cAMP-dependent protein kinase inhibitor isolated from *Physarum polycephalum*. Technical assistance provided in the isolation of nuclei and nucleoli by Carla Armijo and Denise Barela, who were supported by NIH Grant RR-08136, is gratefully acknowledged.

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# Single Subunits of Sepharose-Bound Pyruvate Kinase Are Inactive†

David H. Porter and Janet M. Cardenas*

ABSTRACT: Bovine skeletal muscle pyruvate kinase was covalently coupled to Sepharose that had previously been activated by low concentrations of cyanogen bromide. Reaction conditions were chosen such that essentially all tetrameric enzyme molecules were covalently bound via a single subunit. Denaturation of the immobilized enzyme with guanidine hydrochloride followed by removal of noncovalently bound subunits and denaturant resulted in essentially no enzymatic activity remaining bound to the resin. Thus, single immobilized subunits of bovine pyruvate kinase were inactive. Sepharose-bound enzymatic activity could be recovered by adding soluble renaturing enzyme subunits to the immobilized monomers. The former combine noncovalently with the latter, presumably resulting in re-formation of bound tetramers, and an average recovery of 61% of the original matrix-bound activity was observed. While interactions with other enzyme subunits appear to be necessary for catalytic activity of bovine muscle pyruvate kinase, these subunit interactions apparently can be provided by chemically modified subunits. Soluble, renaturing subunits from enzyme that had been inactivated by treatment with trinitrobenzenesulfonic acid were able to interact with matrix-bound single subunits, thereby restoring the enzymatic activity of the latter.

Bovine muscle pyruvate kinase (EC 2.7.1.40) consists of four subunits of 57 000 daltons each (Cardenas et al., 1973). Four phosphoenolpyruvate binding sites occur per tetramer.

Pyruvate kinase can be reversibly renatured after its denaturation in solutions of urea or guanidine hydrochloride (Johnson et al., 1969; Cottam et al., 1969; Cardenas & Dyson, 1973; Strandholm et al., 1976; Cardenas et al., 1977). Recovery of 65–90% of the original enzymatic activity occurs when the urea or guanidine-HCl is removed, via either dilution or dialysis, and the enzyme is incubated under mild conditions in the presence of mercaptoethanol or dithiothreitol.

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Irreversible inactivation occurs when pyruvate kinase is treated with trinitrobenzenesulfonic acid (Hollenberg et al., 1971; Hess & Röschlau, 1972; Hubbard & Cardenas, 1975). Inactivation is thought to occur via covalent modification of a residue located in or near the ADP binding site, as ADP protects against inactivation. Johnson et al. (1979) have demonstrated via peptide sequence analysis that modification occurs via trinitrophenylation of the $\epsilon$-amino group of a lysine. Although trinitrophenylated subunits have lost most of their enzymatic activity, they retain their overall hydrodynamic structure and their ability to interact with other subunits (Hubbard & Cardenas, 1975).

While all native pyruvate kinases characterized to date have been found to be tetramers, reports exist suggesting catalytic activity in dimeric forms (Steinmetz & Deal, 1966; Cottam et al., 1969). High concentrations of urea or guanidine-HCl both dissociate pyruvate kinase into monomers and eliminate enzymatic activity (Steinmetz & Deal, 1966; Cottam et al., 1969; Cardenas et al., 1973). However, the concentrations of urea and guanidine-HCl used to dissociate the enzyme also cause major disruptions in the secondary and tertiary structure (Cardenas et al., 1977) and therefore provide no information regarding possible enzymatic activity of a folded monomer. We therefore were interested in applying the techniques for immobilizing enzyme subunits in order to determine the effect of subunit interactions on the catalytic activity of pyruvate kinase isozymes under mild conditions.

## Experimental Procedures

Substrates, Sepharose 4B-200, cyanogen bromide, and lactate dehydrogenase were obtained from Sigma Chemical Co. Bovine muscle pyruvate kinase was prepared by the method of Cardenas et al. (1973). Pyruvate kinase activity was measured by the method of Bücher & Pfleiderer (1955) in a Beckman Acta III spectrophotometer equipped with an automatic sample changer and a stirring accessory. All enzyme assays were performed at 25 °C in 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 (PEP), 0.16 mM NADH, and 27.5 units/mL lactate dehydrogenase. Standard assay volumes were 1.0 mL for soluble enzyme and 3.0 mL for immobilized enzyme to permit stirring of the suspension during the assay of the latter form of the enzyme. Enzyme activity of immobilized samples was expressed as units per milliliter of packed resin, i.e., that which was sedimented by centrifugation at low speed in a clinical centrifuge. Enzyme units are defined as the amount of enzyme that will catalyze the disappearance of 1 $\mu$mol of substrate per minute under our assay conditions.

*Coupling of Pyruvate Kinase to Sepharose.* Sepharose was activated with cyanogen bromide by using the buffer method described by Parikh et al. (1974). Sepharose was washed with 8 volumes of 1 M NaCl followed by 8 volumes of distilled, deionized water, collecting the resin between washes via vacuum filtration in a funnel with a sintered glass disk. After the last wash, the resin was suspended in distilled, deionized water and collected by centrifugation in a clinical centrifuge at the lowest setting.

To the packed, washed Sepharose was added an equal volume of 2 M sodium carbonate, and the slurry was cooled to 5 °C. Cyanogen bromide (1–2 mg/mL packed resin) was dissolved in acetonitrile and added with swirling to the resin slurry. After 2 min, the slurry was washed with 20 volumes of 0.1 M sodium carbonate buffer (pH 9.0) under vacuum in a funnel with a sintered glass disk.

Pyruvate kinase was coupled to the activated Sepharose by the method of Chan (1976a). The washed Sepharose was added to an equal volume of 5 mg/mL pyruvate kinase that had previously been dialyzed against 0.1 M carbonate buffer, pH 9.0, 1.0 mM EDTA, and 10 mM 2-mercaptoethanol. This mixture was incubated at 3 °C with occasional gentle swirling. After 24 h, unreacted groups were inactivated, and unbound protein was removed by washing the Sepharose in 10 volumes of 0.1 M ethanolamine-HCl, pH 7.5. The resin was then suspended in 2.5 volumes of 0.1 M ethanolamine-HCl, pH 7.5, and 10 mM 2-mercaptoethanol and incubated at 3 °C with occasional gentle swirling. After 24 h, the Sepharose was washed with and stored in a renaturation solution containing 0.05 M Tris-HCl, pH 7.5, 0.1 M KCl, 0.5 M sucrose, 5 mM $MgCl_2$, 0.1 mM EDTA, and 5 mM dithiothreitol at 4 °C.

*Trinitrophenylation of Pyruvate Kinase.* Samples of pyruvate kinase were inactivated by treatment with 2,4,6-trinitrobenzenesulfonic acid as described by Hubbard & Cardenas (1975). After inactivation, the reaction was stopped by the addition of 0.1 volume of 1 M Tris-HCl, pH 7.5. The protein was precipitated by the addition of saturated ammonium sulfate and collected by centrifugation (20 min at 12000$g$, 0 °C). The precipitate was dissolved in 1 mL of 0.05 M phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol. This precipitation and centrifugation procedure was repeated 2 more times. The final precipitate was dissolved in 0.05 M Tris-HCl, pH 7.5, 0.1 M KCl, 0.5 M sucrose, 5 mM $MgCl_2$, 0.1 mM EDTA, and 5 mM dithiothreitol, dialyzed against the same buffer for 3 h at 4 °C in the dark, and stored at 4 °C in the dark. The enzyme was 90–98% inactivated by this treatment.

*Removal of Noncovalently Bound Subunits from Sepharose-Immobilized Enzyme.* Prior to the removal of noncovalently bound subunits, Sepharose-immobilized enzyme was washed 3 times by alternately collecting the resin by centrifugation in a clinical centrifuge and resuspending it in 0.05 M Tris-HCl, pH 7.5, 0.1 M KCl, 0.5 M sucrose, 5 mM $MgCl_2$, 0.1 mM EDTA, and 5 mM dithiothreitol at 4 °C. The washed resin was suspended in an equal volume of 8 M guanidine-HCl and incubated for 15 min on ice. Noncovalently bound subunits were removed by filtration, washing the resin with 100 volumes of 4 M guanidine-HCl, 0.025 M Tris-HCl, pH 7.5, 0.05 M KCl, 0.25 M sucrose, 2.5 mM $MgCl_2$, 0.05 mM EDTA, and 2.5 mM dithiothreitol. Enzyme renaturation was effected by transferring the Sepharose-immobilized protein into 25 volumes of 0.05 M Tris-HCl, pH 7.5, 0.1 M KCl, 0.05 M sucrose, 5.0 mM $MgCl_2$, 0.1 mM EDTA, and 5.0 mM dithiothreitol and incubating at 16 °C for 24 h. The gel could be stored at 4 °C.

*Reconstitution of the Quaternary Structure of Sepharose-Bound Subunits.* The procedures described here were based on those developed earlier for the reversible renaturation of soluble pyruvate kinase (Johnson et al., 1969; Cottam et al., 1969; Cardenas & Dyson, 1973; Strandholm et al., 1976; Porter & Cardenas, 1980a,b). Sepharose-immobilized enzyme from which noncovalently bound subunits had been previously removed was collected by centrifugation and suspended in 2.3 volumes of the renaturation solution described above. A solution of soluble, non-Sepharose-bound pyruvate kinase, either native or 90–98% inactivated by trinitrophenylation, was mixed 1:1 with a solution containing 8 M guanidine-HCl and incubated for 15 min on ice in order to generate unfolded monomers. A 40-$\mu$L aliquot of this solution containing approximately 1.1 mg of protein was diluted into 1 mL of the Sepharose-immobilized slurry. This mixture was incubated at

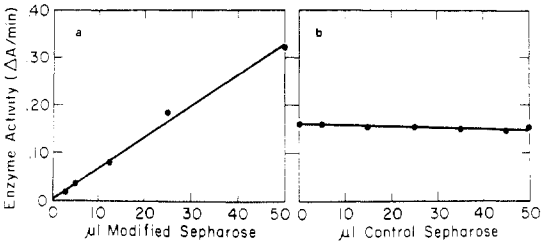


FIGURE 1: (a) Assay of immobilized pyruvate kinase activity as a function of the amount of immobilized enzyme added. Methods are given under Experimental Procedures. (b) Effect of control Sepharose added to assays containing soluble pyruvate kinase. Enzyme assays were performed as described under Experimental Procedures for immobilized enzyme.

Table I: Analysis of the Assay Procedure for Immobilized Pyruvate Kinase[a]

| sample added to assay | observed rate ($\mu$mol min$^{-1}$ assay$^{-1}$) |
|---|---|
| 25 $\mu$L of soluble enzyme | 0.057 |
| 25 $\mu$L of immobilized enzyme-buffer slurry (1:1) | 0.023 |
| 25 $\mu$L of soluble enzyme + 25 $\mu$L of immobilized enzyme-buffer slurry | 0.081 |

[a] Enzyme assays were performed with soluble enzyme, Sepharose-immobilized enzyme, and/or control Sepharose in the assay medium.

16 °C for 24 h. During the first 1.5 h, the renaturation vessel was gently swirled every 5–10 min in order to keep the Sepharose suspended.

*Amino Acid Analysis.* Amino acid analysis was used to quantitate the amount of protein bound to Sepharose before and after the removal of noncovalently bound enzyme subunits. Samples of Sepharose-immobilized enzyme were washed by suspending 0.5 mL of packed resin in 20 volumes of distilled, deionized water, centrifuging to collect the resin. The wash procedure was repeated 4 times, and the resin was finally dried by lyophilization. One milliliter of constant-boiling HCl was added to the dried resin, and the samples were hydrolyzed at 110 °C for 24 h in sealed, evacuated vials. The hydrolysate was dried under vacuum, extracted with Durrum's pH 2.0 sample diluent (Dionex Corp.), filtered through a Millipore filter, and analyzed on a Dionex No. 26415 amino acid analyzer equipped with fluorescence detection.

## Results

Immobilized enzymatic activity measured as described under Experimental Procedures was directly proportional to the amount of immobilized enzyme added (see Figure 1a). The addition of up to at least 50 $\mu$L of packed, unmodified Sepharose had no significant effect on assays performed with soluble enzyme (see Figure 1b). Furthermore, assays performed with mixtures of immobilized and soluble enzymes produced overall enzyme rates that were the sum of rates obtained when the soluble and immobilized enzymes were assayed individually (see Table I). Thus, enzyme assays performed as described here appear to yield results that are proportional to the amount of enzyme added to the assay, whether it be in soluble form or immobilized.

Immobilization by the procedures described under Experimental Procedures resulted in the incorporation of 2.0 ± 1.0 units of pyruvate kinase per milliliter of packed Sepharose. The immobilized enzyme was extremely stable when stored as described above. No loss of enzymatic activity could be seen after 3–4 weeks of storage; even after 3–4 months of storage, the immobilized enzyme still retained 60% of its initial activity.

Table II: Amino Acid Content of Sepharose-Immobilized Pyruvate Kinase[a]

| amino acid | nmol of amino acid/mL of packed Sepharose: immobilized enzyme | immobilized enzyme from which noncovalently bound subunits were removed | nmol of amino acid/nmol of hydrolyzed native enzyme[b] |
|---|---|---|---|
| Asp + Asn | 3.61 | 1.09 | 194 |
| Glu + Gln | 5.25 | 1.24 | 200 |
| Gly | 4.61 | 1.30 | 162 |
| Ala | 5.00 | 1.58 | 219 |

[a] Samples were prepared, hydrolyzed, and analyzed as described under Experimental Procedures. Values are given as nanomoles per milliliter of packed Sepharose, corrected for the amino acid content of hydrolyzed samples of control Sepharose. [b] From Cardenas et al. (1973).

Of importance to our studies was the determination of whether the immobilized enzyme became solubilized under mild conditions, either during enzyme assays or upon storage. Checks for enzyme solubilization during the assay were performed by initiating an assay with stirring in the spectrophotometer, quickly removing the immobilized enzyme by low-speed centrifugation before all the substrate was consumed, and then returning the supernatant to the spectrophotometer to determine whether residual enzymatic activity remained. No residual activity in the assay medium was found after centrifugal removal of the resin, indicating that all the enzymatic activity remained immobilized during the course of the assay.

Centrifugation of suspensions of stored immobilized enzyme suspensions followed by enzyme assays of the supernatant revealed some solubilization over very long storage times; after 3–4 months of storage, 25% of the total remaining enzyme activity was found to be solubilized.

Table II lists the amounts of some amino acids as quantified from analyses of acid hydrolysates. These particular amino acids were chosen for quantification because of their stabilities and because, at the very low quantities of protein used, quantification of these amino acids can be performed most accurately. Sepharose-immobilized protein from which noncovalently bound subunits had been removed contains an average of 28% as much of the amino acids as did the immobilized tetramer. The relative quantities of these amino acids agree reasonably well with the ratios of the same amino acids reported for soluble, purified bovine muscle pyruvate kinase (Cardenas et al., 1973). Comparison of the quantities of these amino acids with the published amino acid content for the hydrolyzed native enzyme indicates that immobilized preparations contain 5.53 ± 0.99 $\mu$g of protein per mL of packed resin. With the assumption that each tetramer contains four subunits of identical amino acid composition, then the agarose from which noncovalently bound enzyme subunits have been removed would contain 1.56 ± 0.25 $\mu$g of protein per mL of packed resin, or approximately 28% that of the tetramer. The assumption regarding identical amino acid compositions for the four subunits appears valid in view of their identical molecular weights and the appearance of one-fourth the number

Table III: Enzymatic Activities of Sepharose-Bound Tetramer and Monomer[a]

| expt no. | activity (units/mL of packed Sepharose): bound tetramer | bound "monomer" | expected for fully active monomer | bound monomer (% activity of that expected for fully active monomer) |
|---|---|---|---|---|
| 1 | 1.07 | 0.030 | 0.268 | 11.2 |
| 2 | 3.06 | 0.049 | 0.765 | 6.4 |
| 3 | 2.52 | 0.052 | 0.63 | 8.2 |

[a] Sepharose activation was performed with 1.2 mg of cyanogen bromide per mL in experiment 1 and with 2.0 mg of the reagent per mL in experiments 2 and 3.

of tryptic peptides that would be predicted from amino acid analyses (Cardenas et al., 1973; S. C. Johnson and J. M. Cardenas, unpublished experiments). The results therefore indicate that most of the tetramers are covalently bound to the resin via a single subunit and that removal of noncovalently bound subunits produces mainly immobilized monomers.

Table III shows the quantity of enzymatic activity actually obtained for immobilized tetramers. From the amount of activity determined for the bound tetramer and the average amount of protein bound to the resin as determined from amino acid analysis, one can calculate a specific activity of about 400 units of pyruvate kinase activity per mg of bound protein. This specific activity for immobilized pyruvate kinase compares very well with that obtained in our preparations of soluble enzyme.

Also listed in Table III are the actual enzymatic activities obtained for immobilized monomers, as well as the calculated amounts of activity that would be expected for fully active monomers, i.e., one-fourth of the activity of the tetramers. Averaging results of the three experiments indicates that the bound monomer has no more than 8.6% of the activity that it would be expected to have if it were fully active. Even this activity may well be an overestimate of the activity of the immobilized monomers. Results of the amino acid analyses given in Table II show that immobilized monomers contain 28% as much protein as is found in samples of immobilized tetramers. The discrepancy between this value and the expected 25% may indicate that some enzyme tetramers were covalently bound to the resin via more than one subunit.

Shown in Table IV are results obtained when immobilized tetramers are reconstituted by adding soluble, renaturing subunits to the immobilized monomer. Reconstitution with potentially fully active subunits results in recovery of approximately 61% of the initial bound activity.

Over the range of soluble enzyme concentrations used (32–44 μg/mL), the recovery of bound enzymatic activity is essentially independent of the initial concentration of soluble enzyme. Addition of a second aliquot of soluble monomers after the first reconstitution was complete (24 h) produced no further increase in the yield of bound activity.

Reconstitution of bound tetramer by the addition to the bound monomer of soluble subunits from enzyme that had been 90–98% inactivated by trinitrophenylation resulted in recovery of 16–19% of the original bound activity (see Table IV).

In order to interpret the reconstitution results obtained when inactivated, soluble subunits are added, one must consider the expected renaturation yield, the potential activity of the immobilized subunits, and the residual activity contributed by the "inactivated" trinitrophenylated soluble subunits. With

Table IV: Reconstitution of the Quaternary Structure of the Sepharose-Bound Subunits with Native or Trinitrophenylated Soluble Subunits

1. Reconstitution with Native Subunits

| expt no. | activity (units/mL of Sepharose): initial bound tetramer | reconstituted bound tetramer | % recovery | initial concn of soluble enzyme in reconstitution mixture (μg/mL) |
|---|---|---|---|---|
| 1 | 1.07 | 0.68 | 63 | 44 |
| 2 | 3.01 | 1.81 | 60 | 42 |
| 3 | 2.87 | 1.70 | 60 | 32 |

2. Reconstitution with Inactive Subunits

| expt no. | activity (units/mL of Sepharose): bound tetramer | reconstituted | % recovery | initial concn of soluble enzyme in reconstitution mixture (μg/mL) |
|---|---|---|---|---|
| 1 | 1.07 | 0.169 | 15.9 | 44 |
| 2 | 3.01 | 0.585 | 19.4 | 42 |

the assumption that renaturation yields are the same when trinitrophenylated soluble subunits are used as when unmodified, soluble subunits are added (about 61%), and with the assumption that the bound monomer is potentially fully active, contributing 25% of the activity of the original bound tetramer, then the expected activity recovery for immobilized tetramers formed with soluble, trinitrophenylated subunits is equal to (bound activity of original tetramer)(% recovery)[0.25 + 0.75(residual activity of trinitrophenylated tetramer)].

For experiment 1 of Table IV, part 2, the original bound tetramer had an activity of 1.07 units/mL of packed resin, and the trinitrophenylated enzyme had a residual activity of 2.1% that of native enzyme. From these data, one would expect a "reconstituted" activity of 0.18 unit/mL of packed resin. The actual amount of activity recovered after reconstitution was 0.169 unit/mL of packed resin. For experiment 2 of Table IV, part 2, 3.01 units of enzyme activity were originally bound to the resin. Removal of noncovalently bound subunits and reconstitution with trinitrophenylated subunits from soluble enzyme containing 9.7% of the original activity yielded a reconstituted immobilized activity of 0.585 unit/mL of packed resin. This value compares very well with a theoretical activity recovery of 0.584 unit/mL of packed resin if the bound monomer is fully reactivated (with a 61% yield) upon addition of trinitrophenylated subunits.

## Discussion

Since the conditions required to disrupt the quaternary structure of multisubunit proteins often disrupt the secondary and tertiary structures as well, it is often difficult if not impossible to obtain structured, partially assembled, and stable soluble systems for study. Enzyme immobilization provides a convenient means of studying the properties of single subunits and of their interactions with other components in the system.

Removal of noncovalently bound subunits of immobilized pyruvate kinase by treatment of the immobilized enzyme with guanidine-HCl leaves 28% of the protein that was originally associated with the resin (see Table II). This value is close to that expected if only one subunit per tetramer had been covalently linked to the resin. While treatment with guanidine-HCl removes approximately 72% of the protein from the resin, incubation of the immobilized protein under renaturation conditions restores only about 2% of the original matrix-bound

activity. Thus, immobilized monomers have little, if any, enzymatic activity. One must further consider that some enzyme molecules would probably be covalently bound via more than one subunit. The small amount of residual activity remaining after the removal of noncovalently bound subunits may well be entirely due to the existence of a few molecules that retain at least some quaternary structure, and hence the true monomer may have no enzymatic activity at all.

The results presented above are consistent with a requirement of subunit interactions for catalytic activity in pyruvate kinase. However, other possibilities could account for the lack of enzymatic activity of the immobilized monomer, and these possibilities must be considered. One possibility is that the immobilized subunit was prevented from folding correctly because of interactions with the matrix. Another possibility is that the immobilization procedure modified an amino acid residue that is required for enzymatic activity. It is particularly important to consider the latter possibility since cyanogen bromide activated Sepharose immobilizes proteins by reacting with the $\epsilon$-amino group of lysine residues, and bovine muscle pyruvate kinase is known to have an essential lysine residue at or near the active site (Hubbard & Cardenas, 1975; Johnson et al., 1979).

The reason for the essential lack of activity in immobilized monomers can be examined by attempting to reconstitute the quaternary structure of the immobilized subunit by using active and inactive subunits. If the immobilized subunit is capable of refolding correctly, it should be able to interact with other subunits, thereby mediating the reappearance of Sepharose-bound enzymatic activity. The results given above indicate that recovery of matrix-bound enzymatic activity does indeed occur if renaturing soluble subunits are added to the covalently bound monomer. It is interesting to note that immobilized monomers can still interact with renaturing soluble monomers even after incubation of the former for 24 h under renaturing conditions. The efficiency of recovery of immobilized enzyme (61%) compares reasonably well with that reported for others for the renaturation of soluble pyruvate kinase (Cottam et al., 1969; Johnson et al., 1969; Cardenas et al., 1977; Porter & Cardenas, 1980a,b).

If reconstitution is performed with inactivated subunits, then the appearance of Sepharose-bound activity presumably would result from reactivation of the immobilized subunit via its interaction with inactivated, soluble subunits provided that no critical active-site residue in the covalently bound subunit was modified during immobilization. One would predict that very little immobilized activity would result from reconstitution of the bound tetramer with inactivated subunits if critical residues in the immobilized subunit have been modified. If essential active-site residues were not modified during covalent attachment of the subunit to Sepharose, than reconstitution of matrix-bound tetramers via the addition of inactivated subunits should yield an immobilized activity equal to one-fourth of that of the original bound native tetramer. When one assumes the same efficiency of activity recovery (61%) for reconstitution with native and chemically inactivated subunits and further considers the residual activity of chemically modified subunits, one calculates an activity recovery that is extremely close to that obtained experimentally.

These results are consistent with the hypothesis that Sepharose-bound subunits of pyruvate kinase are inactive in the monomeric state but can be reactivated by interaction with either native or inactive, trinitrophenylated soluble subunits. However, the possibility exists that recovery of Sepharose-bound activity during reconstitution with trinitrophenylated subunits may arise through preferential reassociation of a small number of subunits that are not inactivated by trinitrophenylation (trinitrophenylated enzyme retains 2–10% of its original activity). We feel it to be unlikely that native and trinitrophenylated subunits have significantly different affinities for immobilized subunits in view of past work from our laboratory concerning hybridization of trinitrophenylated type M pyruvate kinase with the native type L isozyme (Hubbard & Cardenas, 1975). The results of these earlier experiments demonstrated that similar ratios of hybrids were produced regardless of whether native or trinitrophenylated type M pyruvate kinase is used for hybridization. It should be pointed out that denaturation of trinitrophenylated pyruvate kinase followed by renaturation under conditions such as those described here does *not* result in reversal of the inactivation produced by trinitrophenylation (Hubbard & Cardenas, 1975).

While the present work provides no information as to the enzymatic activity of the dimer, it strongly suggests that the monomer possesses little or no activity. This conclusion is supported by the existence of a concentration dependence in the renaturation kinetics of bovine muscle pyruvate kinase (Porter & Cardenas, 1980b), which indicates that the monomer cannot be fully active. Rabbit muscle and yeast pyruvate kinase appear to dissociate into dimers in 2.4 M urea. The dimers formed from the rabbit muscle enzyme are reported to be partially active (Cottam et al., 1969) while those from yeast are inactive (Boiteux et al., 1978).

While this is the first study of the subunit activity of immobilized pyruvate kinase, subunit immobilization has been used to study other partially assembled systems. Isolated monomers of aldolase and transaldolase are partially active but less stable than the native enzyme. On the other hand, monomers of lactate dehydrogenase, like those of pyruvate kinase reported here, are essentially inactive [see Chan (1976a,b)].

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# Effects of 1,2-Dimethoxyethane on the Catalytic and Coenzyme Properties of Glycogen Phosphorylase†

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ABSTRACT: Dimethoxyethane, a good activator of phosphorylase *b*, has been used to study mechanisms of phosphorylase activation and the catalytic reaction. Activation can be explained best by an alteration of the allosteric equilibrium in favor of the active R conformation. Lesser effects are seen with phosphorylase *a*, and activation does not alter appreciably the equilibrium between the dimeric and tetrameric forms. With 20% 1,2-dimethoxyethane, the $V_m$ value of phosphorylase *b* is 74% of that obtained in the presence of adenosine monophosphate. In the presence of 10% 1,2-dimethoxyethane, the $K_i$ value for glucose inhibition is increased 3-fold, but inhibition by 1,5-gluconolactone is increased. The allosteric activation of glycogen phosphorylase results in a change in $pK_1$ for the pH–activity profile. The formation of the dianionic form of the phosphoryl group of the coenzyme, pyridoxal phosphate, may account for this change. By analogy to the effects of anions and a change in dielectric on the acid hydrolysis of glucose 1-phosphate, it is suggested that the dianion of the coenzyme could stabilize the developing positive charge of an oxonium ion intermediate. Dimethoxyethane also affects the interaction of pyridoxal phosphate with phosphorylase. It influences the rates of both resolution and reconstitution. Good preparations of apophosphorylase *a* can be made by using 1,2-dimethoxyethane in the resolution medium.

The activity of glycogen phosphorylase is controlled by an equilibrium between inactive and active conformations. The equilibrium is shifted toward an active form by substrates, phosphorylation, and the allosteric activator AMP (Madsen et al., 1978; Kastenschmidt et al., 1968; Helmreich et al., 1967). Specific conformational changes in the protein result in activation (Madsen et al., 1978; Weber et al., 1978), and changes include the active site, which contains the coenzyme pyridoxal phosphate (Parrish et al., 1977; Sygusch et al., 1977). Although there is considerable information about the phosphorylase molecule and the activation process, there is some uncertainty about how these changes in protein structure cause activation of the catalytic reaction. Kinetic studies of the reaction suggest that α-glucan phosphorylases from skeletal muscle (Engers et al., 1969), liver (Maddaiah & Madsen, 1966), and *E. coli* (Chao et al., 1969) proceed via a rapid equilibrium random Bi–Bi mechanism. 1,5-Gluconolactone is a potent inhibitor of glycogen phosphorylase (Tu et al., 1971; Gold et al., 1971). Because of its inhibitory action and the fact that this compound possesses a structure similar to the half-chair conformation of an oxonium ion (Hackert & Jacobsen, 1969), it has been suggested that the transition state involves formation of an enzyme–glycosyl complex in which the glucosyl residue is in the half-chair conformation (Tu et al., 1971). Similar suggestions about the reaction were made by Gold et al. (1971).

Recently it has been shown that organic solvents cause activation of glycogen phosphorylase (Dreyfus et al., 1978; Uhing et al., 1979). This report characterizes the properties of muscle phosphorylase in the presence of an organic solvent, 1,2-dimethoxyethane, in order to further investigate the process of activation and the nature of the catalytic process. The cofactor of glycogen phosphorylase, pyridoxal phosphate, has been determined to be an indispensable component of the enzyme because its removal results in the loss of catalytic activity (Hedrick et al., 1966). Both structural (Feldmann et al., 1976; Shimomura & Fukui, 1977, 1978) and catalytic (Feldmann & Hull, 1977; Palm et al., 1979) roles for the coenzyme have been suggested. The effects of 1,2-dimethoxyethane on the coenzyme site in phosphorylase and its uses in the resolution of rabbit skeletal muscle phosphorylase *a* are described in this report.

## Experimental Procedures

*Preparation of Enzymes.* Rabbit skeletal muscle glycogen phosphorylase *b* was prepared according to the method of Fischer & Krebs (1962) and recrystallized at least 3 times before use. Residual AMP was removed by treatment with Norit A. Muscle phosphorylase *a* and liver phosphorylase *a* were prepared from the respective phosphorylase *b* forms by phosphorylation with rabbit skeletal muscle phosphorylase kinase (Krebs, 1966). Apophosphorylase *b* was prepared according to Shaltiel et al. (1966) and passed over a Sephadex G-25 column that had been equilibrated with 0.2 M imidazole and 0.05 M L-cysteine at pH 6.0.

Apophosphorylase *a* was prepared from phosphorylase *a* by modifying the procedure of Shaltiel et al. (1966). Phosphorylase *a* (5 mg/mL) is incubated for approximately 12 h at room temperature (22–23 °C) in the presence of 0.4 M imidazolium citrate–0.1 M L-cysteine, pH 6.0, which also contains 10% (v/v) 1,2-dimethoxyethane (the solvent is added to the deforming buffer immediately before use to minimize the precipitation of salts). After the incubation period, an equal

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