Analysis of the phosphofructokinase subunits and isoenzymes in human tissues

George A. DUNAWAY,*† Thomas P. KASTEN,* Thomas SEBO* and Robert TRAPP†

Departments of *Pharmacology and †Medicine, Southern Illinois University School of Medicine, Springfield, IL 62794-9230, U.S.A.

The 6-phosphofructo-1-kinase (PFK) subunits and isoenzymes were studied in human muscle, heart, brain, liver, platelets, fibroblasts, erythrocytes, placenta and umbilical cord. In each tissue, the subunit types in the native isoenzymes were characterized by immunological titration with subunit-specific antibodies and by column chromatography on QAE (quaternary aminoethyl)-Sephadex. Further, the subunits of the partially purified native isoenzymes were resolved by SDS/polyacrylamide-gel electrophoresis, identified by immunoblotting, and quantified by scanning gel densitometry of silver-stained gels and immunoblots. Depending on the type of tissue, one to three subunits were detected. The M_r values of the L, M and C subunits regardless of tissue were $76\,700 \pm 1400$, $82\,500 \pm 1640$ and $86\,500 \pm 1620$. Of the tissues studied, only the muscle PFK isoenzymes exhibited one subunit, which was the M-type subunit. Of the other tissues studied, the PFK isoenzymes contained various amounts of all three subunits. Considering the properties of the native PFK isoenzymes, it is clear that, in human tissues, they are not simply various combinations of two or three homotetrameric isoenzymes, but complex mixtures of homotetramers and heterotetramers. The kinetic/regulatory properties of the various isoenzyme pools were found to be dependent on subunit composition.

INTRODUCTION

It is well known that PFK (EC 2.7.1.11) is a key regulatory enzyme which is exquisitely sensitive to the cellular microenvironment through complex responses to a variety of reversibly bound modulators. Further, PFK activity, as well as isoenzyme types, are affected by alterations of nutritional, hormonal, developmental and pathological states. Tissue-specific changes in PFK activity and isoenzymic content contribute significantly to the diversities of glycolytic and gluconeogenic rates which have been observed for different tissues (for review, see Dunaway, 1983).

In rats and rabbits, three distinct PFK subunit types have been directly demonstrated by SDS/PAGE (Foe & Kemp, 1984, 1985; Dunaway & Kasten, 1985a,b, 1986, 1987). Although not directly demonstrated by SDS/PAGE, three subunit types were predicted to occur in human tissues (Kahn *et al.*, 1979; Meienhofer *et al.*, 1979; Vora *et al.*, 1980). These deductions were correctly extrapolated from immunological, chromatographic and electrophoretic data. Further, the gene for each subunit in humans has been located on separate chromosomes. That is, the genes for the L-type, M-type and C-type subunits have been assigned to chromosomes 21, 1 and 10 respectively (Vora & Francke, 1981; Vora *et al.*, 1982; Weil *et al.*, 1980). In the present paper, L-type and M-type subunits refer to the subunits that were originally identified in adult muscle and liver. Although the third type of subunit has been referred to as the F-type, P-type or C-type subunit, we prefer to use 'C-type' subunit, which conforms with the nomenclature that has been used in previous studies of rabbit brain (Foe & Kemp,

1984, 1985) and rat tissues (Dunaway & Kaster, 1985a,b, 1986, 1987).

To summarize the earlier work with human PFK isoenzymes (Kahn *et al.*, 1979; Meienhofer *et al.*, 1979; Vora *et al.*, 1980), the consensus of opinion by these investigators is that only the M-type subunit is present in adult skeletal muscle and heart. On the basis of immunological evidence, it has been speculated that the M-type subunit is also the major type in brain, erythrocyte and testes (Meienhofer *et al.*, 1979; Cottreau *et al.*, 1979). The L-type subunit was thought to be the predominant form in liver, placenta, lymphocytes, platelets, erythrocytes, testes, kidney and hepatomas (Meierhofer *et al.*, 1979; Kahn *et al.*, 1979). By using an antibody prepared from brain PFK, which had been immunoabsorbed with liver and erythrocyte PFKs and which was assumed to be specific for the C-type subunit, it was found that this antibody neutralized most of the PFK activity from platelets, fibroblasts, kidneys and hepatomas and part of the PFK activity in brain and testes (Meienhofer *et al.*, 1979; Cottreau *et al.*, 1979). These data strongly suggest the existence of three subunit types in human tissues. However, estimation of subunit proportions which are based on immunological data is often unreliable and dependent on specificity and affinity. Antibody specificity for individual subunits can best be appreciated by immunoblotting, which none of the previous work employed. Also, data based on immunoprecipitation of enzyme activity may vary, since the affinity of each type of antibody for a specific subunit may not be sufficient to permit complete precipitation of heterotetramers containing only one or two of the specific subunits. Interpretation of chromatographic

Abbreviations used: PFK, 6-phosphofructo-1-kinase; QAE, quaternary aminoethyl; PAGE, polyacrylamide-gel electrophoresis.

† To whom correspondence and reprint requests should be addressed.

data is also extremely difficult for tissues where complex subunit hybridization exists, since the various isoenzymes may not be resolvable. In order to understand better the nature of the PFK isoenzymes in human tissues, we have examined their subunits by SDS/PAGE and by immunoblotting with subunit-specific antibodies. Contrary to earlier concepts, these data demonstrate that, with the exception of skeletal muscle, PFK isoenzymes in human tissues are complex mixtures of homotetramers and heterotetramers which apparently were formed by random subunit association. Also, an examination of the kinetic properties of the isoenzyme pools from different tissues suggests that the kinetic/regulatory properties of the isoenzyme pools in various tissues are dependent on subunit types and amounts.

EXPERIMENTAL

Tissues and organs

Heart, cerebral cortex and liver were obtained from a 19-year-old male approx. 6 h after death. Muscle tissue was obtained from a left-deltoid-muscle biopsy and processed within 15 min after removal. Although it was found that this patient suffered from McArdle disease, the PFK activity was normal. Erythrocytes and platelets, which were obtained from the Blood Bank of Central Illinois, were pooled from several individuals. Placenta and umbilical cord were obtained immediately after delivery and were processed within 15 min after complete expulsion. Human skin fibroblasts were obtained from the Skin Culture Laboratory, Division of Plastic Surgery. These cells were grown at 37 °C in Dulbecco's Modified Eagle's Medium with 10% (v/v) fetal-calf serum, 100 units of penicillin/ml and 100 µg of streptomycin/ml, and confluent cells were processed immediately after harvest.

Extraction, purification and activity measurement

Human tissues or cells were homogenized in 50 mM-Tris/phosphate buffer, pH 8.0, containing 25 mM-NaF, 1 mM-ATP, 10 mM-dithiothreitol, 0.1 mM-EDTA, 0.5 mM-phenylmethanesulphonyl fluoride, 1 mM-*p*-aminobenzamide, leupeptin (250 µg/l), 0.1 mM-tosyl-lysylchloromethane hydrochloride, bestatin (500 µg/l), phosphoramidon (1 mg/l), pepstatin (1 mg/l) and 0.1 mM-tosyl-phenylalanylchloromethane hydrochloride. Activity was assayed as previously described (Kasten *et al.*, 1983). Protein was determined by the method of Bradford, with bovine serum albumin as a standard (Read & Northcote, 1981). PFK supernatant fluids which were prepared from each tissue were directly chromatographed on Cibacron Blue F3GA gel as described by Kasten *et al.* (1983). Nearly complete recovery of activity was obtained, and the partially purified isoenzymes had specific activities as shown in the legend of Fig. 1. The partially purified isoenzymes were used for the SDS/PAGE and kinetic studies.

Immunological techniques

Antibodies to the rat PFK subunits were employed, since, as revealed by immunotitration and immunoblotting, they react specifically and quantitatively with human PFK subunits. To prepare antibodies specific for L-type and M-type subunits, rabbits were immunized with their respective homotetramers, L₄ or M₄, and each

immune IgG was prepared as previously described (Dunaway & Weber, 1974; Thrasher *et al.*, 1981). The antibody for C-type subunit was prepared from rabbits which were immunized with the brain PFK isoenzymes. The purified anti-(brain PFK) antibodies were subsequently absorbed with L₄ and M₄ subunits, and the anti-(L-type subunit) antibodies were absorbed with M₄ subunits and M-C hybrids which were isolated from purified brain PFK isoenzymes. The M-C hybrids from the brain PFK isoenzymes were exclusively eluted from QAE-Sephadex in the first 100 ml of the 150 mM-NaCl-containing buffer [see Dunaway & Kasten (1985a,b) and the next section for more information]. Immunotitrations with each type of antibody were conducted as previously outlined (Dunaway & Weber, 1974; Thrasher *et al.*, 1981).

Ion-exchange column chromatography

QAE-Sephadex was equilibrated with starting buffer, comprising 50 mM-Tris phosphate, pH 8.0, 25 mM-NaF, 1 mM-ATP, 0.1 mM-EDTA, 25 mM-(NH₄)₂SO₄ (ultra-pure; Schwartz/Mann), 0.1 mM-phenylmethanesulphonyl fluoride and 10 mM-dithiothreitol. For resolution of PFK isoenzymes, 10 units of total activity were introduced on to a 1.5 cm × 30 cm column. Activity was resolved by discontinuous-gradient elution, and 1 ml fractions were collected. For the step-gradient elution, the first elution step was 100 ml of starting buffer plus 150 mM-NaCl. The final elution employed 30 mM-NaCl in starting buffer and was completed after 100 ml.

SDS/PAGE

Electrophoresis on 6%-polyacrylamide slab gels was performed as described by Dunaway & Kasten (1985a,b, 1987). Slab gels were silver-stained by the method of Wray *et al.* (1981). The amount of subunit protein in each band was determined with a Zeineh soft-laser scanning densitometer (SL-504-XL; Biomed Instruments, Fullerton, CA, U.S.A.) which was interfaced with an Apple IIe computer. Data were analysed by using Zeineh electrophoresis software (Videophoresis I). Standard curves for the L and M subunits were developed by plotting the densitometric areas versus amount of L₄ and M₄ (50–600 ng). The PFK subunits in each tissue were identified by electrophoretic mobility with brain PFK as a standard, and each subunit was quantified by assuming equal intensity of absorption. The apparent *M_r* values of each subunit were determined by using the standards β-galactosidase (*M_r* 116 000), phosphorylase *b* (97 400), bovine serum albumin (66 000) and ovalbumin (45 000). With these standards, regression analysis was used to obtain a correlation coefficient for the *M_r* of each subunit.

Immunoblotting procedure

Transblotting was performed in a LKB-200S Transphor system. The gel was placed in contact with the nitrocellulose sheet, and protein was transferred at 35 V for 2 h at room temperature, by using 25 mM-Tris/HCl (pH 8.3)/192 mM-glycine/20% (v/v) methanol. After blotting, the nitrocellulose sheet was blocked by shaking overnight in 1% bovine serum albumin in 10 mM-Tris/HCl (pH 7.4)/0.9% NaCl/0.1 mM-EDTA. Subsequently, the blocked nitrocellulose sheet was washed with 10 mM-Tris/HCl (pH 7.4)/0.9% NaCl. Each type of antibody was diluted (1:250) in 10 mM-Tris/HCl

(pH 7.4)/0.9% NaCl and incubated with the blocked nitrocellulose sheet for 2 h at room temperature. The antibody-treated nitrocellulose sheet was washed and subsequently incubated for 30 min in 10 mM-Tris/HCl (pH 7.4)/0.9% NaCl/0.1% Triton X-100. The Triton-treated nitrocellulose sheet was washed in 10 mM-Tris/HCl (pH 7.4)/0.9% NaCl and then incubated for 1 h in affinity-purified goat anti-rabbit IgG-horseradish peroxidase conjugate which had been diluted as per the manufacturer's (Bio-Rad) directions. Then the nitrocellulose sheet was washed with 10 mM-Tris/HCl (pH 7.4)/0.9% NaCl/0.1% Triton X-100 and finally with 50 mM-Tris/HCl, pH 7.4. The detection process was initiated with a 'visualization' solution and stopped by rinsing in deionized water. To prepare the 'visualization' solution, 60 mg of 4-chloro-1-naphthol was dissolved in 10 ml of methanol, and this solution was added to 90 ml of 50 mM-Tris/HCl, pH 7.4. Before use, 20 μ l of 30% (v/v) H₂O₂ was added to the 100 ml of 'visualization' solution.

RESULTS AND DISCUSSION

The PFK isoenzymes in human tissues were characterized by immunotitration with rat subunit-specific antibodies, separation by QAE-Sephadex column

chromatography, and measurement of the proportion of each subunit after resolution by SDS/PAGE (Table 1 and Fig. 1). Each subunit was quantified from silver-stained gels, and the results are shown in Table 2. Also, subunit percentages were confirmed from immunoblots which had been developed with subunit-specific antibodies (Table 2). Since these percentages were not different from those from the silver-stained gels, the partially purified PFK isoenzyme preparations did not contain significant amounts of proteins which co-migrated with any of the PFK subunits. Consequently, the subunit amounts obtained from the silver-stained gels were valid measurements.

Electrophoretic resolution on slab gels indicated that the apparent M_r values for the L-type, M-type and C-type subunits were $76\,700 \pm 1400$, $82\,500 \pm 1600$ and $86\,500 \pm 1500$ respectively. These values were not significantly different from prior reports of the apparent M_r values of the human PFK subunits (Karadshah *et al.*, 1977; Cottreau *et al.*, 1979; Kahn *et al.*, 1980).

Since the PFK activity in skeletal muscle was only titrated by anti-M-subunit IgG and contained only the M-type subunit, it is a simple extrapolation to confirm that skeletal muscle contains only the homotetramer of the M-type subunit. However, interpretation of the PFK isoenzyme profiles in other tissues is not so easy. For

Fig. 1. PFK subunits in human tissue

PFK isoenzymes were partially purified from each tissue, treated with SDS, and subjected to SDS/PAGE on slab gels. Subunits were detected by silver staining and quantified by scanning gel densitometry. The specific activities of the PFK isoenzymes from muscle, heart ventricle (vent.), heart atria, brain, liver, platelet, placenta, erythrocytes (RBC) and fibroblasts (Fibro.) were 78, 64, 58, 109, 25, 40, 125, 25 and 30 units/mg of protein respectively.

Table 1. Characterization of PFK isoenzymes in different tissues by immunotitration with subunit-specific IgG and by stepwise elution from QAE-Sephadex

The supernatant fluids for each tissue were incubated with graded amounts of subunit-specific IgG for 2 h at 37 °C and then centrifuged. The amount of specific IgG and the maximum percentage titration of total activity were determined and recorded. Also, each supernatant fluid was introduced on to a QAE-Sephadex column, which was then washed sequentially with 100 ml of buffer containing 150 mM-NaCl and then with 100 ml of buffer containing 300 mM-NaCl. This method completely resolves mixtures of the homotetramer of the M (150 mM-NaCl) and L subunits (Dunaway & Kasten, 1985a,b).

Tissue	Subunit-specific IgG...	Titration of activity (%)			Activity eluted from QAE-Sephadex (%)	
		Anti-L	Anti-M	Anti-C	150 mM-NaCl	300 mM-NaCl
Skeletal muscle		0	100	0	100	0
Heart ventricle		5	95	12	98	2
Heart atria		12	88	25	91	9
Cerebral cortex		13	89	50	71	29
Liver		90	48	30	15	85
Fibroblast		25	17	95	15	85
Erythrocyte		70	70	16	29	71
Platelet		62	10	76	10	90
Placenta		96	12	24	5	95
Umbilical cord		83	15	60	7	93

Table 2. Quantification of the PFK subunits in each tissue

The percentages of each subunit were determined from silver-stained polyacrylamide gels and from transblots which were developed with subunit-specific antibodies. The silver-stained gels shown in Fig. 1 were scanned, and the percentages of each subunit in each tissue determined as described in the Experimental section. Polyacrylamide gels which were identical to those shown in Fig. 1 were transblotted, and subunits visualized as described in the Experimental section. By scanning of the developed transblots, the absorbance of each subunit, which was detected by the appropriate subunit-specific antibodies, was measured. The percentages of each subunit were calculated by dividing the absorbance for each subunit by the total absorbance and multiplying by 100; ND, not detected. The erythrocytes and platelets were pooled from more than one individual, whereas the other tissues were from only one source (see the Experimental section). At least five separate determinations for each tissue were made, and the results are expressed as means $\pm$ S.D.

Tissue	Subunit ...	L-type		M-type		C-type	
		Stain (%)	Blot (%)	Stain (%)	Blot (%)	Stain (%)	Blot (%)
Skeletal muscle		ND	ND	100	100.0	ND	ND
Heart ventricle		3 $\pm$ 0.5	2.5	91 $\pm$ 7	93.0	7 $\pm$ 2	4.5
Heart atria		14 $\pm$ 2	15.9	76 $\pm$ 3	77.7	10 $\pm$ 2	6.4
Cerebral cortex		15 $\pm$ 2	14.2	55 $\pm$ 3	58.4	30 $\pm$ 4	27.4
Liver		62 $\pm$ 5	61.6	29 $\pm$ 3	28.9	10 $\pm$ 2	9.5
Fibroblast		21 $\pm$ 2	21.8	16 $\pm$ 3	17.0	63 $\pm$ 6	61.2
Erythrocyte		54 $\pm$ 4	56.0	43 $\pm$ 3	40.3	5 $\pm$ 1	3.7
Platelet		42 $\pm$ 3	39.8	19 $\pm$ 3	22.5	38 $\pm$ 2	37.7
Placenta		81 $\pm$ 6	83.0	12 $\pm$ 2	10.5	8 $\pm$ 2	6.5
Umbilical cord		57 $\pm$ 4	60.0	16 $\pm$ 2	10.0	27 $\pm$ 3	30.0

example, PFK activity from the other tissues was titrated to various degrees of each type of subunit-specific antibodies, and all three types of subunits were expressed, and exhibited a wide range of relative amounts (see Tables 1 and 2). It is clear that in these tissues heterotetramers are present and that the relative amount of each type is dependent on the relative subunit amounts. In the heart atria and ventricles as well as the cerebral cortex, the extent of titration of the PFK total activity by the anti-M-subunit antibodies and the elution of considerable activity by the 150 mM-NaCl wash suggested that the predominant isoenzymes were those rich in this subunit (Table 1). As shown in Fig. 1 and Table 2, the M-type subunit was the predominant subunit in these tissues. It has been previously thought that, in addition to skeletal muscle, adult human brain and heart contained primarily M_4 (for review see Dunaway, 1983). The present data indicate that indeed M_4 is the only isoenzyme in adult muscle and the primary isoenzyme in heart atria and ventricles. However, in the human heart atria, as in rat heart atria (Dunaway & Kasten, 1985b), large amounts of M-rich hybrids also exist and could convey different regulatory properties to the PFK-catalysed reaction. Also, it is clear that PFK isoenzymes in the human cerebral cortex contained a respectable percentage (30%) of C-type subunits and consequently significant amounts of the M-C hybrids such as those found in rat and rabbit brain (Dunaway & Kasten, 1985a; Foe & Kemp, 1984, 1985).

In liver, placenta and umbilical cord, immunotitration with anti-L-subunit antibody caused a loss of most of the PFK activity in these tissues, and SDS/PAGE revealed that the L-type subunit was the major type in these tissues. Earlier immunological studies indicated that L_4 is the predominant human liver PFK isoenzyme (Kahn *et al.*, 1979). However, we have shown that 62% and 29% of the subunits in the liver isoenzymes are L-type

and M-type respectively, and that 90% and 48% of the total activity was precipitated by anti-L IgG and by anti-M IgG respectively. Consequently, we would predict that significant amounts of the L-M hybrids are also present in the human liver. Similar conclusions have been reached for rat liver PFK isoenzymes (Dunaway & Kasten, 1987). Interestingly, a higher proportion of L-type subunit was found in the placenta than in the liver.

PFK isoenzymes from fibroblasts and platelets contained the highest proportion of C-type subunit, and the isoenzymes from brain and umbilical cord exhibited a significant percentage of this subunit. As expected, total PFK activity from these tissues reacted strongly with the antibody that was specific for the C-type subunit. Also, the platelet PFK isoenzymes consisted of nearly equal amounts of the L-type (42%) and C-type (38%) subunits, as well as a lesser amount of M-type subunit (20%). The fibroblast PFK isoenzymes contained approximately equal amounts of L-type (21%) and M-type (16%) subunits, and, of the tissues examined, the greatest proportion of C-type subunit (63%). These data extend previous reports that fibroblasts and platelets contain PFK isoenzymes with high proportion of the C-type subunits (Meienhofer *et al.*, 1979; Kahn *et al.*, 1979, 1980; Vora *et al.*, 1980; Davidson *et al.*, 1983). Previous analysis by SDS/PAGE of PFK isoenzymes in platelets suggested that only the C-type and L-type subunits were present (Kahn *et al.*, 1980). As shown in Fig. 1 and Table 2, these subunits are the major types in platelets, but the M-type subunit is also expressed to a significant extent. Apparently, the immunological and chromatographic techniques that were previously used were not sensitive enough to detect the M-type subunit in these tissues (Kahn *et al.*, 1980; Vora, 1981).

Erythrocytes contained predominantly L-type (54%) and M-type (43%) subunits, but also contained detectable amounts of C-type subunit (3%). Also, the M-type

subunit appears to be composed of two partially resolvable forms, since both react after transblotting only with the anti-(M-type subunit) antibodies. It is likely that the lower- M_r M-type subunit was produced by proteolysis during storage of the erythrocytes. As would be expected, most of the total PFK activity in erythrocytes was susceptible to titration by either anti-L or anti-M antibodies and was very refractory to titration by anti-C antibodies. The PFK isoenzymes of the human erythrocytes have received considerable attention, since approx. 50% of the total PFK is lost in patients with muscle PFK deficiency (Tarui *et al.*, 1969). In human erythrocytes, at least two subunits (L and M) were detected by SDS/PAGE (Kaur & Layzer, 1977; Karadsheh *et al.*, 1977). As measured by scanning gel densitometry, the L:M ratio was reported to be 2:1 or less by Kaur & Layzer (1977), who used 5%-acrylamide tube gels, and 2.9:1 by Karadsheh *et al.*, 1977, who used 4.5%-acrylamide tube gels. Our results indicated that the C-type subunit was found in low amounts (5%) and that the proportion of L-type subunit was only 25% greater than for the M-type subunit. The greater amounts of the L-type subunits reported in the previous studies may have been the result of co-migrating proteins, such as M-type or C-type subunits that have undergone proteolysis. Immunoblotting, as we have done, would have demonstrated in the early work the presence of co-migrating altered M-type or C-type subunits. When proteolytic inhibitors were not used, we have found proteolysed PFK subunits (Dunaway & Kasten, 1987).

The present data extend the previous studies of PFK isoenzymes in human tissues, which were described in the Introduction, by presenting the relative PFK subunit composition of the isoenzyme pool in each tissue, which provides better insight into the actual types of isoenzymes that are present. It is clear that a more complete understanding of the PFK isoenzymes in different tissues, regardless of the source, requires a knowledge of the subunits that are present in the isoenzyme pool, and that

one cannot rely solely on immunological, chromatographic and electrophoretic analyses of the native isoenzymes. As shown previously with the PFK isoenzymes in rat tissues, the PFK isoenzyme pools in human tissues can be very complex. Also, the PFK isoenzyme pools in human skeletal muscle, heart ventricles and atria, cerebral cortex and liver are qualitatively similar to the pools in comparable rat tissues (Dunaway & Kasten, 1987).

In order to appreciate better the physiological roles of the various PFK subunits, the kinetic/regulatory properties of the PFK isoenzymes which exhibited the greatest variation of subunit composition were studied. For example, we have studied the regulatory properties of the PFK isoenzymes from skeletal muscle, which contain only the M-type subunit, from placenta, which is predominantly L-type subunit, and from platelets as well as fibroblasts, which exhibit the highest contents of C-type subunit. The kinetic/regulatory properties of the PFK isoenzymes are complicated, and can be graphically represented in many ways. In order to present the most relevant material, we have selected the fructose 6-phosphate saturation curves with (Fig. 2*b*) and without (Fig. 2*a*) fructose 2,6-bisphosphate over the range of maximum change in saturation. Also, the ATP-inhibition curves were performed at an approximately physiological concentration of fructose 6-phosphate (0.1 mM) as well as in the absence (Fig. 3*a*) or presence (Fig. 3*b*) of fructose 2,6-bisphosphate (1.0 μ M). In the absence of activators or in the presence of 1.0 mM-AMP (results not shown), very little PFK activity could be detected at fructose 6-phosphate concentrations less than 1.0 mM or at ATP concentrations greater than 0.5 mM. However, by the addition of fructose 2,6-bisphosphate, significant activity can be detected at concentrations of fructose 6-phosphate less than 1.0 mM and of ATP greater than 0.5 mM. As shown in Fig. 2(*a*), the homotetramer of the M-type subunit (M_4) had the greatest affinity for fructose 6-phosphate (apparent $K_m = 2.0$ mM), and the L-rich isoenzymes from placenta have a decreased affinity for

Fig. 2. Effect of subunit composition on fructose 6-phosphate saturation of PFK

Methods are as essentially described by Dunaway & Kastseim (1985*a,b*), except that the assay enzymes were exhaustively dialysed. In (a) and (b) the concentration of MgATP was 1.0 mM, and in (b) that of fructose 2,6-bisphosphate was 1.0 μ M. Subunit integrity was confirmed by SDS/PAGE. The same partially purified PFK isoenzymes as shown in Fig. 1 were used. The isoenzyme pools from muscle, placenta, platelets and fibroblasts are represented by $\circ$, $\bullet$, $\diamond$ and $\blacklozenge$ respectively. Activity was expressed as a percentage of maximal velocity, which was measured at 1.0 mM-MgATP, 10 mM-fructose 6-phosphate and 1.0 μ M-fructose 2,6-bisphosphate.

Fig. 3. Effect of subunit composition on ATP inhibition of PFK

Methods were essentially as described by Dunaway & Kasten (1985a,b) except that assay enzymes were exhaustively dialysed. In (b) the concentration of fructose 2,6-bisphosphate was 1.0 μ M. For (a) and (b), the concentration of fructose 6-phosphate was 0.1 mM and the concentration of $MgCl_2$ was 1.0 mM at concentrations of ATP less than 1.0 mM, and, at concentrations of ATP greater than 1.0 mM, the concentrations of $MgCl_2$ and ATP were the same. Subunit integrity was confirmed by SDS/PAGE. The same partially purified PFK isoenzymes as shown in Fig. 1 were used. The isoenzyme pools from muscle, placenta, platelets and fibroblasts are represented by $\circ$, $\bullet$, $\diamond$ and $\blacklozenge$ respectively. Activity is expressed as a percentage of maximal velocity, which was measured at 1.0 mM-MgATP, 10 mM-fructose 6-phosphate and 1.0 μ M-fructose 2,6-bisphosphate.

fructose 6-phosphate (apparent $K_m = 5$ mM). Also, the presence of high amounts of C-type subunit, such as those found in platelets (40%) and fibroblasts (63%), promoted even lower affinities for fructose 6-phosphate (apparent $K_m = 3.5$ mM and 4.0 mM respectively). For all of these isoenzymes, fructose 2,6-bisphosphate was required to obtain full activity; and, for human PFK, fructose 2,6-bisphosphate acted as a K-type and perhaps a weak V-type activator. The presence of 1.0 μ M-fructose 2,6-bisphosphate strongly increased affinities for fructose 6-phosphate in all of the isoenzyme pools examined. Fructose 2,6-bisphosphate caused M_4 and the L-rich isoenzymes from placenta to exhibit essentially identical fructose 6-phosphate saturation curves and apparent K_m values of 0.07 mM (Fig. 2b). In contrast, the presence of C-type subunits imposed in a roughly proportional manner lower affinities for fructose 6-phosphate than was observed for the L-type and M-type subunits. That is, in the presence of 1.0 μ M-fructose 2,6-bisphosphate, the apparent K_m values for the platelet and fibroblast isoenzyme pools are 0.35 mM and 0.55 mM respectively. These data indicate that introduction of the C-type subunit in PFK isoenzyme pools leads to a depressed affinity for fructose 6-phosphate and that this decreased affinity is related to the proportion of C-type subunit. The effects of excess ATP on the isoenzyme pools from muscle, placenta, platelets and fibroblasts in the absence of activators (Fig. 3a) and in the presence of 1.0 μ M-fructose 2,6-bisphosphate (Fig. 3b) were also examined. Fig. 3(a) shows that, in the absence of fructose 2,6-bisphosphate, M_4 was the least susceptible to ATP inhibition and that the L-rich isoenzymes were less sensitive to the ATP inhibition than were the isoenzyme pools from platelets and fibroblasts. This same trend was still exhibited in the presence of fructose 2,6-bisphosphate, although this sugar was a very potent antagonist of ATP inhibition for all the isoenzyme pools. Regardless of the presence of fructose 2,6-bisphosphate, ATP inhibition is more pronounced for the isoenzyme pools containing C-

type subunits, and the severity of this inhibition increases as the proportion of C-type subunit increases. These data suggest that the enhanced sensitivity to inhibitory concentrations of ATP is an intrinsic property of this subunit. It has been previously reported that the PFK activity from muscle was more resistant to ATP inhibition than was PFK activity from placenta (Meienhofer *et al.*, 1972). Also, PFK activities from platelets and fibroblasts were found to be similarly affected by ATP but to be more sensitive to ATP inhibition than were the isoenzymes from muscles and placenta (Kahn *et al.*, 1979). It is suggested that the physiological role of the C-type subunit in isoenzyme pools is to promote decreased affinity for fructose 6-phosphate and increased sensitivity to intercellular concentrations of ATP, and that the degree of these effects is related to the proportion of C-type subunit. Compared with other isoenzyme types, this intrinsic property could exert a dampening influence on glycolysis which protects the tissue from excessive lactate accumulation, or perhaps could allow other tissues which are relatively more important for immediate survival to compete more effectively for glucose.

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