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Purification and Properties of Mouse Pyruvate Kinases K and M and of a Modified K Subunit†

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ABSTRACT: The K_4 and M_4 isozymes of mouse pyruvate kinase were purified to homogeneity, and their physical, chemical, and kinetic properties were compared. The K isozyme is slightly larger, but a high degree of homology exists as evidenced by a similar amino acid composition, immunotitration value, and two-dimensional arginine peptide pattern after tryptic digestion. Also, the more active conformational form of the K isozyme has kinetic and chromatographic properties similar to those of the M isozyme. Only K subunit could be extracted with antibody from fresh spleen extracts, but this subunit can be cleaved to form a product with the mobility of the M subunit. The cleavage is accomplished by an endogenous enzyme and appears to be the first step in K-enzyme degradation. This product is called K^{pm}. $K^{pm}K$ hybrid could also be purified to homogeneity. This enzyme has the structure $K_2^{pm}K_2$, and both types of subunit have activity. The K^{pm} form has a higher $K_{0.5S}$ value for phosphoenolpyruvate and a lower $K_{0.5S}$ value for ADP than does either the K or the M type. However, the K^{pm} and M subunits otherwise have very similar properties and it is speculated that the K^{pm} subunit is an M-type precursor.

Four tissue-specific types of pyruvate kinase subunits have been identified. Immunologic criteria divide these into two non-cross-reacting groups: the L and L′ types and the M and K types[1] [see Hall & Cottam (1978) for review]. The L and L′ subunits also have a simlar amino acid composition [see Hall & Cottam (1978)] and yield similar fingerprint patterns (Saheki et al., 1978), and the L subunit has been reported to be formed from the L′ type by proteolysis (Marie et al., 1977). Thus these two forms are probably products of a single gene. The K and M types also have antigenic determinants in common, and the K type has been reported to be converted to the M type (Marie et al., 1976). However, they have also been reported to have very different peptide fingerprint patterns (Saheki et al., 1978), and comparative amino acid composition

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[1] In this investigation the L, K, M nomenclature will be used, with subscripts to identify the quarternary structure when relevant. The homotetrameric K_4 enzyme is also commonly called the M_2 or A isozyme. The L′ subunit is found in red blood cells, and the native isozyme is often called the R or D isozyme [see Ibsen (1977) for review]. The subunit derived from the K form having a mobility like the M subunit is called K^{pm}, i.e., the K subunit pseudo-M-type product.

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data are inconsistent [see Hall & Cottam (1978)]. Most investigators refer to them as products of different genes [see Ibsen (1977) for review].

This paper reports new techniques for purifying the mouse K_4 and M_4 isozymes and compares their physical, chemical, and kinetic properties. They have similar amino acid compositions, yield similar fingerprint patterns, have quantitatively indistinguishable immunological properties, and the active form of the K_4 isozyme has kinetic properties like those of the M_4 type. However, the K subunit is slightly larger.

An endogenous enzyme can cleave the K subunit to form a subunit with the molecular weight of the M subunit. This subunit is called K^{pm}. The $K_2K_2^{pm}$ hybrid was also purified and studied.

Experimental Procedures

Enzymes, substrates, and other biochemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Sepharose and Blue-Dextran were from Pharmacia (Uppsala, Sweden). Ampholytes were from LKB (Rockville, MD). Acrylamide was recrystallized from $CHCl_3$ prior to use. DEAE[2]- and CM-cellulose were from Bio-Rad Laboratories (Richmond, CA). All chemicals were reagent or enzyme grade. Glass-distilled deionized water was used for all solutions. Swiss–Webster male mice (~20 g) obtained from Simenson Labs (Gilroy, CA) were used in all experiments described.

Buffer B (10 mM potassium phosphate, pH 7.0, 5 mM $MgSO_4$, 1 mM EDTA, and 0.5 mM DL-dithiothreitol) was used in most manipulations as indicated. The Cl^- concentration in chromatographic eluants was determined titrametrically (*Sigma Technical Bulletin*, 1965). Phosphoenolpyruvate levels in eluants were estimated by using the lactate dehydrogenase coupled pyruvate kinase assay and calculating the concentration from the amount of NADH used.

The specific activity values reported and kinetic data were obtained by using the coupled lactate dehydrogenase assay as delineated by Ibsen & Trippet (1973). Routine assays used phenylhydrazine in a manner simliar to that described by Imamura & Tanaka (1972). The method of Lowry et al. (1951) was used to determine protein levels. Blue-Dextran Sepharose was prepared as described by Ryan & Vestling (1974). The used resin was regenerated by washing with 0.01 N HCl and 1 M KCl.

The $NaDodSO_4$ electrophoretic method of Laemmli & Favre (1973) using 10% polyacrylamide in 0.9-mm slab gels was employed. The molecular weight was determined by comparison with the following standards: bovine serum albumin, 68 000; catalase, 60 000; rabbit muscle pyruvate kinase, 57 000; aldolase, 40 000; lactate dehydrogenase, 36 000. Polyacrylamide gels were polymerized with ammonium persulfate. Gels without detergents were run at pH 8.9 in the presence of 1 mM Fru-1,6-P_2 and 10 mM mercaptoethanol and were preelectrophoresed for 1–2.5 h. Protein bands were visualized by staining with a 0.05% Coomassie Brilliant Blue-R (B0630) in 10% acetic acid–10% isopropyl alcohol (v/v) solution and destained in 10% acetic acid–10% isopropyl alcohol.

Isoelectrofocusing in gels was performed according to Chua et al. (1978). Ampholines were leached out with 10% trichloroacetic acid, and the gels were stained with Coomassie Blue as before. Isoelectrofocusing in columns was performed as described by Ibsen & Trippet (1972). In this case, proteins were determined by using the Bio-Rad assay (*Bio-Rad Technical Bulletin*, 1979). Electrofocusing was also performed in the presence of 4–6 M urea in gels and in solution. Prior to use, the urea was subjected to vacuum desiccation overnight to remove any cyanate which may have formed. The pH in gels was determined by slicing them at 1-mm intervals and extracting in water.

Antibody was prepared by injecting a New Zealand white rabbit subcutaneously along the spine with 0.3 mg of homogeneous K_4-pyruvate kinase in complete Freund's adjuvant 3 times at weekly intervals during alternate months over a 3-month period. The IgG was purified by subjecting the serum to 40% $(NH_4)_2SO_4$ precipitation followed by DEAE-cellulose chromatography (Fahey, 1967).

In immunotitration studies, antigen was precipitated by adding antibody, incubating for 30 min at 37 °C, allowing the solution to stand at 4 °C overnight, and centrifuging at 10000*g* for 30 min. When collected, the precipitate was washed 3 times with saline buffered with 10 mM phosphate at pH 7.0 containing 0.01% Triton X-100.

Anti-pyruvate kinase IgG columns were prepared by reacting 250 mg of DEAE-purified antibody with cyanogen bromide activated Sepharose 4B in a 1:30 (w/w) ratio for 6 h at 4 °C (Porath et al., 1973). A 4 × 2 cm column bound about 150 IU K isozyme and 200 IU M isozyme. Homogeneous enzyme could be eluted by the following protocol. The column was prewashed with 20 mL of buffer B, 15 mL of 3.5 M thiocyanate in buffer B, and 20 mL of buffer B, loaded with ~100 IU of antigen containing solution, washed with 40 mL of buffer B and 20 mL of buffer B containing 10% glycerol, 1 M KCl, and 0.01% Triton X-100, and finally with 10 mL of buffer B. The enzyme was eluted with 3.5 M thiocyanate in buffer B. After 6 weeks the column rapidly lost its capacity to bind antigen.

Amino acid analyses were performed according to Moore & Stein (1963) on a Beckman Spinco analyzer after hydrolysis for 24 and 48 h in 6 N HCl at 110 °C. The mean values were used for calculation, except those for threonine and serine which were extrapolated to zero time. The number of residues was calculated on the basis of the mean subunit weight obtained by $NaDodSO_4$ electrophoresis. For the purpose of the calculations, it was also assumed there were 3 tryptophans and 10 cysteine residues. These values are similar to that obtained for either isozyme in other species (Hall & Cottam, 1978).

The procedure described by Hall et al. (1978) was followed to obtain fingerprint patterns by using thin-layer cellulose strips (20 cm × 20 cm × 0.1 mm Polygram cel 300, Brinkman, Westbury, NY) and TPCK-treated trypsin. Prior to ninhydrin staining, arginine-containing spots were visualized by using the phenathrenequinone reagent of Yamada & Itano (1966) as described by Phelan et al. (1972). Each isozyme was run 4 times with 0.8–2.0 mg of protein being used per experiment, the optimal amount being ~1 mg. Good resolution was obtained with three of the M-isozyme and two of the K-isozyme preparations when stained for arginine. However, the ninhydrin spots were not sufficiently resolved to give usable comparative results.

Results

Purification of K_4 Isozyme. Mouse spleen extracts were prepared by grinding with 3 volumes of buffer B in ice using a Potter-Elvhjem homogenizer and centrifuging 60 min at 100000*g*. They yield three peaks of activity when electrofocused. The mean p*I* values ($n = 5$) were 5.0 ± 0.22 SD, 6.33

[2] Abbreviations used: DEAE, diethylaminoethyl; CM, carboxymethyl; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; TPCK, tosylphenylalanine chloromethyl ketone; PEP, phosphoenolpyruvate; ADP, adenosine 5′-diphosphate; mRNA, messenger ribonucleic acid.

Table I: Purification of Mouse Spleen K-Type Pyruvate Kinase

step	act. (IU) total (10 g of tissue)	act. (IU) per mg of protein	% act. recovered	purification (x-fold)
S-100[a]	1486	1.3	100	1.0
50–70% $(NH_4)_2SO_4$ ppt	1210	2.4	81.0	1.8
Blue-Dextran Sepharose-KCl gradient peak III	666.0	54.8	44.8	42.2
Blue-Dextran Sepharose-PEP gradient	498.4	397.5	33.5	305.8

[a] The 100000g supernatant of the tissue extract.

± 0.17 SD, and 6.68 ± 0.01 SD. The pH 5.0 form accounts for ~20% of the total activity, while the pH 6.3 form accounts for 66.6–100% of the remaining activity. Previous data suggest the pH 5.0 form is the red cell isozyme and the pH 6.3–6.7 pair is a K_4-isozyme conformational set (Ibsen et al., 1975; Yanagi et al., 1971). Cardenas & Dyson (1978), using electrophoretic zymography, also find only two types of pyruvate kinase in spleen extracts which they believe to be K_4 and the red cell isozymes. It is logical to assume these correspond to the pH 6.3 and 6.7 forms and the pH 5.0 form, respectively. In neither study has evidence for the presence of KM hybrids in mouse spleen extracts been adduced.

The pH 5.0 enzyme was precipitated by adding $(NH_4)_2SO_4$ to 50% saturation, and the remaining activity was precipitated between 50 and 70% $(NH_4)_2SO_4$ saturation. About 75–81% of the total activity in the latter fraction is recovered, and the specific activity is almost doubled (Table I). This recovery corresponds over 90% of the putative K isozyme activity present in the extract. The mean p*I* values obtained in the 50–70% fraction were 6.59 ± 0.10 SD (90.5 ± 12.0% SD of the total activity) and 7.34 ± 0.0 (9.5 ± 8.0% of the total activity) (n = 4). These data indicate pH 6.3 enzyme was converted to the higher p*I* forms. Simlar shifts in p*I* values have previously been observed for the rat K isozymes (Ibsen & Trippet, 1972; Muroya et al., 1976). Indeed, Muroya et al. (1976) observed a much greater fraction of the rat spleen enzyme to convert to the highest p*I* type when treated with 70% $(NH_4)_2SO_4$.

The $(NH_4)_2SO_4$ precipitate is dissolved in a minimal volume of buffer B, exhaustively dialyzed against the same buffer, and applied to a 25 × 1.7 cm Blue-Dextran Sepharose 4-B or 6-B column, washed with 300 mL of buffer B, and eluted with a linear KCl gradient. The majority of activity elutes in a peak (Peak III) which reaches a maximum between 0.22 and 0.32 M KCl (mean value 0.27 ± 0.04 SD, n = 13). This peak is free of most contaminating protein (Figure 1), and collection and concentration of this fraction results in a 20–25-fold increase in specific activity (Table I). Distinct peaks are also obtained at mean concentrations of 0.077 ± 0.02 SD (n = 10) M KCl (peak I) and at 0.18 ± 0.05 SD (n = 6) M KCl (peak II). Figure 1A is typical; however, much variability is obtained in the relative proportions of these peaks.

When peak III enzyme from the first KCl elution is reeluted from a second Blue-Dextran Sepharose column by using a phosphoenolpyruvate gradient in the presence of 5 × 10^{-5} M Fru-1,6-P_2, enzyme is obtained in a sharp symmetrical peak at ~0.4 mM phosphoenolpyruvate in ~75% yield. The mean final specific activity was 346.6 ± 22.7 SE (n = 22) IU/mg of protein, and recovery ranged from 9 to 36% of the initial activity. A typical experiment is summarized in Table I. This

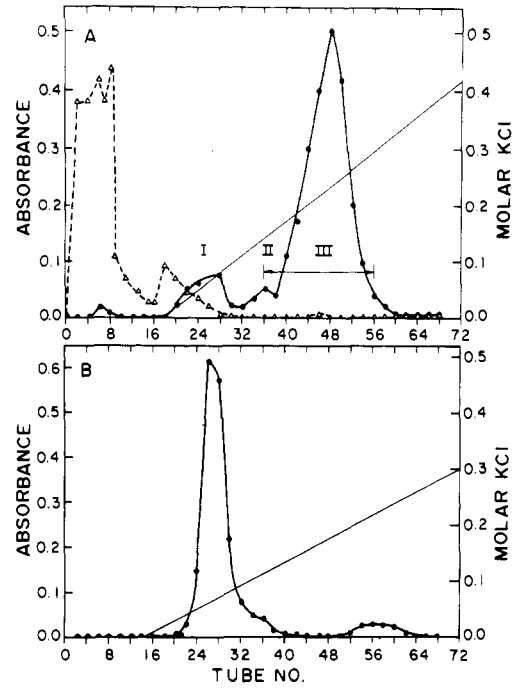

FIGURE 1: Elution of pyruvate kinase K from Blue-Dextran Sepharose with KCl gradients. (Panel A) The elution pattern obtained by using the dialyzed 50–70% $(NH_4)_2SO_4$ precipitate in the absence of Fru-1,6-P. Pyruvate kinase activity (●); absorbtion at 280 nM (Δ). (Panel B) The elution pattern obtained when the samples spanned by the arrow in panel A are collected, concentrated, dialyzed, and then eluted by KCl, all in the presence of 5 × 10^{-5} M Fru-1,6-P_2.

enzyme is homogeneous as judged by the following criteria: native polyacrylamide electrophoresis; isoelectrofocusing in polyacrylamide gels; isoelectrofocusing in columns, in which activity and protein level were found in a single symmetrical peak with a mean p*I* value of 6.22 ± 0.035 SD (n = 3) (Table III); Ouchterlony double immunodiffusion; immunotitration, in which a symmetrical protein precipitation pattern was obtained; immunoelectrophoresis; $NaDodSO_4$–polyacrylamide gel electrophoresis. When fresh tissue was used and the enzyme was rapidly prepared, the $NaDodSO_4$ pattern showed a single band suggesting it was homomeric. A small variable fraction of activity is not eluted with as much as 5 mM phosphoenolpyruvate but can be eluted with 0.5 M KCl.

Homogeneous enzyme has also been prepared from kidney extracts by a simlar technique with one additional step: the enzyme is passed through a DEAE-cellulose column at pH 7.2 prior to the first Blue-Dextran column. This step is introduced as a precaution to remove KL hybrids which may be present in kidney (Cardenas & Dyson, 1978). Under these conditions the L subunits bind but the K subunits do not and KL hybrids are retarded. The purified products from either source have indistinguishable $NaDodSO_4$ electrophoretic mobilities and immunotitration properties.

Fru-1,6-P_2 changed the KCl elution pattern. The enzyme corresponding to the arrow (peaks II and III) shown in Figure 1A was concentrated, dialyzed, added to second Blue-Dextran column, and again eluted with KCl, all in the presence of 5 × 10^{-5} M Fru-1,6-P_2. Over 90% of the added enzymatic activity is recovered as enzyme found in peak I (Figure 1B). Such enzyme is almost, but not quite, homogeneous. Peak III enzyme not treated with Fru-1,6-P_2 is not converted to peak I enzyme.

Purification of M Isozyme. Leg skeletal muscle including bone was homogenized in a Waring blender with 3 volumes of buffer B/g of tissue and centrifuged at 100000*g* for 60 min. Isoelectrofocusing of these extracts results in two activity peaks.

Table II: Purification of Mouse Skeletal Muscle M-Type Pyruvate Kinase

step	act. (IU) total (10 g of tissue)	act. (IU) per mg of protein	% act. recovered	purification (x-fold)
S-100[a]	7636	13.2	100	1.0
60 °C for 10 min	7080	34.5	92.7	2.6
50–70% $(NH_4)_2SO_4$ ppt	4625	47.3	60.6	3.5
Blue-Dextran Sepharose–KCl gradient	2334	279.2	30.6	21.2
Blue-Dextran Sepharose–PEP gradient	884	380.3	11.6	28.8

[a] The 100000*g* supernatant of the tissue extract.

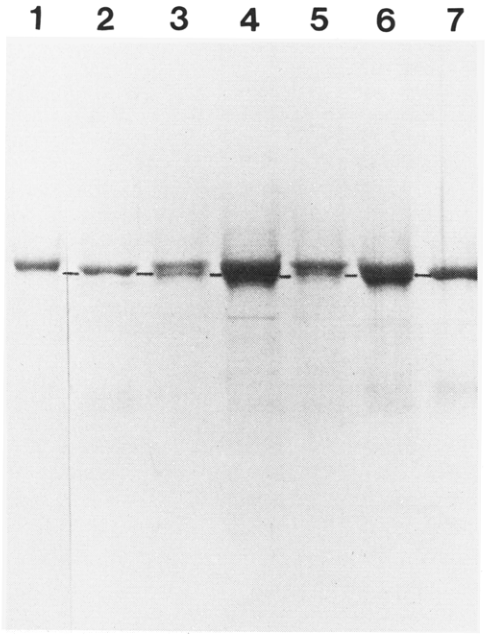

FIGURE 2: Comparison of mobilities of K, M, and K^{pm} subunits in $NaDodSO_4$–polyacrylamide gels. The additions to the various channels are (1) 4.0 μg of K isozyme, (2) 3.6 μg of M isozyme, (3) 2.0 μg of K isozyme plus 1.8 μg of M isozyme, (4 and 5) 7.2 and 3.6 μg of the same preparation of a double-banded preparation of K isozyme, (6) 3.6 μg of the same KK^{pm} isozyme plus 3.6 μg of M isozyme, and (7) 7.2 μg of M isozyme. The penned line is drawn through the M-subunit lines in channels 2 and 7.

A small variable fraction of the total activity (4.9 ± 4.6% SD) has a p*I* value of 6.30 ± 0.00 SD while the remaining activity peaks at pH 7.32 ± 0.04 SD (n = 2). The K isozyme present was inactivated by heating the extract at 60 °C for 10 min. The M isozyme was further purified by fractionating between 50 and 70% $(NH_4)_2SO_4$ saturation and by eluting from two Blue-Dextran columns using KCl and phosphoenolpyruvate in tandem. The enzyme came off in a single peak at ~0.08 M KCl, i.e., at a point approximately corresponding to peak I of the K isozyme. The phosphoenolpyruvate elution was performed in the presence of 5×10^{-5} M Fru-1,6-P_2, and the enzyme was eluted at ~0.4 mM substrate.

This isozyme was obtained at a 9–15% recovery with a mean specific activity of 369.5 ± 11.7 SE (n = 9) IU/mg of protein. Table II summarizes a typical experiment. The enzyme usually appears homogeneous as judged by $NaDodSO_4$ and native gel electrophoresis, Ouchterlony double diffusion, and electrofocusing in gels or columns. The final product has a p*I* value of 7.25 ± 0.07 SD (n = 2) (Table III).

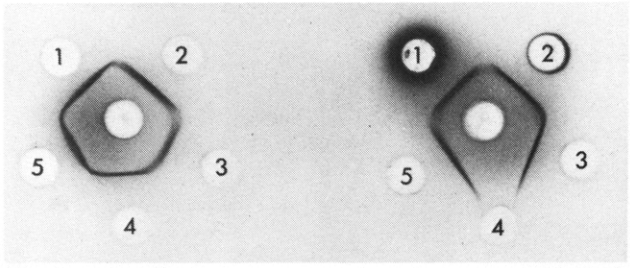

FIGURE 3: Ouchterlony immunodiffusion patterns obtained with K_4 and M_4 isozymes run against anti-pyruvate kinase K IgG. In both sets illustrated, 10 μg of DEAE-purified anti-pyruvate kinase K IgG is in the center well. In the left-hand panel, wells 1 and 3 contain purified M_4 isozyme, while wells 2, 4, and 5 contain purified K_4 isozyme. In the right-hand panel, wells 1, 2, 3, and 5 contain the 100000*g* supernatant of an extract from spleens, the dialyzed 50–70% $(NH_4)_2SO_4$ fraction, peak III from the KCl eluate of the Blue-Dextran Sepharose bound enzyme, and purified K_4 enzyme. Well 4 has buffer B. The absence of true spurs between the K and M enzymes indicates identity of the compared antigenic determinants, whereas the absence of a second precipitation band in any step of purification is another index of homogeneity of enzyme and antibody (Ouchterlony & Nilsson, 1978). The antibody and antigen were allowed to react for 48 h. The gel was then extensively washed in phosphate-buffered saline, pH 7.0, and stained with Coomassie Blue. In all cases ~7 IU of enzyme was used as antigen.

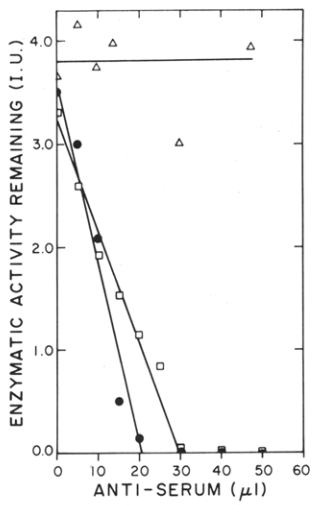

FIGURE 4: Quantitative immunotitration patterns obtained by using purified K_4, M_4, and L_4 isozymes titrated against anti-pyruvate kinase K antiserum. The O-μL value represents the average value obtained when the enzyme sample was run with 10 and 50 μL of neutral rabbit serum. Homogeneous K_4 (□) and M_4 (●) isozyme and partially purified L_4 (△) enzyme were used. Immunotitration was performed as outlined under Experimental Procedures.

Molecular Weight Studies. $NaDodSO_4$ electrophoretic studies show the K isozyme to have a subunit weight of 59 500 daltons and the M-isozyme subunit to weigh 58 600 daltons. Although these differences are of marginal statistical significance (Table III), the K subunit does run slightly slower than the M form when the two are subjected to $NaDodSO_4$ electrophoresis (Figure 2).

The molecular weight of the undenatured isozymes was determined by electrophoresis in 5, 6, and 7% polyacrylamide gels, sedimentation in glycerol gradients, and chromatography on Sephadex G-200 (Table III). The values obtained are ~4 times the subunit weight indicating the enzymes are homotetramers. Although the differences are not statistically significant, the weight of the M_4 isozyme is consistently less than that of the K_4 form.

Immunological Properties. K and M isozymes from partially or fully purified extracts were subjected to Ouchterlony

Table III: Some Physical Properties of the K_4, M_4, and $K_2K_2^{pm}$ Isozymes and Their Subunits

property	mode of determination	K_4 isozyme native	K_4 isozyme subunit K_1	M_4 isozyme native	M_4 isozyme subunit M_1	$K_2K_2^{pm}$ isozyme native	$K_2K_2^{pm}$ isozyme subunit K_1	$K_2K_2^{pm}$ isozyme subunit K_1^{pm}
molecular weight × 10^{-3} ± SE (no. of determinations)	$NaDodSO_4$ electrophoresis	238.0 $K_1 \times 4$	59.5 ± 0.25[a] (17)	234.4 $M_1 \times 4$	58.6 ± 0.40[a] (12)	235.0 $K_1 \times 2 + K_1^{pm} \times 2$	59.3 ± 0.31[a] (6)	58.2 ± 0.33[a] (6)
	mobility in 5, 6, and 7% polyacrylamide gels	230.0 ± 4.31 (7)		225.5 (1)		228.0 (1)		
	glycerol gradient sedimentation	230.0 ± 10.68 (4)		220.0 ± 7.47 (4)		215.0 (1)		
	Sephadex G-200 chromatography	238.0 (1)						
sp act. ± SE (no. of determinations)		346.6 ± 22.7 (22)		369.5 ± 11.7 (9)		228.2 ± 33.5 (5)		
p*I* value ± SE (no. of determinations)	after exposure to Fru-1,6-P_2 run in pH 6–8 ampholine columns	6.22 ± 0.02 (3)		7.25 ± 0.05 (2)		6.75 (1)		
	run with 6 M urea in polyacrylamide gels		6.6		7.4		6.0–6.6	7.3

[a] The single tailed Student's *t* test yielded the following *P* values: the K subunit vs. the M subunit from the respective homotetramers, $0.1 > P > 0.05$; the K subunit of the K_4 isozyme vs. the K subunit from the $K_2K_2^{pm}$ isozyme, $0.6 > P > 0.5$; the M vs. the K^{pm} subunit, $0.4 > P > 0.3$; the K vs. the K^{pm} subunit, $0.05 > P > 0.01$.

Table IV: Some Kinetic Parameters Describing the K_4, M_4, and $K_2K_2^{pm}$ Isozymes

parameter	M_4 isozyme pH 7.5	M_4 isozyme pH 7.0	K_4 isozyme pH 7.5	K_4 isozyme pH 7.0	$K_2K_2^{pm}$ isozyme pH 7.5	
$K_{0.5S}$ PEP (mM)						
no Fru-1,6-P_2	0.035	0.035	0.19	0.09	0.17[a]	1.10[b]
with 5×10^{-5} M Fru-1,6-P_2	0.028	0.033	0.026	0.025	0.023[a]	0.26[b]
$K_{0.5S}$ MgADP (mM)						
no Fru-1,6-P_2	0.28	0.29	0.27	0.32	0.12[a]	0.25[b]
with 5×10^{-5} M Fru-1,6-P_2	0.30	0.38	0.30	0.38	0.12[a]	0.28[b]

[a] From extrapolation of the lower K_m form. [b] From extrapolation of the higher K_m form.

Table V: Amino Acid Composition

amino acid	residues/subunit K type	residues/subunit M type
lysine	38	36
histidine	18	17
arginine	32	31
aspartate + asparagine	51	50
glutamate + glutamine	47	46
serine	32	32
threonine	29	28
proline	23	22
glycine	43	42
alanine	64	60
methionine	12	14
valine	51	51
isoleucine	36	37
leucine	45	45
tyrosine	9	8
phenylalanine	15	14

double diffusion, using anti-pyruvate kinase K IgG. When the K and M forms are added to adjacent wells, a single precipitin band with no spurs is obtained (Figure 3). Thus the K isozyme does not have immunodeterminants which are not shared with the M subunits. Immunological cross-reactivity has been observed in other species [see Hall & Cottam (1978)]. The data of Figure 4 show that the quantitative response to antibody is also very similar. The L isozyme shows little or no cross-reactivity.

Kinetic Characteristics. Reaction rates were determined by using phosphoenolpyruvate and MgADP as the variable substrates, at pH 7.5 and 7.0, in the presence and absence of 5×10^{-5} M Fru-1,6-P_2. The K_4 isozymes exist in two forms which can be distinguished by their relatively high and low affinities for phosphoenolpyruvate. Fru-1,6-P_2 and H^+ favor the higher affinity form. The lower $K_{0.5S}$ form of the K_4 isozyme and the M_4 isozyme have indistinguishable kinetic characteristics (Table IV).

Amino Acid Composition. Table V reports the results of amino acid analyses. The two isozymes have a very similar composition, and the values for the M isozyme are, in general, within the range reported for other species; much greater variation has been reported for the K isozyme [see Hall & Cottam (1978)].

Fingerprint Analyses. The homogeneous homomeric isozymes were carboxymethylated and trypsinized. The peptides

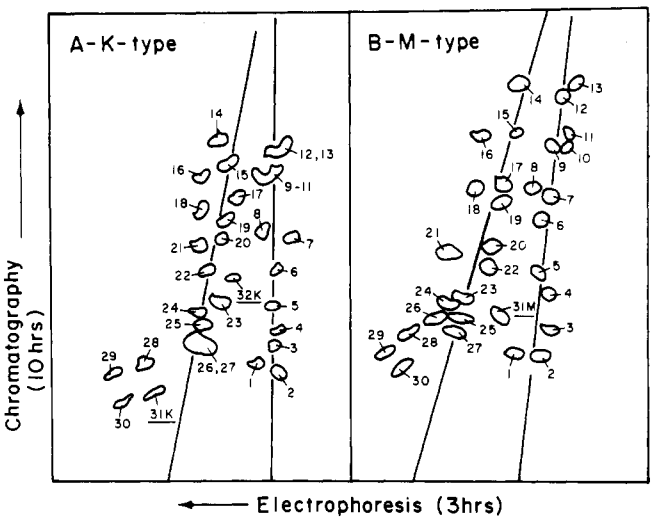

FIGURE 5: Separation of arginine-containing peptides after tryptic digestion of K and M isozymes. The origin lies near the lower right-hand corner. Details are presented in the text.

were separated by using ascending chromatography in one dimension and electrophoresis in the other. The separated peptides were reacted with phenanthrenequinone to visualize the arginine-containing peptides (Figure 5). As an aid in

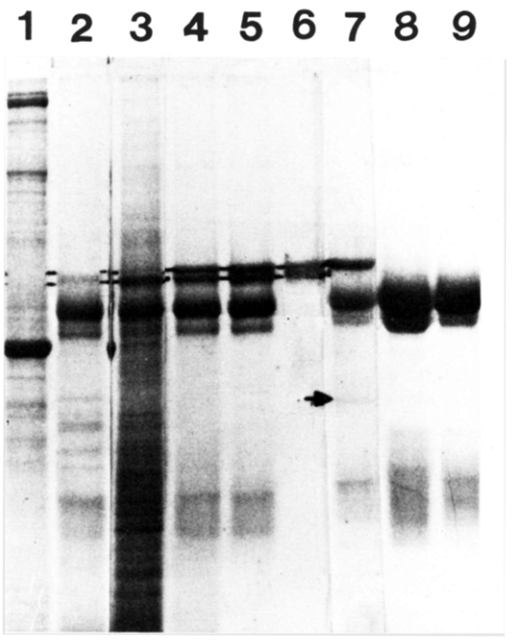

FIGURE 6: $NaDodSO_4$ electrophoretic patterns obtained by using precipitates formed when anti-pyruvate kinase K IgG is reacted with tissue extracts and enzyme at various stages of purification. Channel 6 contains 6 μg of purified double-banded K-type enzyme. The bands corresponding to the K and K^{pm} subunits are identified by the ink markings carried across the slab. Channels 8 and 9 contain 20 μg of DEAE-purified neutral and anti-pyruvate kinase K rabbit IgG, respectively. Channel 1 contains the precipitate obtained when neutral IgG was reacted with a fresh tissue extract. No band corresponding to K or K^{pm} was obtained. The other channels contained suspensions reacted with antipyruvate kinase IgG as follows: (7) the tissue extract previously cleared with netural IgG (only the K subunit, the minor band noted with arrow, and the IgG bands are discernible); (3) the 50–70% $(NH_4)_2SO_4$ fraction from this extract (the K^{pm} band has become predominant); (4) peak II from the KCl elution (equal amounts of K and K^{pm} are present); (5) the purified heteromeric enzyme. Channel 2 contains the same extract as that of channel 7 which was incubated with 0.1 mg (100–150 units) of hog pancreatic trypsin (Nutritional Biochemical Corp.) for 30 min at 37 °C prior to reaction with antibody. The K subunit disappears and a K^{pm}-like one appears.

identification, two axis were drawn through the spots, and they were numbered starting at the bottom of the right-hand axis in an ascending manner and in a descending manner down the left-hand axis. In the case of the K isozyme, it was assumed that three spots were not resolved in the one labeled 9–11 and two were not resolved in the spots labeled 12,13 and 26,27. These assumptions bring the total number to 32, which agrees with the amino acid analyses (Table V). In any case, the homology between the isozymes is obvious. It appears that 30 spots have very similar mobilities, two spots, labeled 31K and 32K, are liberated from the K isozyme but not from the M isozyme, and one spot labeled 31M is found in the M pattern but not in the K pattern. In addition to demonstrating homology, these data confirm that both isozymes are homomers.

Formation, Purification, and Properties of a $K_2K_2^{pm}$ Hybrid Enzyme. Enzyme prepared from spleen or kidney sometimes yielded two closely spaced protein bands when electrophoresed in $NaDodSO_4$ gels even though they otherwise appeared homogeneous. These two bands have the mobilities of the K and M subunits (Figure 2). The less mobile, K subunit, usually predominated, but equivalent amounts of the two bands were found in an occasional preparation. Although, as will be seen below, it is possible to obtain conditions in which the more mobile K^{pm} band predominates, it has not yet been possible to purify enzyme having more of K^{pm} subunit than K type.

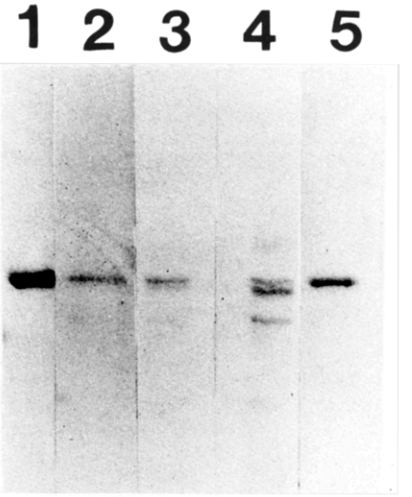

FIGURE 7: $NaDodSO_4$ electrophoretic patterns obtained from eluates of fresh and aged extracts passed through Sepharose antibody columns. Channels 1 and 5 contain 6 and 3 μg of conventionally purified pyruvate kinase K as a marker. Channels 2, 3, and 4 contain the protein eluted when extracts with 100 IU of activity were passed through the column and then eluted as described. Only K subunit was obtained when fresh extract was used (channel 2); K, K^{pm}, and traces of a third band were present in the same extract aged for 5 days at −20 °C with intermittent freezing and thawing (channel 3); these new bands become prominant by the seventh day of aging (channel 4).

Since most tissues expressing K subunit have also been reported to express the M type (Cardenas & Dyson, 1978), it seemed possible that double-banded enzyme represented endogenous KM hybrid which was copurified. However, this seemed unlikely since mouse spleen is, as discussed above, apparently free of hybrid, and in one case seemingly homogenous apparent $K_2K_2^{pm}$ enzyme was purified at a 37% yield. Therefore, the source and nature of this M subunit like protein band was further investigated as described below.

Purified double-banded enzyme was precipitated with antibody, and the precipitate was subjected to polyacrylamide electrophoresis in $NaDodSO_4$. Both bands were recovered suggesting they were both immunologically active (Figure 6). This was confirmed by using antibody coupled to Sepharose 4B in columns (Figure 7). Both techniques also indicate that fresh tissue extracts contained only the K-type subunit. Antibody was used to precipitate enzyme at each step during purification (Figure 6) to test the possibility that K^{pm} was formed during the purification. Although K^{pm} was not present in the original extract, it predominated in the $(NH_4)_2SO_4$ fraction, and the two forms were present in equivalent amounts in subsequent fractions. If the more mobile band in the $(NH_4)_2SO_4$ fraction was due to an unobserved endogenous protein, the shift in the relative proportions of the two bands in extracts and in the $(NH_4)_2SO_4$ fraction could only occur if a large amount of K isozyme were lost. This did not occur; enzymatic activity was recovered in ~75% yield. Thus, the more mobile band must be derived from the K subunit, and it must either have enzymatic activity itself or be modified between the times of assay of the $(NH_4)_2SO_4$ fraction and the running of the electrophoretic gel.

Proportionally, more K^{pm} is present in the $(NH_4)_2SO_4$ fraction than in subsequent steps (Figure 6). This could reflect further conversion during the period which elapsed between isolation of this fraction and treatment with antibody (~7 days), during which time the sample was refrigerated, selection against K^{pm} during subsequent steps, or both.

Incubation of the tissue extract with trypsin caused the K subunit to disappear from extracts and the K^{pm} one to appear

Table VI: Effect of Aging on Elution Profiles of the K Isozyme from Blue-Dextran Sepharose Columns[a]

days aged	Blue Dextran I,[b] % total recovered act.[c]		Blue Dextran II, % total recovered act.[d]	
	peak II	peak III	PEP elution	0.5 M KCl elution
0	12.5	87.5	100.0	0.0
4	21.0	79.0	89.6	10.4
10	57.9	42.1	75.0 (50.0)[e]	25.0 (50.0)[e]

[a] The data were derived with a single spleen extract which was fractionated with $(NH_4)_2SO_4$ and stored at −20 °C. The first portion was immediately applied to a Blue-Dextran Sepharose 4-B column and the remainder frozen. After 4 days the whole sample was thawed; that portion not used was refrozen and thawed on the tenth day. [b] 0–0.5 M KCl elution. [c] Total activity recovered in the KCl eluants. In this particular experiment peak I accounted for <1% of the total activity in all three preparations. [d] Total activity recovered in the PEP and the KCl elutions from the second Blue-Dextran Sepharose column. [e] The numbers in parentheses indicate results obtained with peak II enzyme.

(Figure 6, channel 2). This and their relative mobilities suggest K^{pm} is generated by proteolytic cleavage of the K subunit.

It was noted that while enzyme precipitated from fresh tissue extracts was single banded, K^{pm} was often observed in stored extracts, even when frozen at −20 °C. In the case illustrated in Figure 7, a spleen extract was run through an antibody column on the day it was prepared and 5 and 7 days later. In between it was stored at −20 °C, but it was subjected to thawing and refreezing twice, between days 1 and 5 and again on day 5. Figure 7 clearly shows that the fresh extract yields only K subunit, but K^{pm} is evident by the 5th day and predominates on the seventh day. Additional bands also appear in the aged extracts, one of which became very distinct by day 7. The molecular weight of this band fell between 50K and 51K daltons. Although this third band was unusually distinct in this case, such "extra" bands were not uncommon. An example of another still more mobile product is the minor band marked by the arrow in Figure 6. Similar immunologically cross-reactive bands with a molecular weight of ~50 000 can sometimes be observed when K isozyme was mildly trypsinized. Thus, their relative mobilities and the immunological relationships all suggest these bands represent K-subunit degradative products.

Retrospective analyses of the data suggest a direct correlation exists between the amount of peak II observed in the first Blue-Dextran elution and the presence of K^{pm} subunit in the purified product. In the cases where K^{pm} was present in the purified enzyme, peaks II and III overlapped and the sample taken for further purification included peak II enzyme. Therefore, it seemed possible that peak II was the source of K^{pm} in the final product. However, it is logical to assume that K_2M_2 would be eluted at a KCl concentration which is the mean of that needed to elute K_4 and M_4 isozyme, i.e., peak II. A 50–70% $(NH_4)_2SO_4$ fraction from a spleen extract was divided into three parts in order to test the possibility that peak III is homomeric K_4 enzyme and to see if peak II is endogenous K_2M_2 or if it is derived $K_2K_2^{pm}$ enzyme. The first portion was immediately and quickly purified. The second portion was purified after being stored at −20 °C for 4 days, and the third portion was purified after 10 days of aging. It was thawed and refrozen on the fourth day. The data summarized in Table VI show that the proportion of peak II clearly increases with aging. By the tenth day the majority of the activity was found in this peak.

Peak II from the 10-day-old extract, as well as peak III from all three fractions, was further purified by a phosphoenol-

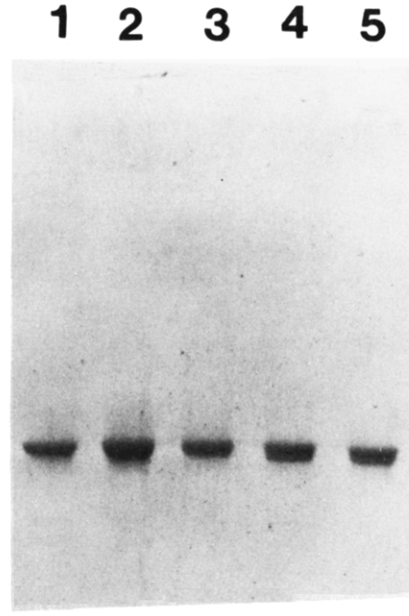

FIGURE 8: $NaDodSO_4$ polyacrylamide electrophoretic comparison of enzyme purified from peaks II and III of the first blue-Dextran elution. Channels 1, 2, and 3 contain pyruvate kinase K purified from peak III of the KCl elution after 0, 4, and 10 days of aging; channels 4 and 5 contain enzyme purified from peak II after 10 days of aging.

pyruvate elution from a second Blue-Dextran column, as usual. The products obtained were subjucted to $NaDodSO_4$ electrophoresis as illustrated in Figure 8. Peak III enzyme is clearly single-banded K type, while peak II contained equal quantities of the K and K^{pm} subunits.

After elution with phosphoenolpyruvate, the columns were further eluted with 0.5 M KCl. As the preparation aged, a greater proportion of the enzyme remained bound after the 0–5 mM phosphoenolpyruvate elution. This was true even though the phosphoenolpyruvate-eluted enzyme came off in a single sharp peak below 1 mM phosphoenolpyruvate. This tightly bound enzyme was found in peak II as well as in peak III (Table VI). The nature of this enzyme is still unclear.

Peak II enzyme could now be obtained in sufficient yield to permit further study of the homogeneous double-banded enzyme by elution from Blue-Dextran Sepharose with phosphoenolpyruvate as before. The data (Tables III and IV) show that the enzyme present is $K_2K_2^{pm}$. That is, the subunits present have the molecular weights expected of the K and M subunits, but the kinetic properties show it is not K_2M_2 isozyme. Moreover, the homogeneous peak II enzyme contains equal amounts of the two bands (Figures 6 and 8). Since this enzyme was separated from K_4 and M_4 enzyme by chromatographic means (peak II free of peak I or peak III), it should contain neither of the homomeric forms, i.e., it is not a mixture of K_4 and M_4 homomers. This possibility is also excluded by the unique kinetic properties described below. That the putative $K_2K_2^{pm}$ hybrid is not a mixture of another sort is also indicated by the facts that it appears homogeneous upon disc gel electrophoresis, gel isoelectrofocusing, and gel exclusion chromatography and that the K^{pm} subunit has enzymatic activity (see below).

The specific activity of the $K_2K_2^{pm}$ enzyme is significantly lower than that of either the K_4 or M_4 isozymes (Table III), being about two-thirds of either. This suggests the subunit has activity but activity is reduced. That K^{pm} has a lesser affinity for phosphoenolpyruvate was demonstrated by kinetic analysis using phosphoenolpyruvate as the variable substrate. A plateau region was observed, indicating the presence of high- and low-affinity forms. Extrapolation of the data indicates

that part of the activity is due to an enzyme form having $K_{0.5S}$ values identical with that found with K_4 enzyme, while the remaining activity is due to a form with a 10-fold lower affinity. Fru-1,6-P_2 has the same proportional effect on both types of subunit (Table IV). These types of response would not be expected if the hybrid were K_2M_2 since the M subunit prefers the active conformation (Table IV) and previous work has shown that the M subunit does retain its kinetic characteristics when in hybrids with either the L (Cardenas & Dyson, 1973) or K (Ibsen et al., 1976) subunits.

A small discontinuity was observed when MgADP was used as the variable substrate, the $K_{0.5S}$ values obtained were about 0.26 and 0.12 mM ADP. One of these is equivalent to those of the K or M isozymes and one is lower (Table IV).

Discussion

The data adduced in this study show that homotetrameric K isozyme can be purified in high yield by using fresh spleen or kidney extracts, by working rapidly, and/or by carefully excluding peak II material from the first KCl elution. Conversely, aged extracts have significant quantities of peak II enzyme which can be used to isolate a heterotetramer called $K_2K_2^{pm}$. The K and M subunits have similar properties, many of which can be expected to be shared by the K^{pm} subunit which is derived from the K form. Salient aspects are considered in more detail below.

Amino Acid Composition. The mouse K and M isozymes have an almost indistinguishable amino acid composition. Similar correspondence was reported for the isozymes when isolated from pig tissues (Berglund et al., 1977). The human isozymes have been reported to have a much greater divergence (Corcoran et al., 1976; Harkins et al., 1977a,b). However, the two different analyses of the human K isozyme are inconsistant (Corcoran et al., 1976; Harkins et al., 1977a). Even though both isozymes have not been analyzed from any other one species, the M isozyme of several mammalian species has been studied [reviewed by Hall & Cottam (1978)]. The values reported herein for the mouse enzyme fit into the general M-type pattern.

Molecular Weight. A composite of the data shows the K and M isozymes to generally have a similar weight (Hall & Cottam, 1978); however, the human M isozyme (Harkins et al., 1977a) or K isozyme from lung (Corcoran et al., 1976) has been reported to be significantly larger than the human K isozyme isolated from kidney (Harkins et al., 1977b). Our studies indicate the mouse K subunit to be the larger by some 900 daltons (Table III). Although this difference is not highly statistically significant, the K isozyme is confirmed the larger by subjecting the two to $NaDodSO_4$ electrophoresis on the same polyacrylamide gel slab (Figure 2). In the only other study noted in which the two isozymes were subjected to simultaneous $NaDodSO_4$ electrophoresis (Saheki et al., 1978), the carboxymethylated rat K and M subunits were found to weigh 61 000 and 59 000, respectively. Therefore, the collective data, exclusive of human studies, suggest that the K isozyme of any given species is slightly larger than that of the M isozyme, but the difference is too small to be detected unless the isozymes are studied simultaneously. A difference of 900–2000 daltons would correspond to some 7–18 amino acid residues. The relatively small size reported for the human kidney K isozyme might result from extensive autolysis resulting in a fragment smaller than the M subunit. Smaller immunologically active fractions were observed in these studies (Figure 6 and 7). The most commonly observed form had a molecular weight of ~50 000. This triad of molecular weight forms (~59 000, 58 000, and 50 000) is analogous to the three forms of phosphofructokinase found upon treatment with subtilisin (Kemp et al., 1980). Variable degrees of degradation may also account for the differences in reported amino acid composition noted above.

Immunological Properties. The K and M isozymes have quantitatively similar immunological properties, and their Ouchterlony double-diffusion patterns show no spurs while the L isozyme does not cross-react (Figures 3 and 4). These data are consistent with the pattern of cross-reactivity found by others [see Hall & Cottam (1978) for review]. In addition, these data show a quantitative similarity, suggesting the homology is extensive. Interestingly, both the immunotitration data (Figure 4) and the binding capacity to the anti-pyruvate kinase K IgG columns indicate the M isozyme to have a slightly greater affinity to the antipyruvate kinase K than does the original pyruvate kinase K antigen.

Fingerprint Analysis. Saheki et al. (1978) found the rat K and M isozymes yield very different $NaDodSO_4$ electrophoretic patterns after hydrolysis with papain or *Staphylococcus V-8* protease. This finding does not agree with the two-dimensional fingerprint analyses reported herein (Figure 5). These data show a close correspondence between the two isozymes with respect to arginine-staining spots and are, in this respect, consonant with the immunological and amino acid composition studies. However, this similarity could result from either the action of two distinct but closely related genes or from the postranslational conversion of one form to the other. If the latter mechanism were operational, the apparent existence of a unique M-subunit peptide (31M, Figure 5) would indicate the conversion was not a simple cleavage at a site cleaved by trypsin.

Kinetic Properties. The data of Table IV show that the active forms of the K and the M isozymes have similar kinetic properties as has been reported for the purified pig isozymes (Berglund & Humble, 1979) and the partially purified rat isozymes (Ibsen & Trippet, 1973). The major kinetic differences between the two isozymes lie in the ease with which they assume the less active T conformation. In the absence of effectors a greater fraction of the K-isozyme molecules are in the T conformation. This isozyme is also susceptible to a greater number of negative effectors. However, even the M isozyme can assume a T conformation, and it probably has the same sites for binding negative and positive effectors as does the K isozyme (Ibsen & Marles, 1976). These properties are compatible with there being a minimal structural difference between the isozymes which stabilizes different conformations. The idea of basic structural conformity accompanied by potential conformational difference is also supported by the observations that the K isozyme elutes like the M isozyme from Blue-Dextran Sepharose in the presence of Fru-1,6-P_2.

pI Values. Enzymes were prepared in the presence of Fru-1,6-P_2. Therefore, the p*I* values obtained should be those of the Fru-1,6-P_2 complexed enzymes. These values were 6.2, 6.7, and 7.2 for the K_4, $K_2K_2^{pm}$, and M_4 isozymes, respectively (Table III). This suggests the K^{pm} and M subunits have similar p*I* values, i.e., the K_2M_2 hybrid would also have a p*I* value of 6.7. This was confirmed by running the $K_2K_2^{pm}$ hybrid in urea isoelectric focusing gels. Two protein band were obtained, one being broad in the pH 6.0–6.6 range and the other being at pH ~7.3.

After $(NH_4)_2SO_4$ treatment, most of the K isozyme has a p*I* value of 6.6, but some pH 7.2 enzyme was also generated by $(NH_4)_2SO_4$ treatment. A similar triad of isoelectric forms of the K isozyme has been observed in previous studies of impure rat preparations [see Ibsen (1977) for review]. Ibsen

& Trippet (1972) suggested these three forms were conformational variants and that the middle-p*I* form was complexed with Fru-1,6-P_2. These conclusions were based on the observations that incubation of the lowest p*I* form in the absence of Fru-1,6-P_2 generated the highest p*I* form, but incubation of any of the p*I* forms in the presence of Fru-1,6-P_2 generated the middle-p*I* form. In contrast, Muroya et al. (1976) concluded that the three p*I* forms represented enzyme saturated with, half-saturated with, and stripped of Fru-1,6-P_2.

The data obtained in this study offer an alternative explanation, namely, the high-p*I* form is K^{pm}. This conclusion is based upon their similar p*I* values and $K_{0.5S}$ values for ADP and phosphoenolpyruvate. Moreover, both K^{pm} and the "highest p*I* variant" are readily formed in frozen or $(NH_4)_2SO_4$-treated extracts. Also, it was noted that the high-p*I* variant subunit was smaller than middle- or low-p*I* forms even though the difference in molecular weight was considered not to be significant (Ibsen & Trippet, 1972). This is analogous to the relationship between mouse K and K^{pm} subunits.

The free K_4 isozyme and the Fru-1,6-P_2 complexes of K^{pm} and of KK^{pm} hybrids will have p*I* values in the range of 6.6–6.8. It is inevitable that these forms will be confused in the absence of data other than isoelectrofocusing patterns. Therefore, the middle-p*I* form previously observed by Ibsen & Trippet (1972) and Muroya et al. (1976) could be free K_4 enzyme, Fru-1,6-P_2-complexed K^{pm} or KK^{pm} hybrid. This would explain the apparent interconvertability of the forms, e.g., how low-p*I* enzyme can be converted to the high-p*I* variant by incubation without Fru-1,6-P_2 and then to the middle-p*I* form by electrofocusing with Fru-1,6-P_2.

Although $K_2K_2^{pm}$ is a tetramer (Table III), the high-p*I* variant was dimeric (Ibsen & Trippet, 1972). Possibly the inclusion of more than two K^{pm} subunits causes the tetramer to dissociate.

Relationship between K, K^{pm}, and M Subunits. The K^{pm} subunit is not present in fresh extracts, but it may appear during purification, as a consequence of aging or upon the action of trypsin (Figures 6 and 7). That the K and K^{pm} subunits have a precursor–product relationship is suggested by the following: the K subunit is the only form initially present (Figures 6 and 7); as K or K_4 decreases, K^{pm} or $K_2K_2^{pm}$ increases (Figure 6, channel 2, and Table VI); K^{pm} accumulates as a KK^{pm} hybrid (Table VI); the K^{pm} subunit has enzymatic activity and is therefore not a chance contaminant; K^{pm} and K are immunologically similar (Figures 6 and 7); the kinetic properties of K^{pm} parallel those of K and are different from those of M (Table IV). Except that K^{pm} has catalytic activity, the K–K^{pm} relationship is analogous to the L-isozyme cleavage which occurs in fasted liver (Hall et al., 1979).

That K^{pm}-subunit accumulation was most readily observed in impure preparations kept refrigerated or frozen suggests that under these conditions the K^{pm} formed is spared further degradation. That is, at 37 °C the formation of K^{pm} is rate limiting and therefore any K^{pm} formed is immediately destroyed. This could occur if the activity of the enzyme(s) of further degradation is more susceptible to cold or if the enzyme responsible for K^{pm} formation is activated by the cold or by freezing and/or by $(NH_4)_2SO_4$.

Since K^{pm} is derived by a minimal change from K and the K and M subunits have similar chemical properties, K^{pm} and M must also be similar. Indeed, K^{pm} and M must have identical or confusingly similar molecular weights, amino acid compositions, tryptic digestion arginine fingerprint patterns, immunological determinants, pattern of elution from Blue-Dextran Sepharose (i.e., $K_2K_2^{pm}$ elutes like K_2M_2), and p*I* values. Yet the kinetic data show K^{pm} is not the M subunit. Moreover, it is well-known that both the carboxyl and amino termini of the M isozyme are modified [see Hall & Cottam (1978) for review]. Therefore, cleavage alone could not convert K to M subunits. That a unique M-subunit peptide is observed in fingerprint studies (Figure 5) also indicates that if cleavage was responsible for a conversion of K to M subunit, it either occurs at a site different from the tryptic cleavage or the carboxyl terminus is further altered, or both.

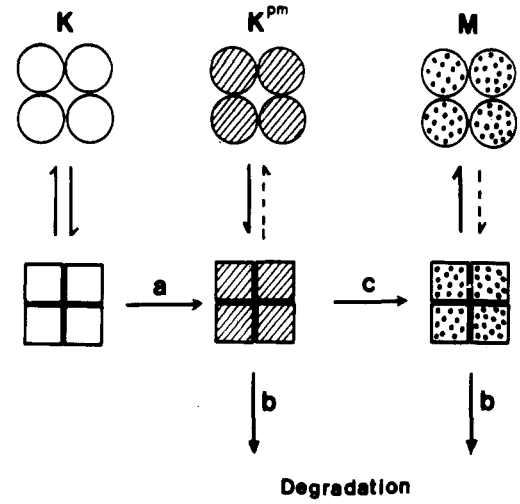

FIGURE 9: Proposed relationship between K, K^{pm}, and M subunits and their role in degradation. The squares (■) designate the T conformation while the circles designate the R conformation. The level of expression of carboxy terminal modifying enzyme c determines the proportion of M subunit formed. The modification causes the M isozyme to accept the R conformation which makes it less available for degradation by proteolytic enzyme b. Normal development would be accompanied by activation of the gene coding for enzyme c rather than by the concomitant repression and derepression of the genes coding for the K and M subunits. Enzyme c might also modify other proteins characteristically expressed in brain and muscle extracts. In this way several gene products could be modified by a single change in gene expression.

It is hypothesized that the K^{pm} subunit is a precursor to the M form and the metabolism of the K and K^{pm} subunits is branched, in order to explain these relationships. That is, in K isozyme expressing cells the K^{pm} subunit is further degraded, but in M isozyme expressing cells the carboxyl terminus is modified. Since the trypsin data suggest the carboxyl terminus of K^{pm} is liberated when K is cleaved and this change decreases the affinity for phosphoenolpyruvate, it is logical to further assume that blockage of the carboxyl terminus stabilizes the R conformation and bestows the distinctive kinetic properties of the M subunit. Since M isozyme expressing tissues have a high specific activity, it may be the M subunit is protected against degradation, particularly if both enzymes are translated by a common mRNA. That is, the transformation from K to M subunit via the K^{pm} product may inhibit the rate of degradation, thereby raising the steady-state level. The enzyme(s) of degradation would be likely to recognize the T conformation or even the dissociated forms (see p*I* values above). These proposed relationships are summarized in Figure 9.

The hypothesized conversion of K to M subunits via a K^{pm} intermediary explains the data obtained. However, the data available do not prove the proposed precursor–product relationship. Marie et al. (1976) claim to have observed such a conversion; that is, they observed that treatment with muscle extracts changed the p*I* value of purified K isozyme to that of the M form. Ironically, the data obtained in this study offer

an alternative explanation for this change in p*I* value, namely, a conversion from K to K^{pm}. Thus, the one-gene hypothesis still needs to be more vigorously tested.

The proposed precursor–product relationship also offers an alternative explanation to that of "leaky genes" for the observation that KM hybrids are found in almost all tissues whether they predominantly express the K_4 or the M_4 isozyme (Cardenas & Dyson, 1978). That is, presumed M subunits found in K_4-rich tissue extracts would be K^{pm} subunits, while K subunits found in M_4-rich tissues would represent unconverted precursor.

Whether the M form proves to be a product of the K-subunit gene or not, the presence of an endogenous enzyme system able to form K^{pm} and other degradative products has significance in its own right. For example, as discussed above, this phenomena can serve to explain the multiplicity of p*I* forms and the variability in physical properties reported by different investigators. The in vivo formation and/or accumulation of K^{pm} may also have physiological significance apart from the possible formation of the M subunit.

Similar site-specific cleavages have been reported for the L isozyme (Hall et al., 1979) and for phosphofructokinase (Kemp et al., 1980), indicating the phenomena may be a mechanism of general significance. In the case of the L isozyme the cleavage clearly serves as a functional degradative step regulating the specific activity. Thus, K^{pm} formation may be a rate-limiting step serving to maintain the steady-state level of the K isozyme.

Moreover, K^{pm}, recognized as the high-p*I* variant, accumulates in some tissue types, tumors in particular, and has been speculated to play a role in anaerobic glycolysis (Ibsen, 1977). Thus, in these tissues an imbalance between the endogenous rate of K^{pm} formation and removal exists and the accumulated K^{pm} may serve a role in metabolism.

Acknowledgments

We express our appreciation to Drs. George Gutman and Nigel Skipper for their help and advice concerning the preparation of the antibody columns, to Darlene Wise for her competent typing and patience, and to Vladimir Sturm for doing the amino acid analyses.

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