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Biochemistry

Isozymes of human phosphofructokinase: Identification and subunit structural characterization of a new system

(hemolytic anemia/myopathy/*in vitro* protein hybridization/column chromatography)

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ABSTRACT **The existence of a five-membered isozyme system for human phosphofructokinase (PFK; ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) has been demonstrated. These multimolecular forms result from the random polymerization of two distinct subunits, M (muscle type) and L (liver type), to form all possible tetrameters—i.e., M_4, M_3L, M_2L_2, ML_3, and L_4. Partially purified muscle and liver PFKs were hybridized by dissociation at low pH and then recombination at neutrality. Three hybrid species were generated in addition to the two parental isozymes, to yield an entire five-membered set. The various species could be consistently and reproducibly separated from one another by DEAE-Sephadex chromatography at pH 8.0 with a concave elution gradient of salt. Under similar experimental conditions, erythrocyte PFK from hemolysates was also resolved into five species chromatographically indistinguishable from those produced in the above experiment. Immunological and kinetic studies of the isozymes provided corroborative evidence to support the proposed subunit structures. Erythrocyte PFK was found to have kinetic properties intermediate between those of muscle and liver PFK and was neutralized only 50% by an antiserum against muscle PFK that completely neutralized muscle PFK. These data demonstrate that muscle and liver PFKs are distinct homotetramers—i.e., M_4 and L_4, respectively—whereas erythrocyte PFK is a heterogeneous mixture of all five isozymes. The structural heterogeneity of erythrocyte PFK provides a molecular genetic basis for the differential organ involvement observed in some inherited PFK deficiency states in which myopathy or hemolysis or both can occur.**

Like most allosteric enzymes, phosphofructokinase (PFK; ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), a key enzyme of glycolysis, is a multimeric protein, the smallest active oligomer being a tetramer. The enzyme from a large number of plant, bacterial, nonvertebrate, and vertebrate species has been purified and studied. The physicochemical and kinetic properties of PFKs from various mammalian tissues have been investigated, and in a few instances, multimolecular forms have been noted (1). At least four forms of PFK have been reported in tumors and normal tissues of rats on the basis of ion-exchange chromatography elution profiles and of antibody precipitation tests (2, 3). The extensive studies by Tsai and Kemp (4–7) have demonstrated the presence of a multilocus isozyme system for PFK in the rabbit. In this species, three separate genetic loci code for a structurally different polypeptide subunit of PFK; either one or two of these polypeptides are expressed in each tissue, resulting in the formation of either single (homotetrameric) or multiple (homo- and heterotetramertic) isozymes.

The existence of an isozyme system for PFK in man was first suggested in 1965 by the observation of a recessively inherited muscle disease associated with PFK deficiency in three siblings of a Japanese family (8). In these individuals, PFK activity was entirely absent in muscle and was half normal in erythrocytes. The clinical effects of the enzymatic defect consisted of generalized muscle weakness and compensated hemolysis. The differential tissue involvement led to the hypothesis that the erythrocyte isozyme is composed of two types of subunits, one of which is the sole subunit present in muscle PFK (9, 10). The proposed structural heterogeneity of erythrocyte PFK protein was supported by immunochemical neutralization experiments (11, 12). Karadsheh *et al.* (13) and Kaur and Layzer (14) have recently presented data to support the suggested hybrid structure for erythrocyte PFK.

The present study addressed the following three basic questions: (*i*) Could erythrocyte PFK be a mixture of all five theoretically possible tetramers composed of two distinct subunits, rather than a single hybrid species? (*ii*) What is the tissue of origin of the nonmuscle subunit identified as an "E" or "R" subunit (13, 14) by other workers? (*iii*) What are the relative proportions of these two subunits in the erythrocytes?

Preliminary results of these studies have been presented (15).

MATERIALS AND METHODS

Chemicals and Reagents. Adenosine nucleotides, NAD, NADH, Fru-6-*P*, Fru-1,6-P_2, dithiothreitol, glycylglycine, and sodium glycerophosphate were purchased from Sigma; auxiliary enzymes were from Boehringer Mannheim; Freund's complete and incomplete adjuvants were from Difco; DEAE-cellulose (DE-52) was from Whatman; DEAE-Sephadex A-25 was from Pharmacia; agarose–hexane–ATP (AGATP), type 2, was from P-L Biochemicals; Ionagar was from Colab Labs (Chicago Heights, IL); Nonidet was from Particle Data (Chicago, IL). Human muscle and liver specimens, obtained at surgery and autopsy, respectively, were stored frozen at −20°C until used for extraction. Venous blood was obtained from healthy volunteers.

Assays of PFK Activity. Assays were done with a Gilford model 2400 spectrophotometer at 26°C. The reaction mixture (1.0 ml) contained 50 mM tris(hydroxymethyl)methylglycine at pH 8.4, 5 mM $MgCl_2$, 0.15 mM NADH, 0.1 mM dithiothreitol, 0.25 mM ATP, 2.4 mM Fru-6-*P*, 0.18 unit of aldolase, 0.6 unit of triose phosphate isomerase, and 0.1 unit of glycerophosphate dehydrogenase. The reaction was started by addition of 0.1 ml of the enzyme preparation to 0.9 ml of the assay mixture. The decrease in absorbance at 340 nm was recorded for the next 15–20 min against a blank from which PFK or Fru-6-*P* was omitted. One unit of enzyme is defined as that amount of enzyme that converts 1 μmol of Fru-6-*P* to Fru-1,6-P_2 in 1 min in the above system.

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Abbreviations: PFK, ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11; $K_{0.5}$, concentration of the substrate required to achieve half-maximal velocity; M, muscle subunit; L, liver subunit.

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The kinetics of the PFK isozymes were studied at pH 7.2 in 50 mM Tris·HCl/1 mM EDTA/0.2 mM dithiothreitol. The concentrations of $MgCl_2$, NADH, and auxiliary enzymes were the same as described above. Ammonium sulfate was removed from the auxiliary enzymes by dialysis against Tris·HCl buffer at 4°C for 2 hr. All reactions were started by the addition of Fru-6-*P*. To determine the apparent concentration of Fru-6-*P* required to achieve half-maximal velocity ($K_{0.5}$) values, ATP was kept constant (0.1 mM) and Fru-6-*P* concentration was varied from 0.025 to 1.6 mM. V_{max} was measured by activation with 0.2 mM AMP. The $K_{0.5}$ Fru-6-*P* value for each isozyme was calculated from the Hill plots.

Preparation of PFKs from Muscle, Liver, and Erythrocytes. Muscle PFK was partially purified according to the method of Kemp (16) except that the crystallization steps were omitted. For the immunization of rabbits, this preparation was subjected to DEAE-cellulose chromatography (17) followed by affinity chromatography (18). The final preparation yielded a single band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Protein determination was according to Lowry *et al.* (19). Partial purification of liver PFK was carried out according to the method of Brock (20), and that of erythrocyte PFK was according to the procedure described by Wenzel *et al.* (21) except that, in both cases, the final gel filtration steps were omitted.

Chromatographic Separation of PFK Isozymes. All the operations were carried out at 4°C. The columns were loaded with 0.1–0.15 unit of the enzyme preparation. For linear gradient elution, a DEAE-Sephadex A-25 column (1.7 × 30 cm) was equilibrated in 0.1 M Tris phosphate, pH 8.0/0.2 mM EDTA/0.2 mM AMP/0.7 mM dithiothreitol. A 100-ml linear gradient of NaCl from 0 to 0.8 M prepared in the equilibrating buffer was used for elution; 100 fractions were collected. For concave gradient elution, the gel was equilibrated in the buffer mentioned above, to which 0.025 M NaCl was added. Elution was done with a 300-ml concave gradient of NaCl from 0.025 to 0.525 M in the same buffer; 100 fractions were collected.

Dissociation and Reassociation Experiments with PFKs from Muscle and Liver. These were performed according to the procedure described by Tsai and Kemp (4) with some modifications as described below. Equal amounts (in units) of partially purified muscle and liver PFKs were dissociated separately by dissolving them in small volumes of 0.025 M and 0.1 M glycylglycine/β-glycerophosphate buffers (pH 5.2), respectively, each containing 1 mM EDTA and 0.25 mM dithiothreitol. Muscle and liver PFK solutions were incubated at 26°C for 2 and 10 min, respectively. The duration of exposure to low pH and the strength of the buffer for each enzyme were determined by trial and error, so as to achieve >95% loss of activity, suggesting maximal dissociation. An aliquot of each solution was removed for assay, and equal volumes (one-half of the original volumes) were then mixed. Reassociation of both the mixture and the remaining original solutions was brought about by adjusting the pH to 7.2 with 0.5 M Tris phosphate at pH 8.5. The solutions were incubated at 26°C for another 10 min and assayed again. The reassociation of isozymes was indicated by the recovery of enzymatic activities. Independently reassociated muscle and liver PFKs served as controls. In a series of similar experiments, muscle and liver isozymes were hybridized in varying proportions (3:1 and 1:3).

Dissociation and Reassociation of Erythrocyte PFK. Partially purified erythrocyte PFK was subjected to dissociating and reassociating conditions as described above for liver PFK.

Preparation of Anti-Human Muscle PFK Antibody. For primary immunization, each of the five New Zealand White female rabbits received 250 μg of purified muscle PFK suspended in 0.5 ml of potassium phosphate, pH 8.0/0.2 mM EDTA/10 mM $(NH_4)_2SO_4$/0.1 mM ATP/0.1 mM dithiothreitol and emulsified with 0.5 ml of Freund's complete adjuvant; 0.25 ml was injected into each footpad (1 ml per rabbit). Two booster doses were given subcutaneously 2–3 weeks apart, containing the same amounts of antigen but in Freund's incomplete adjuvant. Test bleedings were done 4–6 weeks after the primary

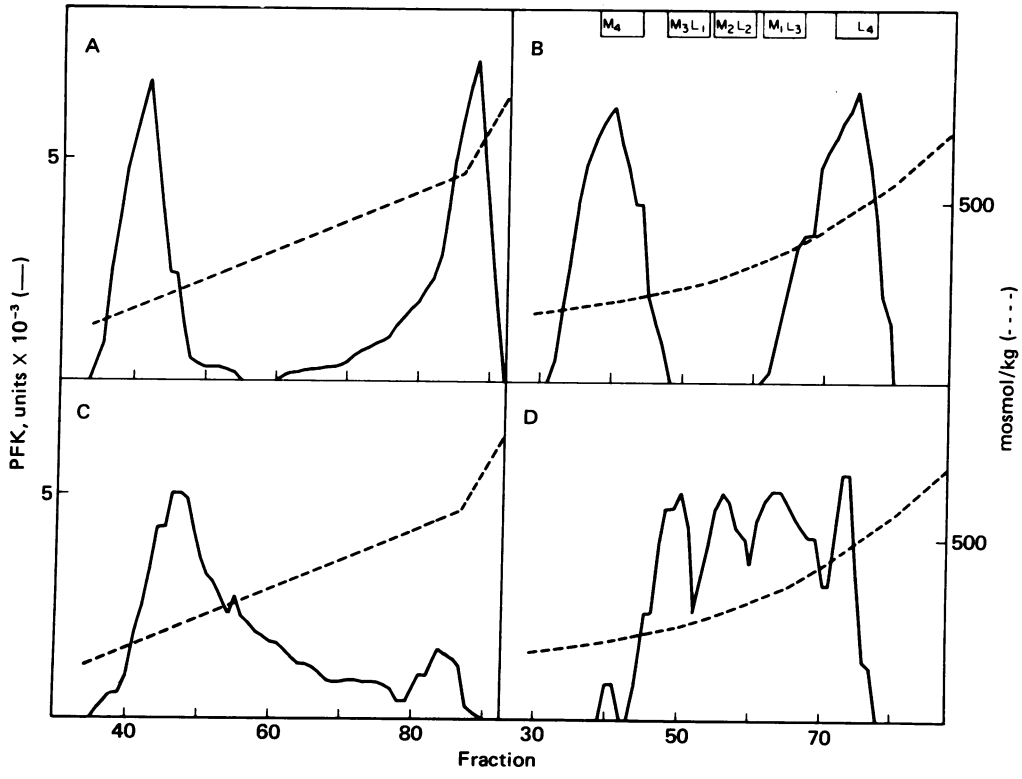

FIG. 1. Chromatographic separation of PFKs from muscle, liver, and erythrocytes. (*A* and *B*) Resolution of a mixture of muscle (M_4) and liver (L_4) isozymes with the linear and concave elution gradients, respectively. (*C* and *D*) Resolution of erythrocyte PFK with the linear and concave elution gradients, respectively.

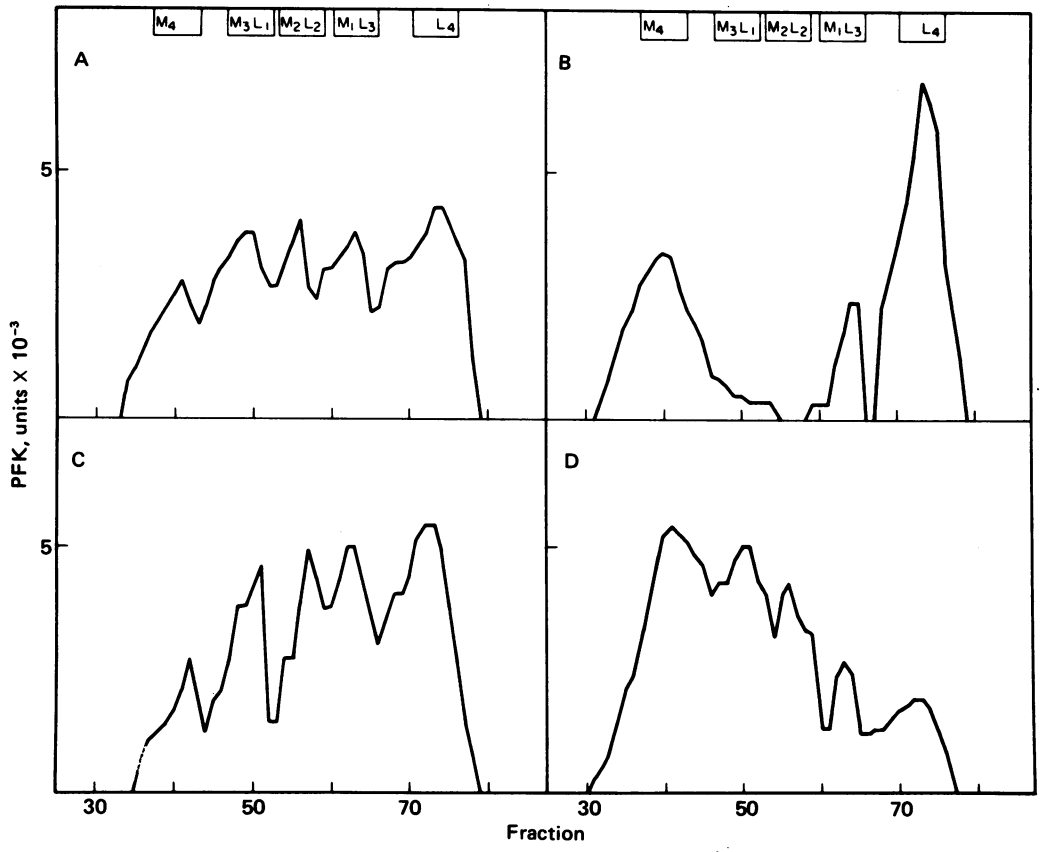

FIG. 2. Chromatographic resolution of dissociated and reassociated PFK isozymes. (*A*) Hybridized solution of muscle and liver PFKs in 1:1 ratio. (*B*) Mixture of control solutions of muscle and liver from *A*. (*C*) Dissociated and reassociated erythrocyte isozymes. (*D*) Hybridization of muscle and liver PFKs in 3:1 ratio.

immunizations and 1 week after the booster doses, and two animals that showed high antibody titer were bled further. Control sera were obtained by bleeding the animals prior to immunizations.

Immunologic Studies. Precipitation of enzyme with antiserum was carried out as follows. Partially purified PFK preparations were diluted with 50 mM Tris·HCl, pH 8.0/1 mM EDTA/1 mM ATP/1 mM ϵ-aminocaproic acid/1 mM dithiothreitol/0.2% bovine serum albumin in a final concentration of 0.2–0.6 unit/ml. In a series of test tubes, 0.3 ml of diluted enzyme was mixed with 0.1 ml of antibody diluted serially with control rabbit serum. The mixtures of enzymes and diluted antisera were incubated for 30 min at 37°C and then centrifuged for 20 min at 15,000 × *g*. The enzyme activity in the supernatant was assayed at pH 8.4. Ouchterlony analysis was carried out according to the method described by Tsai and Kemp (5) with minor modifications. Double-diffusion plates contained 0.8% agar in 50 mM Tris phosphate, pH 8.0/2 mM EDTA/0.1 mM ATP/0.1 mM $(NH_4)_2SO_4$/0.1 mM dithiothreitol/1% Nonidet P-40. The gel was prepared by heating 1 vol of 1% agar solution to boiling and then rapidly mixing in 0.25 vol of buffer containing all the reagents listed above but at 5 times the concentrations. The agar was immediately poured into petri dishes. Wells were punched and plates were used within 24 hr. The wells (15 μl) were filled and diffusion was carried out at 37°C for 24 hr.

RESULTS

Chromatographic Separation of Native PFKs on Linear Elution Gradients. Muscle and liver PFK each eluted as a single, sharp, symmetrical peak widely separated from the other (Fig. 1*A*). This technique, however, could not resolve muscle and erythrocyte PFKs distinctly from each other. Under identical experimental conditions, erythrocyte PFK was resolved into a broad major fraction with its peak in a position just overlapping the right descending limb of muscle PFK and a minor fraction with its peak in the position of the liver isozyme (Fig. 1*C*).

Muscle and liver preparations were recovered quantitatively (90 ± 10%) when either crude tissue homogenates or partially purified preparations were chromatographed. Crude erythrocyte hemolysates yielded quantitative recovery but partially purified ones did not. The poor recoveries (approximately 30%) of the latter may be explained by the tendency of human erythrocyte PFK to undergo self-aggregation to high molecular weight species, with a concomitant decline in specific activity, at high protein concentrations (22).

Chromatographic Separation of Native PFKs on Concave Elution Gradients. The broad major peak of erythrocyte PFK was resolved into four peaks when the early phase of the linear eluting gradient was expanded by using a concave gradient (Fig. 1*D*). The first and last peaks eluted in the same positions as muscle and liver isozymes, respectively (Fig. 1*B*).

The muscle- and liver-type species of native erythrocyte PFK could be quantitated with some degree of accuracy and were found to comprise 1% and 6–11%, respectively, of the total enzyme. However, the hybrids could not be quantitated, due to the overlap of their peaks. There were minor variations in the isozymic profile of erythrocyte PFK from one individual to another but in any given individual the profile was found to be consistent and reproducible.

Dissociation and Reassociation of PFKs from Muscle and Liver. Muscle and liver PFKs lost more than 95% of their activities after standing at pH 5.2 for variable lengths of time. On reassociation at pH 7.2, the muscle, liver, and hybridized solutions regained approximately 40, 60, and 50% of their activities, respectively. The loss of enzymatic activity is probably due to irreversible denaturation of the subunits at low pH. Five

isozymes were produced when hybridization was performed with equal amounts of muscle (M) and liver (L) enzymes (Fig. 2*A*). The three hybrid species produced *in vitro*, as well as the two parental isozymes, were eluted at the same positions in the gradient as the naturally occurring isozymes from erythrocytes. The selective loss of M subunits was reflected in the relative proportions of these five species. The chromatogram resembled an ascending cascade rather than a symmetrical binomial distribution. The control solutions, which were individually reassociated and mixed just prior to chromatography, showed the presence of two parental isozymes and a third very small peak in the position of ML_3 (Fig. 2*B*). This small peak, also obtained when the liver preparation alone was dissociated or reassociated, probably was derived from blood contamination or was indigenous to nonparenchymal cells (e.g., Kupffer cells) and fibrous stroma.

In a series of hybridization experiments, when the M:L ratio was increased from 1 to 1.5, a symmetrical binomial pattern was obtained (data not shown). Fig. 2*D* illustrates the descending cascade-like chromatogram when the M:L ratio was further increased to 3:1.

Dissociation and Reassociation of Erythrocyte Isozymes. Partially purified erythrocyte PFK lost more than 95% of its activity at low pH and regained approximately 50% at neutrality. The chromatographic profile of dissociated and reassociated erythrocyte PFK was nearly identical to that produced by the hybridization of muscle and liver PFKs in 1:1 proportion (Fig. 2*C*).

Immunologic Studies. In double-diffusion studies, muscle and erythrocyte PFK each yielded a single precipitin line, and there was a reaction of identity; liver isozyme failed to react altogether. An excess of antibody precipitated >90% of muscle PFK and approximately 50% of normal erythrocyte PFK but <5% of liver PFK.

Kinetic Studies. All three isozymes exhibited sigmoidal kinetics in the absence of an activator, with different affinities for Fru-6-*P*. The $K_{0.5}$ Fru-6-*P* value of the erythrocyte PFK (0.46 mM) was double that of the liver isozyme (0.24 mM) and half of that of the muscle isozyme (0.89 mM). Upon addition of AMP, the sigmoidal curves shifted to hyperbolic ones, showing classical substrate-saturation kinetics. The $K_{0.5}$ values in the presence of AMP were 0.11 mM for muscle PFK and 0.03 mM for liver PFK. The $K_{0.5}$ Fru-6-*P* of erythrocyte PFK (0.06 mM) was again intermediate between those of the other two enzymes.

DISCUSSION

Considerable metabolic diversity is observed in the regulation of carbohydrate metabolism in various tissues of a given species (23). Control of glycolysis is usually explained in terms of the allosteric properties of three enzymes: PFK, hexokinase, and pyruvate kinase. Generally, the PFK step is considered to be the major rate-limiting reaction (24, 25). The intracellular catalytic activities of the enzymes are profoundly influenced by various metabolites. Thus, diverse metabolic regulation in various tissues can be achieved by differences in effector concentrations or by the presence of tissue-specific isozymes with different physicochemical, kinetic, and regulatory properties. Our studies indicate that both of the above mechanisms are operative with respect to human PFK.

The original description of inherited muscle PFK deficiency—glycogen storage disease, type VIII (26)—in 1965 by Tarui *et al.* (8) prompted various investigators to examine the physicochemical and kinetic properties as well as the genetic control of this enzyme (27–32). The hypothesis of a common muscle-type subunit in both muscle and erythrocyte PFKs was initially suggested by immunochemical neutralization studies (11, 12). Muscle and erythrocyte enzymes were also shown to be separable by DEAE-cellulose chromatography and a stepwise elution gradient of salt (12). Recent studies have shown that human erythrocyte PFK is composed of two nonidentical subunits, of 85,000 and 80,000 daltons, respectively. The 85,000-dalton subunit is designated "muscle type" because it is found to be identical to the sole subunit present in muscle PFK on the basis of their tryptic peptide maps (13). The 80,000-dalton subunit is assumed to be specific for erythrocytes and is designated as "E (erythrocyte)" or "R (red cell)" subunit. The relative proportions of these two have been variably reported to be 1:2.9 (13) and 1.2 or less (14). However, none of these studies identified the nature of the nonmuscle subunit or addressed the question of why only a single heterotetrameric molecule would be assembled in erythrocytes, rather than the entire five-membered set, if two distinct subunits were present.

Our data demonstrate that the nonmuscle subunit of erythrocyte PFK is similar to the subunit present in liver PFK, the L type. With *in vitro* molecular hybridization experiments, human muscle and liver enzymes have been identified as distinct homotetramers (M_4 and L_4), whereas erythrocyte PFK has been shown to be a heterogeneous mixture of five isozymes (M_4, M_3L, M_2L_2, ML_3, and L_4), with the two subunits being present in approximately equal proportions.

We initially chose to determine whether human skeletal muscle and liver PFKs were truly distinct homotetrameric isozymes or not, as they are in the case of the rabbit (4, 33, 34). Although indistinguishable by electrophoresis, these two were readily resolved by DEAE-Sephadex chromatography. The resolution of erythrocyte PFK into two peaks suggested that the minor peak may be similar to the liver isozyme and the major one may consist of either one or many hybrid isozymes, composed of both muscle and liver type subunits.

When the broad peak of erythrocyte PFK was further resolved by using a concave gradient, it separated into four peaks. The first peak eluted in the position of muscle PFK, three others appeared in positions intermediate between muscle and liver isozymes, and the last peak was in the position of liver PFK. This finding appeared consistent with the presence in the erythrocyte of five different tetrameric isozymes, resulting from all the possible combinations of two distinct subunits (M and L): two parental homotetramers, M_4 and L_4, and three hybrids, M_3L, M_2L_2, and ML_3.

The hypothesis that these five chromatographically distinguishable isozymes were members of a single isozyme family was tested by the hybridization experiments, which exploit the general property of mammalian PFK to undergo reversible dissociation (1, 35). The dissociation of tetramers into dimers and monomers can be brought about by lowering the pH of the enzyme solution to below 5.8 and is accompanied by a loss of enzyme activity to 2–5% of the original. A subsequent increase in pH to above 7.0 leads to reassociation into tetramers with partial recovery of enzyme activity (33).

Dissociation and reassociation of human muscle and liver PFKs yielded three additional hybrid species, as well as the original homotetramers. In the control experiment, when muscle and liver PFKs were mixed after independent dissociation and reassociation, the two original homotetramers were obtained, along with a small peak in the ML_3 position. This ML_3 peak was due to muscle subunits contaminating the liver preparation.

These results were consistent with the concept that muscle and liver PFKs are different proteins with homotetrameric structures.

The random recombination of equal numbers of two distinct subunits (M and L) is expected to yield five isozymes in a 1:4:

6:4:1 ratio, as predicted by the binomial distribution, provided that both subunits exhibit similar intra- and interspecies affinities *in vitro*. The deviation from a binomial distribution of five peaks in our study reflects the excessive loss of M subunits during low pH treatment. In fact, during hybridization, the M:L ratio was increased to 1.5:1, and a symmetrical distribution was evident. A further excess of M or L subunits yielded isozyme profiles that were highly skewed toward M- or L-containing species, respectively. These data indicated that *in vitro*, interspecies and intraspecies affinities of each subunit were equal.

Partially purified erythrocyte PFK, after dissociation and reassociation, yielded an isozymic profile nearly identical to that produced by muscle and liver PFKs hybridized in equal proportions. This finding suggested that, in native erythrocyte PFK, the ratio of M to L subunits cannot be very different from unity. This conclusion is also supported by the clinical observation that patients genetically lacking M subunits exhibit half-normal levels of erythrocyte PFK (8, 9, 36).

Our immunological and kinetic studies provide further support to the proposed subunit structures of PFK isozymes. An excess of anti-human muscle PFK serum precipitated >90% of muscle PFK and approximately 50% of erythrocyte PFK, as shown by other investigators (11, 12). However, our experiments indicated that this antiserum failed to precipitate liver enzyme. In Ouchterlony analysis, muscle and liver PFK each yielded a single precipitin line which showed a reaction of identity, but liver PFK failed to react. The absence of cross-reactivity between muscle and liver PFK suggested the presence of large structural differences between M and L subunits. This is in agreement with the reported differences in the tryptic peptide maps and amino acid compositions between the two subunits of erythrocyte PFK (13) and supports our hypothesis that the nonmuscle subunit of erythrocyte PFK is similar to the liver isozyme. The erythrocyte isozymes as a group exhibited a $K_{0.5}$ Fru-6-*P* value (in the presence or absence of AMP) intermediate between those of muscle and liver PFKs.

From the evidence presented, we conclude that human muscle and liver PFKs are distinct homotetramers—i.e., M_4 and L_4—distinguishable by their physicochemical, kinetic, and immunological properties; erythrocyte PFK, until now considered a single hybrid isozyme, is a mixture of two homotetramers and three hybrid isozymes. Our studies are consistent with the existence of two structural loci coding for M and L subunits, which give rise to a five-membered isozyme system. In accordance with the recommendation of the International Union of Biochemistry Enzyme Commission (37), we propose that liver isozyme be referred to as PFK-1, the muscle isozyme as PFK-5, and the hybrids as PFK-2, -3, and -4.

The genetic heterogeneity of erythrocyte PFK may explain the reduction in the erythrocyte enzyme activity by approximately 50% observed in inherited PFK deficiency associated with myopathy. If this syndrome is due to complete lack of the muscle-type PFK, the residual erythrocyte enzyme is expected to be liver-type. We have recently demonstrated the exclusive presence of the liver-type isozyme in the erythrocytes of such an individual (36). It can be speculated that the heterogeneous group of hemolytic syndromes associated with partial erythrocyte PFK deficiency without myopathy (38–41) may be secondary to the total absence of L subunits or qualitative defects of M or L subunits.

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