Bovine Pyruvate Kinases

II. PURIFICATION OF THE LIVER ISOZYME AND ITS HYBRIDIZATION WITH SKELETAL MUSCLE PYRUVATE KINASE*

(Received for publication, October 30, 1972, and in revised form, February 21, 1973)

JANET M. CARDEÑAS† AND ROBERT D. DYSON
From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

SUMMARY

There are at least three electrophoretically distinguishable mammalian isozymes of pyruvate kinase (EC 2.7.1.40). One of these, the type L isozyme, has been associated with the parenchymal cells of rodent livers. We have isolated and purified the equivalent isozyme from bovine liver. Like other mammalian type L pyruvate kinases, this enzyme exhibits cooperative kinetics with its substrate, phosphoenolpyruvate, and is activated by fructose 1,6-diphosphate. We find a Michaelis constant for P-enolpyruvate of about 0.08 mM in the presence of 1 mM fructose 1,6-diphosphate, and half-maximal activity at 0.5 mM P-enolpyruvate in the absence of fructose 1,6-diphosphate.

Bovine type L pyruvate kinase has a sedimentation coefficient of 9.5 S at a concentration near 0.5 mg per ml in the analytical ultracentrifuge, and a molecular weight from sedimentation equilibrium of about 215,000 based on a partial specific volume (estimated from amino acid analyses) of 0.737 ml per g. It is dissociated by guanidine hydrochloride into subunits with a molecular weight of approximately 54,000 using the same partial specific volume. Measurements of the Hill coefficient are consistent with the presence of four substrate binding sites on the liver isozyme, or an average of one per subunit. Thus, the liver isozyme appears to have the same subunit structure and number of binding sites as the skeletal muscle isozyme.

Hybrids of the skeletal muscle and type L isozymes are of interest because the former enzyme exhibits Michaelis-Menten kinetics with its substrates while the liver isozyme behaves allosterically. Hybrids of these two enzymes were produced in vitro by denaturing them together in guanidine hydrochloride, followed by dilution into a renaturing medium containing a reducing reagent and Tris-HCl at pH 7.5. One-half or more of the original activity was recovered. After electrophoresis of the renatured mixture, an enzyme-specific detection procedure revealed five isozymes, consisting of Lα, Lβ, LαMβ, LβMα, and Mαβ where Lα and Mα are the native tetrameric liver and skeletal muscle isozymes, respectively. Densitometric measurements of the electrophoretic pattern suggest hybrid amounts that are consistent with a random redistribution of parental subunits. This result implies that, in spite of significant differences in physical and chemical properties, the liver and skeletal muscle isozymes are very similar, at least near regions of intersubunit contact.

There are at least three major, electrophoretically distinguishable mammalian isozymes of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40). These isozymes have been designated M2, M1, and L, or C, A, and B, respectively, in order of increasing anodic mobility during electrophoresis at alkaline pH values (1, 2).

Type L pyruvate kinase is found mostly in liver and kidney. Recent work indicates that it may be the only isozyme present in liver parenchymal cells (3, 4). This isozyme is activated by fructose 1,6-diphosphate, exhibits cooperative kinetics with respect to phosphoenolpyruvate, and moves toward the positive electrode during electrophoresis at neutral pH (1, 2, 5-9).

Type M1 pyruvate kinase, which is found by itself in adult mammalian skeletal muscle, is also the major isozyme of cardiac muscle and brain. It is insensitive to fructose 1,6-diphosphate, exhibits Michaelis-Menten kinetics with phosphoenolpyruvate (although it seems to be subject to cooperative inhibition by L-phenylalanine (10, 11)) and moves toward the negative electrode during electrophoresis at neutral pH.

The M2 isozyme, which is present in all mammalian tissues examined except adult skeletal muscle and erythrocytes, exhibits cooperative kinetics with phosphoenolpyruvate, but is clearly distinguishable from M1 and L on the basis of other parameters (9, 12-14).

A possible fourth adult pyruvate kinase isozyme, similar or perhaps identical with the liver isozyme, is found in erythrocytes (1, 2).

Since the mammalian liver (type L) isozyme of pyruvate kinase is clearly allosteric, with known modulators, while the skeletal muscle isozyme (type M1) exhibits Michaelis-Menten kinetics with substrates, hybrids of these two isozymes should be valuable in studies on the general mechanism of allosteric and in studies designed to elucidate the different physiological roles of the isozymes. While M1-M2 and M2-L hybrid sets exist in vitro...
hybrids of M₁ and L do not, because the M₁ and L isoforms are not present together in the same tissues. Nor, to our knowledge, are there published reports of the in vitro hybridization of these two isoforms, although preliminary studies have been carried out by Susor and Rutter, who have also published a description of M₁-L hybrid sets produced in vitro.

The paper preceding this one (15) describes the isolation and characterization of bovine skeletal muscle (type M₁) pyruvate kinase. The present paper deals with the purification of the type L isozyme from bovine liver, presents physical evidence for kinase. The present paper deals with the purification of the type L isozyme from bovine liver, presents physical evidence for kinase. In addition, we discuss the problem of measuring the relative amounts of the hybrids using a slightly modified version of Susor and Rutter's method for detecting pyruvate kinase activity after electrophoresis (2) and used this procedure to provide statistical evidence for a random redistribution of subunits from the allosteric and non-allosteric parental enzymes during hybridization.

**EXPERIMENTAL PROCEDURE**

Distilled, deionized water was used for making all solutions. Guanidine hydrochloride was obtained from Heico (Delaware Water Gap, Pa.). Ammonium sulfate and sucrose were special enzyme grade from Schwarz-Mann. DEAE-cellulose (DE-52) and phosphocellulose (P-11) were obtained from Reeve-Angel. Common chemicals were standard reagent grade. Substrates and lactate dehydrogenase were obtained from Sigma Chemical Co.

Enzyme assays were carried out at 25° on a Beckman Acta III spectrophotometer equipped with a automatic sample changer and a circulating constant temperature bath. The standard assay consisted of 1 ml of 0.05 M imidazole-HCl buffer, pH 7.0, with 2.0 mM ADP, 1.0 mM phospho-1,6-diphosphopyruvate, 0.16 mM NADH, 1.0 mM fructose 1,6-diphosphate, and 20 units (micromoles per min) of lactate dehydrogenase. Enzyme activity was followed by observing the decrease in absorbance at 340 nm.

Protein determinations on crude preparations were made by the Polin-Ciaccalute method, as described by Clark (16), using bovine plasma albumin as a standard. On purified material an extinction coefficient of ε<sub>280</sub> = 0.57 was used to establish protein concentration. This value was obtained by assuming that a 1 mg per ml solution produces 3.9 fringes during a synthetic boundary experiment in the analytical ultracentrifuge, which is the fringe displacement observed earlier (15) for the muscle isozyme.

**Purification of Bovine Liver Pyruvate Kinase**

The procedure is summarized in Table I.

**Step 1: Extraction**—Liver from a recently killed animal was either used immediately (as in Table I) or stored at -60°. Portions were passed through a meat grinder and homogenized at pH 7.5 in 3 volumes of 0.15 M KCl, 0.02 M Tris-HCl, 2 mM dithiothreitol, 5 mM MgCl₂, and 1 mM EDTA. Debris was removed by centrifugation at 14,000 x g for 30 min at 0°. The supernatant was carefully decanted and filtered through glass wool.

**Step 2: Acetone Cut**—The extract from Step 1 was adjusted, if necessary, to pH 7.5 at 0°. Acetone (precooled to -20°) was added to a final concentration of 33% (v/v). The resulting precipitate was removed by centrifugation at 10,000 x g for 20 min at -10°. Additional acetone (also at -20°) was added to a final concentration of 50% (v/v) and the second precipitate collected by centrifugation as above. This precipitate was dissolved in a minimal volume of the buffer described in Step 1 and gently stirred overnight in an open container at 4° to remove the remaining acetone.

**Step 3: Ammonium Sulfate Cut**—The acetone cut was fractionated by adding saturated ammonium sulfate, discarding the precipitate formed at 35% of saturation and collecting (by centrifugation at 10,000 x g for 30 min) the precipitate formed at 45%. This latter material was dissolved in and dialyzed against 10 mM potassium phosphate buffer, pH 7.1, containing 0.5 mM sucrose and 10 mM β-mercaptoethanol.

**Step 4: Chromatography on DEAE-cellulose**—A column (5.0 x 60 cm) of DEAE-cellulose (DE-52) was equilibrated with the phosphate buffer described in Step 3. About 5 g of dialyzed protein from this step were applied to the column, which was eluted at about 75 ml per hour with a KC1 gradient formed by a Böcher nine-chambered gradient maker containing 500 ml each of the following KC1 concentrations in the equilibrating buffer: 0.09, 0.12, 0.15, 0.165, 0.18, 0.21, 0.24, 0.27, and 0.30 M. Fractions of about 10 ml each were collected. Those with the highest pyruvate kinase activity were pooled and concentrated by the addition of solid ammonium sulfate to 70% of saturation.

**Step 5: Chromatography on Phosphocellulose**—Samples of 100 to 200 mg from Step 4 were dialyzed against 0.02 M sodium acetate buffer, pH 5.2, containing 1 mM dithiothreitol and 0.5 mM sucrose, then applied to a column (2.5 x 40 cm) of phosphocellulose (P-11) equilibrated with the same buffer. The column was eluted at 30 to 40 ml per hour with a linear gradient comprised of 400 ml of the equilibrating buffer and 400 ml of 0.05 M potassium phosphate buffer, pH 7.5, containing 0.5 mM sucrose and 1 mM dithiothreitol. Fractions with the highest pyruvate kinase activity were pooled and concentrated by the addition of solid ammonium sulfate to 70% of saturation.

**Step 6: Gel Filtration**—A column (1.5 x 75 cm) was packed with Sephadex G-200 previously equilibrated with 0.05 M potassium phosphate buffer, 10 mM β-mercaptoethanol, pH 7.0. The ammonium sulfate suspension from Step 5 was centrifuged at 10,000 x g for 20 min to bring down the pyruvate kinase, which was then dissolved in the equilibrating buffer and applied to the G-200 column. The column was eluted at 10 ml per hour, collecting 1-ml fractions. The peak fractions of pyruvate kinase activity were pooled and precipitated by dialyzing against 80% saturated ammonium sulfate and stored frozen.

When trace amounts of contaminating proteins were found to

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1. W. A. Susor and W. J. Rutter, personal communication.
be present in the pyruvate kinase preparations, they were removed by careful ammonium sulfate fractionation. Small increments of saturated ammonium sulfate were added to pyruvate kinase solutions of at least 5 mg per ml until incipient turbidity occurred. The solution was then allowed to warm up gradually to room temperature before centrifuging at 10,000 X g for 20 min at room temperature. A silky sheen was seen in the ammonium sulfate suspensions, suggestive of crystal formation.

Hybridization of Muscle and Liver Pyruvate Kinases

The procedure for reversible denaturation was adapted, in part, from earlier studies on rabbit muscle (17, 18) and yeast pyruvate kinases (19).

Equal activities of beef muscle and of beef liver pyruvate kinase were centrifuged from their ammonium sulfate suspensions and dissolved together in a sufficient volume of 0.05 M Tris-HCl buffer, pH 7.5, to give a protein concentration of 2.5 mg per ml. This solution was mixed with an equal volume of 8.0 M guanidine hydrochloride containing 0.1 mM β-mercaptoethanol and was allowed to stand in an ice bath for 15 min. The denatured enzyme in guanidine hydrochloride was diluted with gentle swirling into 10 volumes of a renaturation medium consisting of 0.5 M sucrose, 5 mM dithiothreitol, 0.05 M Tris-HCl, 0.1 M KCl, and 5.0 mM MgCl₂, pH 7.5. It was incubated in this medium for about 6 hours at 15°C.

For storage, the hybrid mixture was dialyzed against renaturation medium to further dilute the guanidine HCl, then dialyzed in 1/2-inch caging against a saturated solution of ammonium sulfate, pH 7.5. This latter step reduces the volume and precipitates the protein.

Cellulose Acetate Electrophoresis and Detection of Pyruvate Kinase Activity

Pyruvate kinase at 1 to 5 μmoles per min per ml was dialyzed against 0.02 M Tris-HCl, 0.5 M sucrose, 1 mM EDTA, and 10 mM β-mercaptoethanol, pH 7.5 (measured at room temperature). Electrophoresis on cellulose acetate strips (Sephaphore III, Gelman Instrument Co.) was carried out for 2 to 4 hours at 250 volts (17 volts per cm) and 4°C using the above buffer. Bands of pyruvate kinase activity were visualized by pressing the cellulose acetate strips against an agar film containing components of a lactate dehydrogenase-coupled assay. Exposure to ultraviolet light reveals a strong fluorescence by NADH except in regions where pyruvate kinase produces pyruvate. (Pyruvate allows lactate dehydrogenase to oxidize NADH.) The agar film was prepared and used according to the procedure of Susor and Rutter (2), except for the following modifications. (a) Cleaned glass plates (2 X 10 X 0.04 inches), salvaged from ultracentrifuge use, were pretreated by wiping with 0.5% melted agar instead of a silicone solution. (b) The coupled assay consisted of 1% agarose, 2 mM ADP, 2 mM phosphoenolpyruvate, 1 mM NADH, 0.01 mM MgCl₂, 0.1 M KCl, 10 units per ml of lactate dehydrogenase, and 0.05 mM imidazole-HCl, pH 7.5. (c) The fluorescence pattern was preserved by contact photography on Kodak high resolution or metallographic plates (see “Results and Discussion”). A UVL-21 lamp (Blak-Ray), without additional filters, was used as a light source. For metallographic plates, the light was pointed at the wall or ceiling above the strips for periods of 2 to 4 s. The much slower high resolution plates require direct exposure for about 4 s from a distance of about 3 feet. The photographic emulsion is blackened only in regions of NADH oxidation. (d) For publication purposes, prints were made from a contact negative of the photographic plate. This procedure preserves the original black and white correspondence. To obtain relative activities of the various bands, the original plates were scanned with a Beckman Analytrol densitometer.

Sedimentation Studies and Gel Electrophoresis

Sedimentation studies and gel electrophoresis were carried out as described in the preceding paper (15), except that interference optics were used to determine sedimentation coefficients. Sedimentation equilibrium studies used the meniscus depletion technique of Yphantis (20). Typical conditions are given in the legends to Figs. 3 and 4. The partial specific volume of the protein was estimated from a preliminary study of its amino acid composition, using the techniques described and referenced in the paper preceding this one (15).

RESULTS AND DISCUSSION

Purification of the Liver Isozyme—The isolation procedure presented here produces a single pyruvate kinase with a specific activity of 60 to 75 μmoles per min per mg at 25°C (see Table I). Because the enzyme is unstable, this specific activity is probably low. Nevertheless, it is some 2000 times higher than the crude extract, representing at least that degree of purification. No consistent differences were noted when fresh versus frozen (−60°C) livers were used.

The enzyme that we have isolated is identified by its kinetic and electrophoretic properties as the species associated with liver parenchymal cells (3, 4) and generally known as “the liver isozyme.” Although mammalian liver also contains at least one additional pyruvate kinase in non-parenchymal cells (1-4), our final preparation is free of contaminating electrophoretic forms of the enzyme (see Fig. 1).

In addition to being free of other pyruvate kinase isozymes, our preparation appears to be a homogeneous protein, as judged

Fig. 1. Electrophoresis of liver pyruvate kinase on cellulose acetate. The procedures for electrophoresis and detection are described under “Experimental Procedure.” The origins are marked by dark triangles along the edges of the strips. A, crude extract from liver. The second major isozyme (lower band) is presumably type M₁. B, the purified liver isozyme. Mobility was less in this case because 5 mM MgSO₄ was used in the electrophoresis buffer in lieu of 1 mM EDTA. (The binding of Mg⁶⁺ reduces anodic mobility.) C, the liver isozyme after denaturation in guanidine hydrochloride and subsequent renaturation.
by the following criteria. (a) A single band was found after polyacrylamide disc gel electrophoresis at pH 9.5 (Fig. 2); (b) a linear plot of log concentration versus the square of radial position was obtained after reaching sedimentation equilibrium in phosphate buffer (Fig. 3); (c) a linear plot of log concentration versus the square of radial position was obtained at sedimentation equilibrium in a denaturing medium, 3.56 M guanidine hydrochloride (Fig. 4); (d) a single sedimenting boundary was seen during a velocity experiment in the analytical ultracentrifuge.

**Characteristics of the Liver Isozyme**—Sedimentation equilibrium in phosphate buffer gave a molecular weight of 215,000, with an observed range in five experiments of about ±2%. This value of 215,000 is based on a partial specific volume of 0.737 ml per g, obtained from the amino acid composition. Sedimentation equilibrium in guanidine hydrochloride gave a subunit molecular weight of about 54,000, indicating a tetrameric structure. The molecular weight of the native enzyme agrees fairly well with that reported by Tanaka et al. (6) from sedimentation equilibrium of rat liver pyruvate kinase (208,000) using an unspecified partial specific volume. However, to our knowledge, the only previously reported study on the subunit structure of bovine liver pyruvate kinase, Tamers comprised of 4 similar or identical monomeric units.

We find a sedimentation coefficient of about 9.5 S at concentrations near 0.5 mg per ml. This value is slightly lower than the corresponding value obtained from skeletal muscle pyruvate kinase, $s_{20,w} = 9.94$ S (15), consistent with the 6 to 7% lower molecular weight (215,000 versus 230,000), assuming equivalent frictional coefficients and partial specific volumes. In fact, the partial specific volumes, as estimated from amino acid analyses, do agree very well: 0.737 for the liver isozyme and 0.740 for the muscle isozyme (15).

The extinction coefficient of the liver isozyme appears to be very low ($E_{1%}^{1%} = 0.57$), and nearly the same as the value obtained for the skeletal muscle isozyme (0.55).

In addition to the differences in molecular weight and sedimentation coefficient, bovine skeletal muscle and liver pyruvate kinases have the expected differences in kinetic properties. For example, the activating effect of fructose 1,6-diphosphate on the bovine liver enzyme is shown in Fig. 5. For comparison, a similar velocity profile of the muscle isozyme is shown in Fig. 6. The corresponding Hill plots (not of the same data) are Figs. 7 and 8, respectively.

While the $K_m$ for the muscle isozyme was about 0.03 mM, the $K_m$ for the liver isozyme in the presence of fructose 1,6-diphosphate was about 0.08 mM. In the absence of activator, the liver isozyme reaches half of its maximum velocity at about 0.5 mM phosphoenolpyruvate.

Although there are differences in physical and kinetic parameters, it appears that the liver and skeletal muscle pyruvate kinases are alike at least in subunit structure, both being tetramers comprised of 4 similar or identical monomeric units. (In the case of the liver isozyme, this conclusion is reached by
Comparing molecular weights of the native and guanidine hydrochloride-dissociated enzymes. In addition, the two isozymes are probably alike in having four active sites, or one per subunit. The evidence for four binding sites in the muscle enzyme is given in the preceding paper (15). The evidence for four binding sites in the liver isozyme is less direct and depends on measurements of the Hill coefficient, the values for which range from about 2.4 to well in excess of 3. Since, theoretically, the Hill coefficient is always less than the number of binding sites (see Ref. 22), the most probable number of sites for the liver isozyme is four. We must emphasize, however, that this value is tentative.

Quantitative Detection after Electrophoresis—The active enzyme detection procedure used by Susor and Rutter (2) couples the pyruvate kinase reaction to lactate dehydrogenase. The latter enzyme oxidizes NADH when pyruvate is also available. Hence, illumination by ultraviolet light of 320 to 360 nm produces a fluorescent background (the NADH) with non-fluorescent areas corresponding to the presence of pyruvate kinase (NADH). The technique is insensitive to contaminating proteins but is extremely sensitive to pyruvate kinase. Susor and Rutter record their fluorescence patterns by contact photography on photographic paper. We use emulsion-coated glass plates to preserve the image, thus making it possible to use a scanning densitometer to record (by integration of the traces) the relative amount of activity in each band. However, to use this method to make a quantitative estimate of pyruvate kinase after electrophoresis, one must consider the spectral and exposure characteristics of the photographic emulsion chosen and take certain precautions in its use.

Ideally, one would like to have an emulsion that is sensitive to exciting light at 340 nm and insensitive to NADH fluorescence at 470 nm, or vice versa. The use of filters between the emulsion and agar layer to achieve this characteristic introduces blurring in contact photography due to parallax, although a thin gelatin filter might be acceptable in many applications. Of the readily available photographic emulsions, Kodak’s high resolution plate is an example of one that has highly desirable spectral characteristics (over 10 times more sensitive at 340 than at 470 nm) and, indeed, we find that it can produce a pattern that is generally free of background fogging, even when the exciting light is a Blak-Ray UVL-21 lamp used without filters (see Fig. 1A). However, the extremely high contrast nature of this emulsion makes it difficult to use in quantitative studies. The spectral sensitivity of Kodak’s spectrum analysis Plate No. 1 is even better than the high resolution plate, while projector slide plates are nearly as good, and both allow more latitude for error in exposure. The common panchromatic and metallographic emulsions have less desirable spectral characteristics but, with care, they may still produce highly acceptable patterns in this system, as can be seen from Fig. 9, which was taken on a metallographic plate.

For precise quantitation of the bands, a density versus exposure calibration curve of the emulsion should be available. In addition, one must photograph the developing pattern early enough so that optical density is still decreasing linearly with time. In practice, taking several exposures at different times after initiating the reaction will usually yield at least one plate having a Gaussian distribution of blackening in the region of the band, which is what one would expect from diffusion alone. It can probably be assumed, except for the most critical of measurements, that a Gaussian distribution of densities in the trace means that one has neither exceeded the quasilinear region of the calibration curve, nor allowed the reaction to proceed too close to equilibrium. It is this criterion that we have adopted in the studies reported in the next section of this paper. Those studies were carried out with the activity detection procedure rather than a protein-specific dye because of the very low protein concentrations in our samples. In addition, we can thus be sure that only catalytically active enzyme is being detected.

Hybridization Studies—Both the liver and muscle isozymes of bovine pyruvate kinase lose catalytic activity immediately in 4 M guanidine hydrochloride. Dilution into the renaturation medium described earlier results in the recovery of half or more of the original activity in a 6-hour incubation. Similar recoveries were obtained when either isozyme was examined alone, and when the two were denaturated and renatured together. Denaturation and renaturation of either isozyme alone produced a single electrophoretic band having the same mobility as the corresponding native isozyme (see Fig. 1 for a demonstration using the liver isozyme). In the case of the liver isozyme, sensitivity to fructose 1,6-diphosphate was also regained.

When the L and M isozymes were denatured and renatured together, electrophoretic analysis revealed the production, in addition to the two parental bands of pyruvate kinase, three additional bands with electrophoretic mobilities evenly spaced between the parental forms (Fig. 9). That all bands were

Fig. 7. Hill plot of bovine liver pyruvate kinase with ( ) and without ( ) 1 mM fructose 1,6-diphosphate. Note that fructose 1,6 diphosphate lowers the Hill coefficient, n. PEP, phosphoenolpyruvate.

Fig. 8. Hill plot of bovine muscle pyruvate kinase with ( ) and without ( ) 1 mM fructose 1,6-diphosphate. There is no indication for cooperativity in either case.
Kinases. The hybridization procedure is summarized with heavy origin.

The subunit composition indicated on in vitro from bovine muscle (M) and bovine liver (L) pyruvate kinases. The hybridization procedure is summarized with heavy arrows. (Gu HCl, guanidine hydrochloride.) Dotted arrows indicate the results obtained in other experiments, when one parental isozyme was used alone. The subunit composition indicated on the right is our interpretation of the electrophoresis pattern. The arrow on the right indicates the approximate position of the origin.

FIG. 9. Pyruvate kinase hybrids. The isozyme set was produced in vitro from bovine muscle (M) and bovine liver (L) pyruvate kinases. The hybridization procedure is summarized with heavy arrows. (Gu HCl, guanidine hydrochloride.) Dotted arrows indicate the results obtained in other experiments, when one parental isozyme was used alone. The subunit composition indicated on the right is our interpretation of the electrophoresis pattern. The arrow on the right indicates the approximate position of the origin.

FIG. 10. Densitometer scan of a hybrid pattern. The pattern was photographed somewhat earlier in its development than the one shown in Fig. 9. At these early times, areas under the peaks should represent the relative activities of each band. Note that the ratios approach the theoretical 1:4:6:4:1 expected for a random redistribution of subunits.

Actually pyruvate kinase shown was by the absence of activity when phosphoenolpyruvate was omitted from the detection medium.

Renaturation and hybridization is apparently unaffected by the presence of contaminating proteins, for the same results were obtained when partially purified liver pyruvate kinase was used with the crystalline skeletal muscle isozyme. This observation agrees with the report of Cook and Koshland (23), who concluded that renaturation of a mixture of enzymes results in the renaturation of each one independent of the others.

Densitometric scans (Fig. 10) of a photographic plate produced from an activity assay at an early time of development yielded the approximate ratios 1:4.6:6:0:4.3:1.1 for the activity of the five isozymes. These ratios approach the 1:4:6:4:1 predicted from a random reassortment of subunits obtained from two enzymes, each having four identical subunits.

However, since the detection scheme used after electrophoresis measures activity rather than concentration of protein, several assumptions must be made in order to conclude that the reassortment of subunits was, indeed, random. One must, for example, assume that the equal activities used in the renaturation studies actually represent equal protein concentrations, i.e. that specific activities of the muscle and liver isozymes are the same, so that the lower specific activity of the liver isozyme is due only to its instability. Further, one must assume that the activity of each subunit under the assay conditions used was invariant to the isozyme in which the subunit was found. The apparent randomness of the subunit reassociation could, in other words, be coincidental. Nevertheless, the results so closely approach the theoretical random redistribution ratios that we consider it unlikely that pure chance is responsible.

That the hybrid set shown in Fig. 9 consists of L4, L3M, L2M2, LM3, and M4 can be inferred from the above redistribution data and from the fact that the bands are equally spaced after electrophoresis. In addition, in a preliminary study, we have succeeded in isolating each of the hybrids by isoelectric focusing and have examined them individually, reinforcing the above subunit assignments. Those results will be reported in full in a later communication.

Thus, although the liver and skeletal muscle isozymes have clearly different physical, kinetic, electrophoretic, and immunological characteristics, they have a similar subunit structure and appear to have the same number of binding sites. In addition, the two isozymes must be very similar at their regions of intersubunit contact, for it seems that interactions between like subunits are not favored over interactions between unlike subunits.

Acknowledgments—Appreciation is expressed to Joan Miller for her very able technical assistance, and to Bobbie Heussy for preparing the graphs. We also extend our sincere appreciation to Dr. W. J. Rutter for advice, encouragement, and assistance in the hybridization studies, and to Dr. John A. Black for contributions to the isolation of the liver isozyme.

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Janet M. Cardenas and Robert D. Dyson

J. Biol. Chem. 1973, 248:6938-6944.

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