson, 1941) from guanidine carbonate (Eastman, Rochester, N. Y.) and recrystallized from absolute ethanol. The crystals were then dried at 50°. All pH measurements and buffer adjustments were made at 23°.

Dissociation Procedure. Unless otherwise indicated, the denaturation in all experiments was conducted in the following manner: stock enzyme solution (20 mg/ml, 0.02 imidazole M (pH 7.0)–0.001 M EDTA) was diluted to 4 mg/ml in freshly prepared dissociation solution and allowed to remain there for 1 hr in an ice bath. The dissociation solution was at pH 8 and consisted of 7 M guanidine hydrochloride or 7 M urea, 0.12 M β-mercaptoethanol, 0.04 M Tris-HCl, and 0.001 M EDTA.

Activity recoveries using urea or guanidine were identical. The buffer species or pH of dissociation were not important since dissociation at pH 6.5 (potassium phosphate), pH 7.3 (imidazole), and pH 8.0 (Tris) all gave essentially the same activity recoveries upon reversal. Also, omission of EDTA or β-mercaptoethanol from the dissociation did not affect the reversal recovery. The use of a 100-fold dilution of urea- or guanidine–enzyme, which resulted in only 0.06 M residual denaturing agent, yielded high recoveries. This level of residual denaturing agent had no effect on the activity of native control samples. Although this does not exclude the possibility that the level of residual denaturing agent might interfere with the subunit refolding process, it does seem unlikely.

Reversal Procedure. The renaturation conditions and procedures followed were those previously described for the renaturation of glyceraldehyde 3-phosphate dehydrogenase (Deal, 1967, 1969; Deal and Constantinides, 1967). The optimal conditions for the reversal were: (1) pH 8, 0.05 M Tris-HCl; (2) protein concentration, 0.04 mg/ml; (3) salt, 0.4 M KCl plus 0.1 M $MgCl_2$; (4) reducing agent, 0.06 M β-mercaptoethanol; and (5) temperature, 0° dilution, followed by 6 hr at 16°. These optimal conditions were used for reversal except where designated in the appropriate legends. The dissociated enzyme was diluted with careful swirling into the reversal solution which had been previously cooled in an ice bath to approximately 0°. The samples were then taken to 16° for incubation. Although in some cases the conditions used varied slightly, they remained in the optimal range plateau in all cases. The reducing agent was added to the stock reversal solvent just prior to the protein dilution to prevent undesirable air oxidation of the sulfhydryl groups. In each experiment, control samples were subjected to identical treatment except for the presence of urea or guanidine. Duplicate samples were usually run.

Comparison Studies. The sucrose density centrifugation experiment followed the procedure of Martin and Ames (1961). The Spinco Model L ultracentrifuge was used and the SW 39 rotor was run at 40,000 rpm at 2° for 18 hr.

For the heat-stability and Lineweaver–Burk analyses, the reversed and native enzymes (0.04 mg/ml) in 0.05 M Tris (pH 8.0), 0.3 M KCl, 0.1 M $MgCl_2$, and 0.06 M β-mercaptoethanol were dialyzed 12 hr against 50 volumes of 0.05 M Tris (pH 8.0).

The half-time for the inactivation at 50° under these conditions was about 18 min. This temperature appears to be near the transition point, since at 42° there was only 25% activity loss in 1 hr.

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The Hydrolysis of Adenosine Triphosphate by Purified Components of Nitrogenase*

P. T. Bui† and L. E. Mortenson

ABSTRACT: The hydrolysis of adenosine triphosphate requires both purified components of the N_2-reducing system (nitrogenase): molybdoferredoxin and azoferredoxin. At low pH, the high rate of hydrolysis is not affected by the electron donor dithionite.

This shows that adenosine triphosphate hydrolysis by nitrogenase does not require a source of electrons. Evidence is also presented to suggest that even though the N_2-reducing unit and the adenosine triphosphate hydrolyzing unit are constructed with the same two protein components, these units do not contain an identical number of component molecules.

Nitrogenase, the enzyme system responsible for the biological reduction of N_2, catalyzes a transfer of electrons which is absolutely dependent upon a concomitant breakdown of ATP to ADP and P_i (Kennedy *et al.*, 1968). Thus, in the presence of ATP, nitrogenase can transfer electrons from a suitable reductant, *e.g.*, dithionite (Bulen *et al.*, 1965), to a variety of electron acceptors including N_2 (Mortenson, 1964), $2H^+$ (Bulen *et al.*, 1965), nitrous oxide (Hardy and Knight, 1966b), acetylene (Dilworth, 1966) and homologs (Hardy and Jackson, 1967), azide (Schöllhorn and Burris, 1967), cyanide and homologs (Hardy and Jackson, 1967), and isocyanides (Kelly *et al.*, 1967). On the other hand, the dependence of ATP hydrolysis upon a source of electrons does not appear to be absolute. At high pH, nitrogenase was shown (Mortenson, 1965) to hydrolyze ATP even in the absence of a source of electrons, although at a much reduced rate. The fact that at high pH the rate of hydrolysis is much higher in the presence of an appropriate electron donor has led to the assumption that in N_2 reduction, nitrogenase must get reduced before it can react with ATP. Clearly, if we are to unravel eventually the mechanism of N_2 reduction, it is important to know whether nitrogenase reacts first with the electron donor or with ATP. We therefore reexamined the hydrolysis of ATP in the presence or absence of an appropriate electron donor, using purified components of nitrogenase. We found that at low pH, the rate of ATP hydrolysis was unaffected by the electron donor dithionite. This suggests (1) that the reduction of nitrogenase is not a prerequisite for its reaction with ATP, and (2) that in N_2 reduction, the reaction of nitrogenase with ATP in fact precedes its reaction with the electron donor. These conclusions may make it easier to investigate the role of ATP in N_2 reduction. Earlier attempts to detect exchange reactions of nitrogenase involving

* From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907. *Received February 18, 1969.* Supported by Grant AI 04865-07 from the National Institutes of Health.

† Present address: Biology Division, California Institute of Technology, Pasadena, Calif. 91109.