Purification and Properties of Two Isozymes of Pyruvate Kinase from *Mucor racemosus*

THOMAS M. HOHN[1]† AND JOHN L. PAZNOKAS[1,2]*

Departments of Microbiology[1] and Basic Medical Sciences,[2] Washington State University, Pullman, Washington 99164-4340

The dimorphic phycomycete *Mucor racemosus* was found to contain up to five electrophoretic forms of pyruvate kinase (ATP: pyruvate 2-O-phosphotransferase, EC 2.7.1.40) depending on growth conditions. *M. racemosus* hyphal cells grown on glutamic acid as the carbon source contained only the fastest electrophoretic form, designated PK1, while yeast cells grown on glucose contained only the slowest electrophoretic form, PK5. Intermediate electrophoretic forms PK2, PK3, and PK4 as well as PK1 and PK5 were found in hyphal cells grown on media containing fructose or cellibiose. All five electrophoretic forms had molecular weights of ca. 230,000 as determined from plots of log R_m versus acrylamide gel concentration. Both PK1 and PK5 were purified to homogeneity and determined to be homotetramers, with subunit molecular weights of 54,000 and 58,100, respectively. The amino acid content of PK1 and PK5 was determined and found to be similar but not identical. Analysis of limited tryptic digests and cyanogen bromide cleavage fragments of PK1 and PK5 indicate that the subunits of the two isozymes are significantly different.

The dimorphic phycomycete *Mucor racemosus* is capable of growth in either a yeast or hyphal morphology. Hyphal growth occurs in medium containing a wide variety of carbon sources and an ammonium salt as the sole source of nitrogen (1), whereas growth in the yeast morphology requires the presence of hexose and an organic nitrogen source (2,5). These differences in growth requirements indicate that physiological differences exist between the two growth forms. It is possible that the differential expression of enzymes at control points in carbon and nitrogen metabolism could play a role in maintaining these physiological differences.

Pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40) is considered a likely control point in the glycolytic pathway, since the reaction it catalyzes is unidirectional under most physiological conditions. In addition, pyruvate kinases isolated from a variety of sources exhibit kinetic and allosteric properties that would enable them to function as regulatory enzymes (25).

Earlier studies have provided evidence for the existence of two forms of pyruvate kinase in *M. racemosus* (23). In these studies expression of the two forms of the enzyme was closely correlated with the presence or absence of a hexose in the growth medium. The two pyruvate kinase isozymes identified in *M. racemosus* have kinetic and electrophoretic characteristics similar to isozyme forms I and III reported for *Mucor rouxii* (6).

Escherichia coli is the only other microorganism in which multiple forms of pyruvate kinase have been demonstrated. One of these forms is expressed constitutively (II) while the level of the other (I) changes under differing growth conditions (14). Biochemical and genetic evidence indicates that these two forms of the enzyme result from different genes (24, 30).

In vertebrates, three major noninterconvertible types of pyruvate kinase (L, M_1, and M_2) have been identified (8).

The catalytic and regulatory properties of the L and M_1 types are very different and appear well suited to the specialized tissues in which they occur (14, 25). The M_2 type has a wide distribution and properties intermediate between L and M (10). The expression of these different forms of enzyme appears to be developmentally regulated in many tissues. The M_2 pyruvate kinase constitutes all or most of the detectable activity in embryos, while later in development the various tissues produce predominantly the L or M_1 type of pyruvate kinase (7, 13).

In each of these systems the isozyme types could be generated in a variety of different ways, including multiple structural genes for pyruvate kinase, posttranscriptional modification of transcripts from a single gene, and posttranslational modification of the product from a single transcript.

We report here on the purification and characterization of two forms of *M. racemosus* pyruvate kinase, PK1 and PK5, and provide evidence that these electrophoretic forms are isozymic homotetramers composed of unique subunits.

MATERIALS AND METHODS

Organism and growth conditions. *M. racemosus* ATCC 1216B was grown in 10-liter carboys continuously sparged with either air (PK1) or CO_2 (PK5) and incubated at room temperature (20 to 23°C). For PK1, the organism was grown on a defined medium containing 0.5% glutamate and adjusted to pH 5.0 with H_2SO_4. These cultures were started from sporangiospores grown on YPG medium (0.3% yeast extract, 1.0% peptone [Difco Laboratories], and 2.0% glucose, pH 4.5) in agar petri plates and harvested with distilled water. Sporangiospores were inoculated at a concentration of 5 × 10⁵ spores per ml. The cultures were harvested during the stationary phase of growth (48 h). PK5 was purified from cells grown in YPG medium. An exponential-growth-phase starter culture grown on YPG medium under CO_2 provided a 3% inoculum for these cultures. The cultures were harvested during the late exponential phase of growth (24 h).

Electrophoretic forms PK2, PK3, and PK4 as well as PK5 were observed in extracts of *M. racemosus* hyphal cells

TABLE 1. Purification of PK1 and PK5

Isozyme	Procedural step	Protein (mg)	Total activity (μmol/ min)	Sp act (μmol/min per mg of protein)	Yield (%)
PK1	105,000 × g supernatant	7,040	2,550	0.36	100
	Affigel blue 1	277.2	2,535	9.15	99
	Affigel blue 2	—[a]	1,620	—	64
	Concentrated Affigel blue 2	30	360	12	14
	Ultraogel AcA22	—	240	—	9
	Concentrated Ultrogel AcA22	1.5	122.4	81.6	5
PK5	15,000 × g supernatant	4,850	9,207	1.89	100
	105,000 × g supernatant	3,432	8,774	2.46	92
	45–75% $(NH_4)_2SO_4$	1,538	6,680	4.34	72
	DEAE-cellulose	38.5	2,971	77.1	32
	Sephacryl-200 1	14	2,124	151.7	23
	Sephacryl-200 2	10	1,685	168	18

[a] —, Not determined.

grown in minimal defined medium with fructose as the carbon and energy source.

Preparation of cell extracts. Cells were harvested either by filtration (hyphae) or with a Sharples centrifuge (yeast) and suspended at a concentration of 0.2 g (wet weight) of cells per ml of cold (4°C) buffer C (10 mM Tris hydrochloride, pH 8.0, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 20% glycerol). Cells were broken in a French pressure cell at 15,000 lb/in^2 at 4°C and then centrifuged at 15,000 × g for 15 min at 4°C to remove cellular debris. The resulting supernatant, termed the crude extract, was centrifuged at 105,000 × g for 2 h at 4°C, and the supernatant was retained.

Purification of PK1 from glutamate-grown cells. All procedures were performed at 4°C, and unless otherwise stated, all chromatography steps were conducted in buffer C.

(i) **Affigel blue chromatography 1.** Five hundred milliliters of 105,000 × g supernatant was applied to an Affigel blue column (2 by 10 cm; Bio-Rad, Inc.) and washed with 500 ml of buffer C. The column was eluted with 1,200 ml of a 0 to 400 mM linear KCl gradient (in buffer C) at a flow rate of 0.5 ml/min, and 10.0-ml fractions were collected.

(ii) **Affigel blue chromatography 2.** Pyruvate kinase activity from the Affigel blue column was pooled and applied to a second Affigel blue column (2 by 10 cm). The column was then washed in 500 ml of buffer C and eluted with 1,200 ml of a linear 0 to 300 mM KCl gradient (in buffer C) as described above. The peak pyruvate kinase activity fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine which would be pooled. The pool of peak fractions was concentrated by pressure dialysis (UM-20 filter; Americon Corp.) to 7 ml.

(iii) **Ultrogel AcA22 chromatography.** The concentrated pyruvate kinase from Affigel blue chromatography was applied to an Ultrogel ACA22 (LKB) column (2 by 20 cm) equilibrated with 400 mM KCl (in buffer C). The column was eluted with a flow rate of 0.1 ml/min, and 2.0-ml fractions were collected. The fractions containing peak pyruvate kinase activity were pooled and concentrated by pressure dialysis (UM-20 filter). The purification is summarized in Table 1.

Purification of PK5 from yeast cells. (i) Ammonium sulfate precipitation. Ammonium sulfate precipitation of 465 ml of the 105,000 × g supernatant was performed by the slow stepwise addition of solid ammonium sulfate to 45% saturation. The precipitate was collected by centrifugation at 15,000 × g for 20 min and discarded. The supernatant was then adjusted to 75% saturation with stirring for 1.0 h. The precipitate was collected by centrifugation and suspended in 16.0 ml of buffer C.

(ii) **DEAE-cellulose (DE-52) chromatography.** The 45 to 75% ammonium sulfate fraction was applied to a DEAE-cellulose (DE-52 Whatman microgranular) column (5 by 50 cm). The column was eluted with the starting buffer (buffer C) at a flow rate of 3.3 ml/min, and 10.0-ml fractions were collected. The fractions containing peak pyruvate kinase activity were pooled and precipitated by the slow stepwise addition of ammonium sulfate to 80% saturation. The precipitate was collected by centrifugation at 15,000 × g for 15 min and suspended in 3.0 ml of buffer C.

(iii) **Sephacryl-200 chromatography 1.** The 80% ammonium sulfate precipitate was applied to a Sephacryl-200 (Pharmacia) column (2 by 100 cm). The column was eluted with a flow rate of 0.1 ml/min, and 2.0-ml fractions were collected. Peak fractions were pooled and concentrated by pressure dialysis (UM-20 filter) to 5.0 ml.

(iv) **Sephacryl-200 chromatography 2.** Pyruvate kinase from the Sephacryl-200 chromatography was applied to a second Sephacryl-200 column (2 by 10 cm). The column was eluted as above, and peak fractions were pooled to produce a typical volume of 14.0 ml. A typical purification of PK5 is summarized in Table 1.

Enzyme assays. Pyruvate kinase activity was monitored during the purification procedures by the 2,4-dinitrophenylhydrazone (DNP) method of Leloir and Goldemberg (16). The standard reaction mixture contained 40 mM KCl, 5 mM $MgCl_2$, 13 mM phosphoenol pyruvate (PEP) (Sigma Chemical Co.) 2 mM ADP (Sigma), and 50 mM Tris-maleate, pH 6.5. The reaction was started with PEP and incubated for 10 min at 30°C. One unit will convert 1.0 μmol of PEP to pyruvate per min at pH 6.8 at 30°C.

Pyruvate kinase activity assay in polyacrylamide gels. Following PAGE (as described below), acrylamide gels were removed from their tubes, the dye fronts were measured, and the gels were marked for identification with India ink. The gels were then placed in an Erlenmeyer flask containing a preincubation buffer (60 mM KCl, 10 mM $MgCl_2$, 1 mM fructose 1,6-bisphosphate [FBP], and 50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 6.2) at a ratio of 1 ml of gel per 10 ml of buffer. Incubation was at room temperature for 25 min on a rotary shaker. The assay
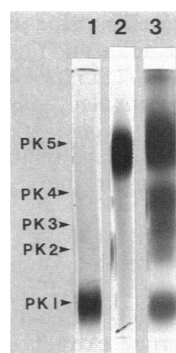
FIG. 1. Multiple electrophoretic forms of pyruvate kinase. Pyruvate kinase enzyme preparations were subjected to PAGE by the method of Laemmli (22) without SDS and stained for enzyme activity as described in the text. Lanes: 1, 0.05 U of purified PK1; 2, 0.10 U of purified PK5; 3, crude extract of fructose-grown cells containing a total of 1.1 U of pyruvate kinase activity plus 0.05 U of purified PK1.

reaction mixture contained 60 mM KCl, 10 mM MgCl$_2$, 10 mM PEP, 4 mM ADP, 1 mM FBP, and 50 mM HEPES, pH 6.2. Gels were suspended at a concentration of 1 ml of gel per 10 ml of reaction mixture and incubated as described above. Pyruvate kinase activity was detected by incubating the gels with 0.1% DNP in 2 N HCl at a concentration of 1 ml of gel per 10 ml of DNP for 7 to 10 min at room temperature on a rotary shaker. The gels were then incubated in 5 N NaOH at a concentration of 1 ml of gel 10 ml of NaOH for 2 to 3 min. Gels were photographed, and migration distances were measured immediately after development.

Immunoelectrophoresis. Rocket immunoelectrophoresis was performed by the method described previously (21). Oblong glass microscope slides (2.5 by 7.5 cm) were painted with a thin coat of 1% low-melting-point agarose (Sigma) and allowed to dry. To this was added a 1.5-mm layer of buffered agarose containing 0.05 M Tris, pH 8.6, with 10 μl of either anti-PK1 or anti-PK5 antibody (rabbit sera) per 5 ml. Samples of 1 to 5 μl of PK1 or PK5 were placed into the wells and electrophoresed at 100 V for 2 to 4 h. Antigen-antibody precipitates were visualized by staining with Coomassie brilliant blue.

Molecular weight determination. Enzyme native molecular weights were estimated by PAGE (11) at gel concentrations of 4.0, 4.5, 5.0, 5.5, and 6.0% with rabbit muscle pyruvate kinase (Sigma) (M_r 235,000), pig heart lactic dehydrogenase LDH1 (Sigma) (M_r 135,000), and bovine serum albumin (Sigma) (M_r 68,000) as standards. Subunit molecular weight was determined by SDS-PAGE with bovine serum albumin (Sigma) (M_r 68,000), rabbit muscle pyruvate kinase (Sigma) (M_r 57,000), ovalbumin (Sigma) (M_r 43,000), trypsinogen (Sigma) (M_r 24,000), and β-lactoglobin (Sigma) (M_r 18,400) as the standards.

PAGE. PAGE with a discontinuous buffer system (Tris-glycine) was performed by the method of Laemmli (15) with or without SDS. When gels were prepared without SDS, the stacking gel was eliminated and samples were applied in buffer C.

Two-dimensional gel electrophoresis was performed by the method of O'Farrell (20) except that a 12.5 or 7.5% polyacrylamide slab was used in the second dimension.

Electrophoresis of cyanogen bromide fragments was performed in gels (23% acrylamide) which contained 7.5 M

urea, 6 mM Nonidet P-40, and 5% acetic acid by the procedure of Borun et al. (3).

Amino acid analysis. The analysis of the amino acid composition of PK1 and PK5 was performed with a Beckman 121 analyzer and a method based on the procedure of Spackman et al. (26). Purified preparations of each enzyme were dialyzed against distilled deionized water overnight and lyophilized. Samples were hydrolyzed in 6 M HCl in sealed evacuated tubes at 110°C for 24 h. Methionine and cysteine contents were determined after oxidation with performic acid at 0°C for 12 h, followed by hydrolysis in 6 M HCl as described above.

Cyanogen bromide treatment. Purified PK1 and PK5 (0.75 mg of each) were carboxy-methylated prior to cyanogen bromide treatment by the modification of the Crestfield procedure described by Hall et al. (9), with the exception of the dimensions of the G-50 column, which had a total bed volume of 4 to 6 ml. The resulting peptide fragments were analyzed by electrophoresis on polyacrylamide gels in 7.5 M urea as described above.

Protein determinations. Protein was measured by using the Coomassie brilliant blue binding reagent (Bio-Rad, Inc.).

RESULTS

Purification of PK1 and PK5. The purification procedure for PK1 described in Materials and Methods and summarized in Table 1 yielded an electrophoretically homogeneous preparation of enzyme (Fig. 1). With the two-dimensional PAGE technique of O'Farrell (20), one protein spot was seen (Fig. 2A). The purification of PK5 also produced an electrophoretically homogeneous enzyme preparation (Fig. 1 and 2B). The purification took advantage of the low affinity of this enzyme for DEAE-cellulose, which resulted in its rapid elution under the column loading conditions. A similar technique has been successfully used with the pyruvate kinase from *Saccharomyces cerevisiae* (12). The purification of PK5 is summarized in Table 1.
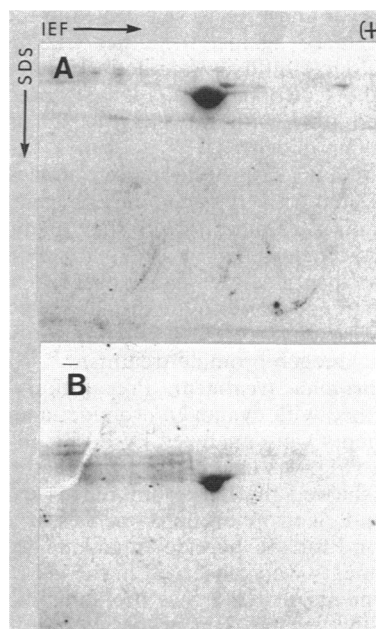

FIG. 2. Two-dimensional PAGE of purified PK1 and PK5. First dimension, isoelectric focusing (IEF); second dimension, SDS-PAGE (7.5% acrylamide). (A) 10 μg of PK1; (B) 10 μg of PK5.
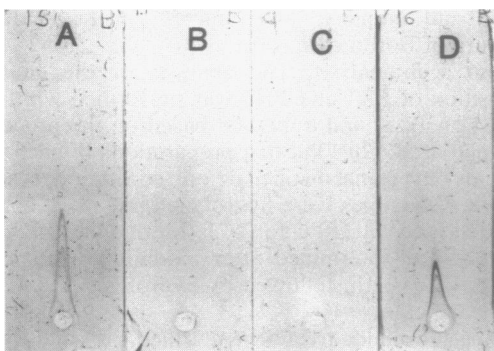
FIG. 3. Rocket immunoelectrophoresis. The agarose on slides A and B contained antibody to PK1. The agarose on slides C and D contained antibody to PK5. Purified PK1 (2 μg) was added to the wells of slides A and C, and purified PK5 (2 μg) was added to the wells of slides B and D. The samples were electrophoresed and stained as described in Materials and Methods.

Molecular weight estimation. The native molecular weights of PK1 and PK5 estimated by the method of Hedrick and Smith (11) were 216,000 and 239,000, respectively. The molecular weight of PK5 was also estimated from a Sephadex G-200 column to be 215,000 (data not shown).

The subunit molecular weights of PK1 and PK5 were estimated by SDS-PAGE. PK1 and PK5 each consist of a single type of subunit with molecular weights of 54,400 and 58,000, respectively.

A comparison of the native and subunit molecular weight estimates for PK1 and PK5 suggests that the most compatible quaternary structure is that of a tetramer. All pyruvate kinases characterized to date have tetrameric quaternary structures (25).

Inhibition of enzyme activity with specific antisera. When either PK1 or PK5 was assayed in the presence of its specific antiserum, greater than 95% inhibition of enzyme activity was observed. Little or no inhibition of activity was observed when PK1 and PK5 were assayed in the presence of antiserum produced against the other enzyme. Using the Ouchterlony double-diffusion method (data not shown) and rocket immunoelectrophoresis (Fig. 3), precipitin bands were observed only with the enzyme against which the antiserum was produced.

Limited tryptic digest peptide maps. Samples of PK1 and PK5 were digested with trypsin in the presence of 0.1% SDS for 2 h. These limited digests were then analyzed by SDS-PAGE and two-dimensional PAGE (20) (data not presented). The pattern of peptide fragments observed indicated that significant differences exist in the primary structure of these two proteins. To confirm these results, the peptides were subjected to cyanogen bromide treatment.

Cyanogen bromide treatment. Preparations of PK1 and PK5 were treated with cyanogen bromide, and the resulting peptide fragments were analyzed by PAGE in the presence of 7.5% urea (Fig. 4). Comparison of the treated enzyme preparations showed that large differences existed in both the number and electrophoretic properties of the fragments produced. For PK1, 16 peptide fragments were detected, while 11 fragments were observed in the gel run with PK5. These experiments indicate that the subunits of PK1 and PK5 differ at the level of their primary structure and do not differ by some simple posttranslational modification.

Amino acid analysis. The amino acid analysis of both enzymes (data not presented), although quite similar for many amino acids, showed considerably more glutamate and lysine in PK5, while histidine and tyrosine were consistently higher in the smaller PK subunit. Although the presence of more residues of a particular amino acid in the lower-molecular-weight subunit would argue strongly for differences in the primary structure of the two enzymes, more data are needed to ascertain the statistical significance of these differences.

Multiple electrophoretic forms. *M. racemosus* was grown under several different nutritional conditions, and the type of pyruvate kinase present was determined in these cells by staining polyacrylamide gels for enzyme activity. PK1 activity was observed in crude extracts from all cultures incubated in air but was not detected in cultures grown anaerobically (data not shown).

PK5 was observed to be the only pyruvate kinase present in anaerobically grown yeast cells. In addition, PK5 was the predominant enzyme activity in cultures grown on a variety of carbohydrates in air (hyphal cells). Cultures grown on glutamate (hyphal cells) contained no detectable PK5. PK2, PK3, and PK4 were detected only in extracts of cultures grown aerobically on carbohydrates. These are also the only growth conditions in which both PK1 and PK5 were observed.

Elution of crude extracts of cells grown in minimal medium supplemented with 1% fructose from a DEAE-cellulose column with a 0 to 85 mM MgCl₂ gradient revealed four peaks of pyruvate kinase activity. These peaks and the void volume material were separated on polyacrylamide gels

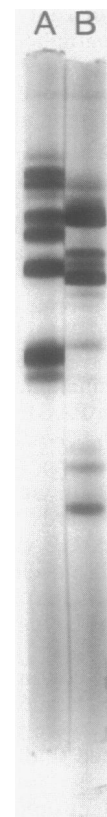

FIG. 4. PAGE of cyanogen bromide cleavage products. (A) 30 μg of PK1; (B) 30 μg of PK5. Following PAGE in gels containing 12% acrylamide, 7.5% urea, 6 mM Nonidet P-40, and 5% acetic acid, the gels were stained as described in Materials and Methods.

TABLE 2. Inhibition of multiple forms of pyruvate kinase with specific antisera

Enzyme source	PK electrophoretic type	Inhibition of activity (%)	
		Anti-PK1[a]	Anti-PK5[b]
PK1	1	94	12
Peak IV[c]	2	85	17
Peak III[c]	3	76	44
Peak II[c]	4	42	79
Peak I	—[d]	3	90
Void volume[c]	5	2	97
PK5	5	0	95
Crude extract[e]	1, 2, 3, and 4	25	68

[a] Rabbit anti-PK1 was prepared by injecting purified PK1 subcutaneously as described in the text.

[b] Rabbit anti-PK5 was prepared by injecting PK5 intravenously as described in the text.

[c] Obtained from DE52 column.

[d] —, Not determined.

[e] Starting material for DE52 column.

and identified by staining for enzyme activity. Of the five electrophoretic forms of pyruvate kinase which can be identified on acrylamide gels, PK2, PK3, PK4, and PK5 were found in fructose-grown cells.

The native molecular weights of PK1, PK2, PK3, PK4, and PK5 were estimated by the method of Hedrick and Smith (11). Electrophoretically homogeneous pools of pyruvate kinase activity obtained by fractionation of crude extracts on a DEAE-cellulose column were used as sources of PK2, PK3, PK4, and PK5. An average molecular weight estimate for all five electrophoretic forms of 227,000 ± 19,000 (standard deviation) was determined.

The ability of antiserum produced against purified preparations of PK1 and PK5 to inhibit the activity of PK1, PK2, PK3, PK4, and PK5 was examined (Table 2). When either PK1 or PK5 was assayed in the presence of its specific antiserum, almost total inhibition of enzyme activity was observed. Little or no inhibition of activity was observed when PK1 and PK5 were assayed in the presence of the antiserum produced against the other enzyme. Both antisera inhibited all three intermediate electrophoretic forms. The amount of inhibition observed for each intermediate electrophoretic form was related to its electrophoretic migration distance (i.e., activity inhibition with anti-PK1: PK2 > PK3 > PK4; with anti-PK5: PK4 > PK3 > PK2).

DISCUSSION

Mucor species contain different isozymes of pyruvate kinase depending on growth conditions and media. In *Mucor* spp. PK5 is always found when carbohydrates are utilized as carbon sources for growth and represents the only isozyme in cells growing anaerobically (e.g., yeast cells) since anerobic growth does not occur in the absence of hexose. PK1, on the other hand, is found in *Mucor* cells growing aerobically on noncarbohydrate substrates (i.e., amino acids), which must be metabolized via a gluconeogenic pathway. PK1 is also found in small amounts in cells grown in air on carbohydrates.

The correlation of PK5 with growth conditions requiring a glycolytic type of metabolism and PK1 with gluconeogenic growth conditions suggests that these enzymes may be uniquely suited for either glycolytic or gluconeogenic metabolism. In this regard, preliminary analysis of the kinetic properties of these isozymes has indicated that PK1 is relatively insensitive to the allosteric activator FBP, while PK5 is activated severalfold by FBP.

Other fungi in which this enzyme has been characterized appear to have a single pyruvate kinase (12, 18, 19). Both biochemical and genetic evidence indicates that *S. cerevisiae* has a single pyruvate kinase with kinetic and regulatory properties (12) very similar to those of PK5 of *M. racemosus*.

The physiological roles of the two forms of pyruvate kinase found in *E. coli* are presently undefined (17). Although their kinetic properties are distinctive and their activity is affected differently by the presence of certain metabolites (29, 30), mutants lacking the constitutive enzyme do not show altered growth characteristics.

The intermediate electrophoretic migration of PK2, PK3, and PK4 to PK1 and PK5 suggests that forms constitute a hybrid set. Supporting evidence for this interpretation is the apparent antigenic similarity of all three intermediate forms to PK1 and PK5 antisera, based on the inhibition of the activities by both PK1 and PK5 antisera. The one intermediate electrophoretic form found in *M. rouxii* (form II) has also been interpreted as being a subunit hybrid. This enzyme has kinetic and immunological properties intermediate between those of forms I and III (6, 22).

Molecular weight estimates would seem to eliminate the possibility that PK2, PK3, and PK4 represent different quaternary structures or aggregation states of PK1 or PK5. PK1 and PK5 are homotetramers with subunits of molecular weights of 54,400 and 58,100, respectively. All pyruvate kinases characterized to date have tetrameric quaternary structures (14, 18, 25).

The presence of hybrid sets of pyruvate kinase subunits in mammals formed between the L and M_2 type subunits and the M_1 and M_2 type subunits have been detected in a wide variety of tissues (4). These hybrid sets have electrophoretic and kinetic properties intermediate between those of the homotetramers from which they were generated; however, they cannot be formed spontaneously from mixtures of the two different forms (27).

As reported for the mammalian enzymes, intermediate electrophoretic forms (PK2, PK3, and PK4) cannot be generated spontaneously. Mixtures of pure PK1 and PK5 did not yield detectable levels of the intermediate forms, and attempts at in vitro hybridization have thus far been unsuccessful. These experiments are complicated by the fact that we observed at best 50% recovery of activity following the harsh treatments used to dissociate tetramers into monomers (19).

Since the intermediate hybrid forms represent only a small portion of the total pyruvate kinase activity in a cell, it is unlikely that they would represent a major mechanism for the regulation of pyruvate kinase activity. It does, however, suggest that when both pyruvate kinase genes (assuming there are two genes) are expressed, the subunits associate as a function of the number of each subunit type present in the cell; i.e., if there are more PK1 subunits than PK5 subunits, there will be proportionately more PK2 and PK3, more PK3 than PK4, and so on.

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Barnicki-Garcia, S.** 1968. Control of dimorphism in *Mucor* by hexoses: inhibition of hyphal morphogenesis. J. Bacteriol. **96:**1586–1594.

2. **Barnicki-Garcia, S., and W. J. Nickerson.** 1962. Nutrition, growth, and morphogenesis of *Mucor rouxii*. J. Bacteriol. **84:**841–858.

3. **Borun, T. W., K. Ajior, A. Sweidler, T. W. Dolby, and R. E. Stephens.** 1977. Studies of human histone messenger RNA. II. The resolution of fractions containing individual human histone messenger RNA species. J. Biol. Chem. **252:**173–180.

4. **Cardenas, J. M., and R. D. Dyson.** 1978. Mammalian pyruvate kinase hybrid isozymes: tissue distribution and physiological significance. J. Exp. Zool. **204:**361–267.

5. **Elmer, G. W., and W. J. Nickerson.** 1970. Nutritional requirements for growth and yeast-like development of *Mucor rouxii* under carbon dioxide. J. Bacteriol. **101:**595–602.

6. **Friedenthal, M., E. Roselino, and S. Passeron.** 1973. Multiple molecular forms of pyruvate kinase from *Mucor rouxii*: immunological relationship among the three isozymes and nutritional factors affecting the enzymatic pattern. Eur. J. Biochem. **35:**148–158.

7. **Guderley, H. E., and J. M. Cardenas.** 1979. Developmental changes in the pyruvate kinase isozymes of Coho salmon. J. Exp. Zool. **208:**1–12.

8. **Hall, E. R., and G. L. Cottam.** 1978. Isozymes of pyruvate kinase in veterbrates: their physical, chemical, kinetic and immunological properties. Int. J. Biochem. **9:**785–793.

9. **Hall, E. R., V. McCully, and G. L. Cottam.** 1979. Evidence for a proteolytic modification of liver pyruvate kinase in fasted rats. Arch. Biochem. Biophys. **195:**315–324.

10. **Harada, K., S. Saheki, K. Wada, and T. Tanaka.** 1978. Purification of four pyruvate kinase isozymes of rats by affinity elution chromatography. Biochim. Biophys. Acta **524:**327–339.

11. **Hedrick, J. L., and A. J. Smith.** 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. **126:**155–164.

12. **Hunsley, J. R., and C. H. Suelter.** 1969. Yeast pyruvate kinase: purification and some chemical properties. J. Biol. Chem. **244:**4816–4818.

13. **Ibsen, K. H., J. P. Cardin, Jr., R. H.-C. Chiu, K. N. Garratt, S. W. Marles, and J. R. Doty.** 1980. Distribution of pyruvate kinase isozymes in adult and developing *Xenopus laevis*. Comp. Biochem. Physiol. **65B:**473–480.

14. **Kane, F. J.** 1973. Pyruvate kinase, p. 353–382. *In* P. D. Boyer (ed.), The enzymes, vol. III. Academic Press, Inc., New York.

15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London)

227:680–685.

16. **Leloir, L. F., and S. H. Goldemberg.** 1960. Synthesis of glycogen from uridine diphosphate glucose in liver. J. Biol. Chem. **235:**919–924.

17. **Malcovati, M., and H. L. Kornberg.** 1969. Two types of pyruvate kinase in *Escherichia coli* K12. Biochim. Biophys. Acta **178:**420–423.

18. **Munday, K. A., I. G. Giles, and P. C. Poat.** 1980. Review of the comparative biochemistry of pyruvate kinase. Comp. Biochem. Physiol. **67:**403–411.

19. **O'Brien, M. D., and M. Kapoor.** 1980. Studies of the structure-function relationships of *Neurospora* pyruvate kinase: denaturation of urea and the effects of ligands on the refolding and renaturation process. Int. J. Biochem. **11:**107–116.

20. **O'Farrell, P. H.** 1975. High-resolution two-dimensional gel analysis of proteins. J. Biol. Chem. **250:**4007–4021.

21. **Ouchterlony, O., and L.-A. Nilsson.** 1976. Rocket immunoelectrophoresis, p. 19.31–19.32. *In* D. M. Weir (ed.), Handbook of experimental immunology. Blackwell Scientific Publications, Oxford.

22. **Passeron, J. L., and E. Roselino.** 1977. A new form of pyruvate kinase in mycelia of *Mucor rouxii*. FEBS Lett. **18:**9–12.

23. **Paznokas, J. L., and P. S. Sypherd.** 1977. Pyruvate kinase isozymes of *Mucor racemosus*: control of synthesis by glucose. J. Bacteriol. **130:**661–666.

24. **Petierra, A. G., and R. A. Cooper.** 1977. Pyruvate formation during the catabolism of simple hexose sugars by *Escherichia coli*: studies with pyruvate kinase-negative mutants. J. Bacteriol. **129:**1208–1214.

25. **Seubert, W., and W. Schoner.** 1971. The regulation of pyruvate kinase. Curr. Top. Cell. Reg. **3:**237–267.

26. **Spackman, D. H., W. H. Stein, and S. Moore.** 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. **30:**1190–1206.

27. **Strandholm, J. J., J. M. Cardenas, and R. D. Dyson.** 1976. Bovine pyruvate kinases isozymes and hybrid isozymes. Arch. Biochem. Biophys. **173:**126–131.

28. **Valentini, G., P. Iadarola, B. L. Somani, and M. Malcovati.** 1979. Two forms of pyruvate kinase in *Escherichia coli*. A comparison of chemical and molecular properties. Biochim. Biophys. Acta **570:**248–258.

29. **Waygood, E. B., M. K. Rayman, and D. Sanwal.** 1975. The control of pyruvate kinase of *Escherichia coli*. II. Effectors and regulatory properties of the enzyme activated by ribose 5-phosphate. Can. J. Biochem. **53:**444–454.

30. **Waygood, E. B., and B. D. Sanwal.** 1974. The control of pyruvate kinases of *Escherichia coli*. I. Physiocochemical and regulatory properties of the enzyme activated by fructose 1,6-diphosphate. J. Biol. Chem. **249:**265–274.