Are the rat tissue/organ proportions of 6-phosphofructo-1-kinase subunits strain-specific?

George A. DUNAWAY* and Tom KASTEN

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

Recent studies suggest that the tissue/organ proportions of 6-phosphofructo-1-kinase (PFK) subunits from diverse strains of rat may be drastically different. To test this possibility rigorously, the PFK isoenzyme populations and subunit contents in muscle, liver, brain and heart were examined in the following strains: Wistar, ACI, Long Evans, Norway Brown and Wag/Rij. Regardless of the strain, adult muscle possessed only the M-type subunit; adult liver contained predominantly the L-type subunit as well as M-type and C-type subunits; and the adult brain and heart exhibited all three subunit types.

INTRODUCTION

It has been strongly suspected that at least three 6-phosphofructo-1-kinase (PFK; EC 2.7.1.11) subunits are expressed in mammalian tissues (for review see Dunaway, 1983). In the present paper, the major forms in adult muscle and liver are referred to as M-type and L-type subunits respectively, and the third subunit type is designated the C-type subunit. It has been directly shown that three subunit types were present in rabbit brain (Foe & Kemp, 1984) as well as in rat brain and heart (Dunaway & Kasten, 1985a,b; Dunaway *et al.*, 1985). Others have studied the nature of PFK subunits in the brain from the Wag/Rij rat, which originated from the Wistar strain (Vora *et al.*, 1985). Using the techniques of chromatographic resolution, immunochemical analysis and kinetic studies, these investigators suggested that their data were consistent with the existence of at least three subunit types in rats (Vora *et al.*, 1985). Further, from their column-chromatography studies, it was suggested that brain PFK in this species of rat was primarily C₄, i.e. a homotetramer of the C-type subunit. Our studies with the Wistar rat (Dunaway & Kasten, 1985a) and similar work with rabbit (Foe & Kemp, 1984) indicate that brain PFK isoenzymes contain three types of subunits and are composed of many, if not all, of the 15 possible tetramer. Since it was suggested that strain-specific differences may exist (especially in the Wag/Rij rat), we directly measured the PFK subunit distributions in different tissues of the Wag/Rij and other strains of rat. If high contents of C₄ enzyme indeed exist in the brain of the Wag/Rij rat or other rat strains, this would facilitate its purification and investigation of its kinetic, regulatory and structural properties.

EXPERIMENTAL

Animals

Male adult rats of diverse strains (Wistar, ACI, Long Evans and Norway Brown) were purchased from Harlan/Sprague-Dawley, Indianapolis, IN, U.S.A. The tissues from the Wag/Rij rats were generously supplied

by Dr. Ralph Oskam, Department of Hematology, Division of Medical Enzymology, Academic Hospital, Catharijnesingel 101, 3500 CG Utrecht, The Netherlands. All rats were housed in cages with filter caps (Lab Products Inc., Maywood, NJ, U.S.A.) and illuminated daily from 07:00 to 19:00 h. Water and Purina Laboratory rat chow were available *ad libitum*. After decapitation and exsanguination of the rats, tissues were rapidly removed and homogenized. PFK activity was extracted and assayed as previously described (Kasten *et al.*, 1983).

Materials

Cibacron Blue F3GA gel was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). QAE-Sephadex A-50 was purchased from Pharmacia Fine Chemicals. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Purification of PFK from liver, muscle and brain

The L₄ and M₄ isoenzymes were purified to homogeneity from liver and muscle respectively, by the method of Kasten *et al.* (1983). Isoenzymes from brain were purified by a slight modification of the technique for L₄ (Dunaway & Kasten, 1985a), and their subunits were used for identification of subunits in other tissues. Protein was determined by the Bradford method, with bovine serum albumin as a standard (Reed & Northcote, 1981).

Immunological techniques

In order to prepare L- and M-type-subunit-specific antibodies, rabbits were immunized with homogeneous L₄ or M₄, and each immune IgG was prepared as previously described (Thrasher *et al.*, 1981; Dunaway & Weber, 1974). Since titration of L₄ with M-type-subunit antibodies did not result in neutralization of activity, these antibodies were not absorbed. However, it was necessary to absorb the L-type-subunit antibodies with M₄ and with M–C hybrids, which were isolated from purified brain PFK isoenzymes. The M–C hybrids in the

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

* To whom correspondence should be addressed.

purified brain PFK isoenzymes were easily isolated, since they are exclusively eluted from QAE-Sephadex in the first 100 ml of the 150 mM-NaCl-containing buffer (Dunaway & Kasten, 1985a). The C-type antibody was prepared from rabbits which were immunized with the brain isoenzymes, and the purified C-type antibodies were subsequently absorbed with L₄ and M₄. Immunotitrations with each type of antibody were conducted as previously outlined (Dunaway & Weber, 1974; Thrasher *et al.*, 1981).

Immunoblotting procedures

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/0.05% SDS. 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 M-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. Detection was initiated with a visualization solution and stopped by rinsing in deionized water. To prepare the former solution, 60 mg of 4-chloro-1-naphthol was dissolved in 10 ml of methanol, and this solution 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.

SDS/polyacrylamide-gel electrophoresis

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 of approx. 50 units/mg of protein. The partially purified isoenzymes were subjected to electrophoresis on 6%-acrylamide slab gels and silver-stained as described by Dunaway & Kasten (1985a,b). 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

Table 1. Measurement of PFK activity and immunological and chromatographic analysis of PFK isoenzymes

Total PFK activity in supernatant fluid was assayed as described in the Experimental section. The supernatant fluids for each tissue were incubated with graded amounts of subunit-specific IgG after 2 h at 37 °C and then centrifuged. The amount of activity remaining after this treatment was measured for each amount of subunit-specific IgG, and the maximum percentage titration of total activity determined and recorded in this Table. 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 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	Strain	Total PFK activity (units/g)	Titration with subunit-specific antibodies (%)			Activity (%) in:	
			L-type	M-type	C-type	150 mM-NaCl	300 mM-NaCl
Muscle	Wistar	80.2	0	100	0	100	0
	ACI	78.2	0	95	0	100	0
	Long Evans	90.9	0	94	0	100	0
	Norway Brown	103	0	96	0	100	0
	Wag/Rij	108	0	94	0	100	0
Liver	Wistar	4.3	88	18	6	10	90
	ACI	5.6	91	35	4	12	88
	Long Evans	4.8	90	46	10	14	86
	Norway Brown	4.8	85	33	19	15	85
	Wag/Rij	3.8	94	32	9	10	90
Brain	Wistar	28.0	19	86	90	40	60
	ACI	21.6	18	84	89	39	61
	Long Evans	24.3	22	86	89	36	64
	Norway Brown	20.9	28	84	90	41	59
	Wag/Rij	22.8	26	89	88	45	55
Heart	Wistar	23.5	12	92	15	95	5
	ACI	20.8	12	91	17	88	12
	Long Evans	21.7	19	92	11	87	13
	Norway Brown	24.2	16	90	16	87	13
	Wag/Rij	23.2	16	92	16	93	7

Fig. 1. PFK subunits in muscle, liver, brain and heart of different species of rat

PFK isoenzymes were partially purified from each tissue for each species of rat, treated with SDS, and subjected to SDS/polyacrylamide-gel electrophoresis. Subunits (C, M, L) were detected by silver staining, and their relative amounts estimated by scanning gel densitometry. The poorer resolution of the C-type subunit in the brain samples as compared with the other tissues is due to the presence of large amounts of both subunits.

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 against various amounts of L_4 and M_4 (50–600 ng). The PFK subunits for each tissue were tentatively identified by electrophoretic mobility by using brain PFK subunits as standards, and each subunit was quantified by assuming equal intensity of absorption. Subunit identity was confirmed by immunoblotting with subunit-specific antibodies.

RESULTS AND DISCUSSION

For different rat strains, properties of the native PFK isoenzymes in muscle, liver, brain and heart were studied by chromatographic resolution on QAE-Sephadex and by immunological titration with subunit-specific antibodies as previously described (Dunaway & Kasten, 1985a,b). The utility of the chromatographic technique using QAE-Sephadex is that, in the 150 mM-NaCl wash, M_4 and M-rich hybrids are found and, in the 300 mM-NaCl wash, L_4 as well as L-rich and C-rich hybrids are eluted (Dunaway & Kasten, 1985a,b). As shown in Table 1, the total PFK activities, immunotitrations of PFK activity with the subunit-specific antibodies, and resolution of PFK isoenzymes by the QAE-Sephadex technique were different for each tissue; but no remarkable strain-related differences were noted. Interestingly, the nearly complete titration of PFK activity in brain by either the M- or C-type-subunit antibodies

suggests that the PFK subunits were extensively hybridized with each other and that homotetramers of each subunit are not the primary isoenzymes.

Since the region of the gel where a subunit is located could contain other proteins, one cannot rely solely on protein-stained gels to reveal correct subunit percentages. To avoid this potential error, an alternative method, immunoblotting, was adopted to confirm the identity and amount of PFK subunits in each tissue. For the tissues from each strain of rat, the subunits from partially purified PFK isoenzymes as detected via silver-stained polyacrylamide gels are shown in Fig. 1, and the subunit percentages as determined by scanning these gels are presented in Table 2. Transblots of gels, which were identical with those shown in Fig. 1, and specific detection with subunit-specific antibodies were used to confirm the identities and percentages of each subunit in each tissue (Table 2). By both criteria, the subunit patterns and percentages were nearly identical for each type of tissue in each strain of rat. Further, the apparent M_r of each type of subunit was the same regardless of the rat strain or tissue. The excellent agreement between the subunit percentages that were obtained from silver-stained gels and from immunoblots that were developed with subunit-specific antibodies strongly supports the accuracy of the subunit percentages presented in Table 2. The validity of these measurements is also substantiated by the close agreement between the subunit percentages for partially purified PFK isoenzymes from heart and brain (Table 2) and those subunit

Table 2. Measurement of subunit percentages in tissues from different rat strains

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 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.

Tissue	Strain	Percentage of each subunit as determined by:					
		Silver staining			Immunoblotting		
		L-type	M-type	C-type	L-type	M-type	C-type
Muscle	Wistar	0	100	0	0	100	0
	ACI	0	100	0	0	100	0
	Long Evans	0	100	0	0	100	0
	Norway Brown	0	100	0	0	100	0
	Wag/Rij	0	100	0	0	100	0
Liver	Wistar	75	21	4	78	16	6
	ACI	70	25	5	73	20	7
	Long Evans	72	28	10	74	19	7
	Norway Brown	67	32	10	70	22	8
	Wag/Rij	78	18	4	77	17	6
Brain	Wistar	18	45	37	19	43	38
	ACI	15	49	36	17	47	36
	Long Evans	15	48	37	13	50	37
	Norway Brown	16	47	37	20	45	35
	Wag/Rij	15	50	35	18	45	37
Heart	Wistar	10	80	10	10	79	11
	ACI	16	72	12	13	75	12
	Long Evans	15	73	8	14	74	12
	Norway Brown	17	73	10	12	75	13
	Wag/Rij	11	79	9	12	76	13

percentages (L:M:C) previously reported for the homogeneous PFK isoenzymes of heart (13:75:12) and brain (14:48:38) (Dunaway & Kasten, 1985a,b). Also, results with purified liver PFK isoenzymes (G. A. Dunaway & T. Kasten, unpublished work) indicated subunit percentages (L:M:C) of 70:24:6, which agrees quite well with those obtained from partially purified liver PFK isoenzymes (Table 2).

These results clearly demonstrate that the amounts of PFK subunits and consequently the distribution of native isoenzymes in each tissue did not exhibit any significant strain-related differences. However, the amount of each PFK subunit were considerably different between the tissues and organs that were studied. For this reason, the relative amounts of each of the native PFK isoenzymes are very likely different in each tissue, and these variations in the amounts of each of the possible isoenzymes contribute to the different total PFK activities as well as the different chromatographic and immunological properties that were observed.

By direct observation of subunits, it is obvious that brain PFK of the Wag/Rij rat is not exclusively or primarily composed of the C-type subunit, as previously reported (Vora *et al.*, 1985). The interpretation which incorrectly suggested that C₄ is the only PFK isoenzyme in Wag/Rij-rat brain was based only on examination of the native isoenzymes by chromatographic resolution and immunochemical analysis. Although earlier work (Tsai & Kemp, 1973; Dunaway & Weber, 1974; Foe &

Kemp, 1984), using linear gradients, tends to agree with their chromatographic results, elution profiles that were generated by using discontinuous-gradient techniques have revealed the true complex nature of the native brain PFK isoenzymes (Foe & Kemp, 1984, 1985; Dunaway & Kasten, 1985a). Subunit analysis of different regions of the resolved activity confirmed that the types of PFK isoenzymes in the brain are many and may approach the maximum possible number of 15 (Foe & Kemp, 1984; Dunaway & Kasten, 1985a).

The immunological studies of Vora *et al.* (1985) with anti-L and anti-M antibodies seemed to confirm their chromatographic results, i.e., only C₄ was present in Wag/Rij brain. They attempted to make their immune serum specific for the M-type subunit by absorption with liver and brain PFK, which was assumed to eliminate reaction with L-type and C-type subunits respectively. These 'absorbed' M-type antibodies were reported to lose reactivity with liver PFK, but continued to neutralize brain PFK. Assuming that brain PFK was only C₄ led to the conclusion that the M-type and C-type subunits must share some common antigenic determinants (Vora *et al.*, 1985). However, the data reported in the present paper clearly demonstrate that most of the reaction of the 'absorbed' anti-M antibodies with brain PFK can be attributed to the presence of high proportions of the M-type subunit in brain PFK isoenzymes. Consequently, the suggestion that M and C subunits share some common antigenic determinants was

not successfully demonstrated. By immunoblotting, our anti-M IgG did not detectably cross-react with the C-type subunit in any of the PFK preparations that were studied. The L-type antibody, which was used by Vora *et al.* (1985), was generated against erythrocyte PFK, which contains L-type and M-type subunit, and was made specific for the L-type subunit by absorption with muscle PFK and perhaps brain PFK (it is not clear to us from the methodological descriptions if brain PFK was used). Since this absorbed antibody reacted only with L₄ and not with brain PFK, it was suggested that brain PFK did not contain the L-type subunit (Vora *et al.*, 1985). However, since the content of this subunit in brain PFK isoenzymes was only 16% of the total amount of subunits (see Table 2), it is very likely that the rat L-type subunit is dispersed in hybrids to the extent that it cannot be easily recognized by antibodies that are directed against the human L-type subunit.

From our own earlier difficulties and those of others, it is evident that more definitive studies concerning the subunit composition of PFK isoenzymes must include direct identification and measurement of each subunit type. Consequently, we recommend that direct observation of subunits be accepted as a major criterion for establishment of the nature of subunit types and consequently potential isoenzyme types. Also, we would concur with Vora *et al.* (1985) that a standard nomenclature for the PFK subunits should be adopted by investigators in this field. We would endorse the widely accepted use of the letters L and M to represent the major subunits that were originally identified in adult liver and muscle respectively. With respect to the other subunit type, the use of any letter which represents an organ, tissue or cell type does not appear to be justified. Our studies (G. A. Dunaway & T. Kasten, unpublished

work) of PFK isoenzymes in a large number of organs, tissues and cell types in a variety of mammals indicate that the third type of subunit, when present, was expressed with one or both of the other subunits and was never the predominant subunit. Our recommendation is that the letter 'C' be used to designate this subunit, as was originally proposed in the pioneering work with rabbit PFK isoenzymes (Tsai & Kemp, 1973)

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