Nature of the subunits of the 6-phosphofructo-1-kinase isoenzymes from rat tissues

George A. DUNAWAY* and Thomas P. KASTEN

Department of Pharmacology, Southern Illinois University, School of Medicine, Springfield, IL 62708, U.S.A.

The nature of the PFK (6-phosphofructo-1-kinase) isoenzymes in many rat tissues was examined by immunological and chromatographic techniques and by measurement of their subunit compositions. It was revealed that, except for diaphragm and skeletal muscle, these complex isoenzymic populations contained different amounts of the three subunit types and were nearly tissue-specific. Apparently this tissue specificity is due to different concentrations of the tetramers, which in turn are controlled by the types and amounts of each subunit that are available to associate randomly.

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 in nutritional, hormonal, developmental and pathological states. Tissue-specific changes in PFK activity and the nature of the PFK isoenzyme pool contribute significantly to the diversities of glycolytic and gluconeogenic rates which have been observed in different tissues (for review see Dunaway, 1983).

Three distinct subunit types have been indicated in mammalian tissues (Tsai & Kemp, 1973; González *et al.*, 1975; Kahn *et al.*, 1979; Meienhofer *et al.*, 1979; Vora *et al.*, 1980; Foe & Kemp, 1984; Dunaway & Kasten, 1985*a,b*, 1986). The nature of the PFK isoenzymes in mammalian tissues has been classically examined by kinetic, immunological, chromatographic and/or electrophoretic techniques (for review see Dunaway, 1983). These types of studies clearly indicated the complexity of the PFK isoenzyme pools, but the subunit composition of the native isoenzyme pools is potentially subject to misinterpretation unless directly measured. More recent studies have remedied this problem by employing the SDS/polyacrylamide-gel electrophoresis method of Laemmli (1970) to evaluate subunit compositions directly and consequently to appreciate better the nature of the native PFK isoenzyme pools (Foe & Kemp, 1984; Dunaway & Kasten, 1985*a,b*). We have employed this technique to study the PFK isoenzymes in liver, muscle, heart and brain from different rat strains; and, although strain differences were not noted, it was found that in each tissue the pool of PFK isoenzymes was composed of differing amounts of the three types of subunits (Dunaway & Kasten, 1986). In the studies reported herein, we extend these studies to other rat tissues and suggest a uniform explanation for the different properties and/or types of PFK isoenzymes which have been reported in various rat tissues.

EXPERIMENTAL

Wistar rats were maintained as previously described (Dunaway & Kasten, 1986). After decapitation and exsanguination, each of the tissues or organs was rapidly removed and homogenized in 50 mM-Tris/phosphate, 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-tosylphenylalanylchloromethane hydrochloride. PFK activity was assayed as previously described (Kasten *et al.*, 1983).

The immunological techniques, immunoblotting procedures and SDS/polyacrylamide-gel electrophoresis methodologies were conducted as previously described (Dunaway & Weber, 1974; Thrasher *et al.*, 1981; Dunaway & Kasten, 1986).

Determination of subunit contents

PFK-containing 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 of approx. 50 units/mg of protein. The partially purified isoenzymes (0.2 unit/gel track) were subjected to electrophoresis on 6% -acrylamide slab gels and silver-stained as described by Dunaway & Kasten (1985*a,b*). The PFK subunits for each tissue were tentatively identified by their electrophoretic mobilities, with brain PFK subunits as standards, and each subunit was quantified by assuming equal intensity of absorption. Also, subunit identity was confirmed by immunoblotting with subunit-specific antibodies. 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 with Zeineh electrophoresis software (Videophoresis I).

Abbreviations used: PFK, 6-phosphofructo-1-kinase; QAE, quaternary aminoethyl.

* To whom reprint requests should be addressed.

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

Total PFK activity in supernatant fluid was assayed as described by Dunaway & Kasten (1986). 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 below. Also, each supernatant fluid was loaded on a QAE-Sephadex column, which was then washed sequentially with 100 ml of buffer containing 150 mM-NaCl and then 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	Total PFK activity (units/g)	Loss of PFK activity (%) with:			Percentage of PFK activity eluted from QAE-Sephadex by:	
		Anti-L IgG	Anti-M IgG	Anti-C IgG	150 mM-NaCl	300 mM-NaCl
Skeletal muscle	80.2	0	99	0	100	0
Diaphragm	80.7	0	99	0	100	0
Brain	25.6	20	76	90	60	40
Heart	23.5	10	95	18	95	5
Thyroid	9.7	62	38	77	28	72
Intestinal muscle	9.2	50	40	91	19	81
Testis	10.8	75	2	92	4	96
Intestinal mucosa	6.3	80	4	94	5	95
Spleen	6.3	67	16	83	9	91
Kidney	5.5	52	50	74	21	79
Lung	5.2	68	57	77	23	77
Liver	4.0	88	20	6	10	90
Adipocyte	0.5	50	70	28	50	50

Standard curves for the L and M subunits were developed by plotting the densitometric areas versus the amounts of L_4 and M_4 . The linear range was found to be 50–600 ng. The subunit amounts were converted into pmol/g by assuming M_r values of the L, M and C subunits to be 80000, 85000 and 87500.

RESULTS AND DISCUSSION

The PFK isoenzymes in many different types of organs and tissues were examined by immunotitration with subunit-specific antibodies and by column chromatography on QAE-Sephadex with step-gradient elution. In the 150 mM-NaCl wash, M_4 and M-rich hybrids were eluted, whereas the more anionic hybrids were eluted by 300 mM-NaCl (Dunaway & Kasten, 1985a,b). The rationale for using these methods has been previously justified (Dunaway & Kasten, 1986). The results reported for muscle, liver, heart and brain essentially confirm our earlier work (Dunaway & Kasten, 1986), but are included so that all tissues examined can be directly compared.

As shown in Table 1, PFK activities in skeletal muscle and diaphragm were only affected by anti-M-subunit IgG and were exclusively eluted from QAE-Sephadex in the 150 mM-NaCl wash. Collectively, these data imply that only M_4 is present in these tissues. In contrast, the PFK activity in the other tissues or organs was titrated to various degrees by each type of subunit-specific IgG and exhibited a variety of elution patterns from QAE-Sephadex. These data provide insight into the nature of the native isoenzymes in each tissues and suggest that the brain isoenzymes were primarily M–C hybrids, that the heart isoenzymes were largely the M-rich forms, that the isoenzymes in testis, intestinal mucosa

and spleen were primarily L–C hybrids, that the tetramers containing the L-type subunit were the major forms in liver, and that in thyroid, intestinal muscle, kidney, lung and adipocytes extensive and complex hybridization of the three types of subunits had occurred. Clearly, the isoenzyme pools in most tissues, except for skeletal muscle and diaphragm, exhibited complex, diverse and nearly characteristic patterns.

With partially purified isoenzymes, the subunits in each tissue were resolved by SDS/polyacrylamide-gel electrophoresis (Fig. 1) and identified both by comparison with the brain standard and from immunoblots of gels which were identical with those shown in Fig. 1. These data confirmed that only the M-type subunit and, consequently, M_4 was present in skeletal muscle and diaphragm. However, the PFK isoenzymes in other tissues and organs exhibited various contents of two or three subunits. If proteolytic antagonists were used, the apparent M_r values of each type of subunit in each tissue, except intestinal mucosa, were very similar, 80000 ± 500 , 85000 ± 500 and 87500 ± 600 respectively for the L, M and C subunits. With respect to the standard curve, the linear regression coefficients for each subunit M_r were no less than 0.995. Further, for all tissues, except intestinal mucosa, no proteins other than those which migrated identically with the standard subunits reacted detectably, as measured by immunoblotting with corresponding subunit-specific antibodies. Characterization of the subunits from intestinal mucosa by immunoblotting (see Fig. 2) revealed that the two subunits, of M_r 82000 and 86000, reacted exclusively with the anti-(C-type subunit) antibodies. As shown by scanning of the silver-stained gel, the two C-type subunits produced 53% of the total absorption. Individually, the higher- and lower- M_r forms were 22% and 31% of the total absorption respectively.

Fig. 1. Resolution of subunits in each tissue

The 6%-acrylamide gels were developed by silver staining. For more details see the Experimental section.

Fig. 2. Characterization of the subunits by immunoblotting

The silver-stained gels are shown on the left, and Anti-L, Anti-M and Anti-C indicate the type of antibody used to detect each immunoblotted subunit. MUC, MUS and BR indicate isoenzymes isolated from intestinal mucosa, intestinal muscle and brain respectively.

Interestingly, nearly equal proportions were obtained by immunoblotting. Assuming that maximum silver staining was obtained, this suggests that the higher- M_r form reacts better with the anti-(C subunit) antibodies. Also, another subunit, of M_r 76 500, reacted specifically with the L-type-subunit antibodies; and no protein from the intestinal-mucosa extract could be detected with anti-(M-type subunit) antibodies, although traces of M-type subunit were suggested by immunotitration with anti-(M-type subunit) antibodies. As revealed by immunoblotting, omission of proteolytic-enzyme inhibitors during purification of PFK isoenzymes can lead to the formation of a spectrum of immunoreactive proteins exhibiting decreased M_r values (G. A. Dunaway & T. P. Kasten, unpublished work). However, the lower M_r values of the subunits found in the isoenzymes from mucosal cells of the small intestine cannot be so readily explained. That is, the proteolytic inhibitors that were used blocked decrease in the M_r of PFK subunits from all other tissues studied, including the intestinal muscle from which the mucosal cells were isolated; and, in the intestinal-mucosal cells, only the lower- M_r forms of each subunit and none of the native subunits were detected. It is possible that in this tissue the subunits with the lower M_r value could exist *in vivo*. However, it cannot be eliminated that a proteolytic enzyme in extracts of intestinal-mucosal cells was not completely antagonized by the inhibitors that were employed, and that the altered subunits were exclusively formed. Although the

lower- M_r forms of the C-type subunit were previously unknown, this is not the first report of the detection of altered L-type subunits. It has been reported that the M_r of the L-type subunit from human leucocytes was decreased by 2000 compared with the same subunit from human liver or red blood cells, which had an apparent M_r of 74 000–75 000 (LaGrange *et al.*, 1981). Also, comparable M_r losses have been obtained after limited proteolysis *in vitro* of PFK subunits from rabbit skeletal muscle and yeast (Taucher *et al.*, 1975; Kemp & Foe, 1983).

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 contents obtained from the silver-stained gels were valid measurements.

The subunits in each tissue vary with respect to the types expressed and amount(s) present in the PFK isoenzyme pools. For example, the skeletal muscle and diaphragm exhibited only the M-type subunit and the highest concentrations of this subunit, or for that matter any type of subunit, in any of the other isoenzyme pools. Interestingly, the amount of the M-type subunit in

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 identical with those shown in Fig. 1 were transblotted, and subunits were detected 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 indicates not detected. At least five separate preparations for each tissue were processed, and the results are expressed as means $\pm$ S.D.

Tissue	L-type subunit			M-type subunit			C-type subunit		
	Silver-stained gel		Immuno-blot (%)	Silver-stained gel		Immuno-blot (%)	Silver-stained gel		Immuno-blot (%)
	(pmol/g)	(%)		(pmol/g)	(%)		(pmol/g)	(%)	
Skeletal muscle	ND	ND	ND	3819 $\pm$ 200	100	100	ND	ND	ND
Diaphragm	ND	ND	ND	3843 $\pm$ 200	100	100	ND	ND	ND
Brain	158 $\pm$ 16	13	16	597 $\pm$ 60	49	46	463 $\pm$ 47	38	38
Heart	123 $\pm$ 13	11	10	850 $\pm$ 80	76	79	145 $\pm$ 15	13	11
Thyroid	185 $\pm$ 20	40	37	134 $\pm$ 12	29	30	143 $\pm$ 14	31	33
Intestinal muscle	140 $\pm$ 14	32	33	105 $\pm$ 10	24	22	193 $\pm$ 18	44	45
Testis	231 $\pm$ 24	47	50	6 $\pm$ 1	1	ND	257 $\pm$ 26	52	50
Intestinal mucosa	141 $\pm$ 14	47	50	ND	ND	ND	159 $\pm$ 17	53	50
Spleen	114 $\pm$ 12	38	40	45 $\pm$ 4	15	10	141 $\pm$ 13	47	50
Kidney	97 $\pm$ 8	37	40	79 $\pm$ 8	30	27	86 $\pm$ 8	33	33
Lung	106 $\pm$ 10	43	42	67 $\pm$ 7	27	26	74 $\pm$ 7	30	32
Liver	143 $\pm$ 14	75	78	40 $\pm$ 4	21	22	8 $\pm$ 1.2	4	0
Adipocyte	9 $\pm$ 2	38	37	12 $\pm$ 2	51	53	3 $\pm$ 0.2	11	10

isoenzymes from intestinal mucosa was very low to undetectable, which suggests that the M-type-subunit gene may not be expressed in these tissues. Of the isoenzyme populations in the various tissues where the M-type subunit was detected, the M-type subunit contents varied over a very wide range, 12–3843 pmol/g. The isoenzymes in liver contained the highest percentage of L-type subunit, but the actual amount of this subunit in the liver PFK isoenzymes was not remarkably different from that in isoenzymes from other tissues, except for muscle and diaphragm, which had none. When present, the L-type subunit in the isoenzyme pools exhibited a range of 9–231 pmol/g. Except the muscle and diaphragm, which expressed no C-type subunit, a concentration range of 3–463 pmol/g for the C-type subunit in the various isoenzyme pools was noted. Clearly, tissues and organs are capable of producing each type of subunit over a wide range, which conveys considerable variability to the types of isoenzyme pools that can exist. In order to understand better how the native isoenzyme pools in each tissue are established, the role of the regulatory processes that control gene expression, mRNA splicing and subunit concentration must be examined.

On the basis of the data presented in Table 2, it is possible to suggest a uniform explanation of earlier work with PFK isoenzymes. For example, the minor hepatic PFK isoenzyme (PFK-L₁) was only partially titrated with the anti-(L-type subunit) antibody and had kinetic properties which were different from L₄ and M₄ enzymes (Dunaway & Weber, 1974). Since the work presented herein indicates the presence of all three subunits in hepatic PFK isoenzymes, this 'isoenzyme' is probably unresolved isoenzymes which are composed of L, M and

C subunits. Other investigators have studied PFK isoenzymes in a variety of rat tissues and have reported different physical, kinetic and regulatory properties. For example, Tejwani & Mousa (1981) have reported that rat lung PFK had different kinetic and regulatory properties compared with L₄ and M₄ enzymes. Rat thyroid PFK activity has been reported to exhibit different kinetic and regulatory properties compared with M₄ enzyme (Meldolesi & Laccetti, 1979). These observations are not surprising, considering the existence of nearly equal amounts of all three subunits in the isoenzymes from lung and thyroid. If the native PFK isoenzymes were formed by random subunit interactions, then a high proportion of the complex hybrids containing all three subunits would be expected to exist in these PFK isoenzyme pools. From testes of mature rats, two PFK isoenzymes have been reported which are distinct from L₄ and M₄ (Lynn & Gomes, 1979). Since very low amounts of M-type subunits are found in testes, these isoenzymes were probably a mixture of the five homotetramers and hybrids containing the L and C subunits. Also, it has been suggested that a unique PFK isoenzyme (S-type PFK) exists in rat spleen, which can be kinetically, immunologically and electrophoretically discriminated from L₄ and M₄ (Bittner *et al.*, 1978; Levin *et al.*, 1982). Since all three subunits are found in PFK isoenzymes from spleen, it is very likely that the S-type PFK is composed of homotetrameric and hybrid PFK isoenzymes. Some very interesting studies by Kellett and associates suggested the presence of an unusual PFK isoenzyme in the mucosal cells of the rat small intestine (Khoja *et al.*, 1983; Khoja & Kellett, 1983). This 'isoenzyme', PFK-D in their terminology, was kinetically, electrophoretically and chromatographically dis-

tinct from the L_4 and M_4 isoenzymes. On the basis of our data, this is not surprising, since PFK-D is very likely PFK isoenzymes which are composed of varying amounts of the lower- M_r forms of the L- and C-type subunits.

In rat tissues, we have demonstrated that total PFK activity, immunological and chromatographic properties of the native isoenzymes, and the subunit compositions of the PFK isoenzyme pools, are considerably varied. Except for skeletal muscle and diaphragm, the PFK isoenzyme pools in rat tissues are clearly not one or more of the homotetramers of the three subunit types. On the contrary, they are postulated to be complex mixtures of different amounts of homotetrameric and heterotetrameric isoenzymes, which are formed randomly depending on the types and amounts of each subunit that are expressed.

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