Molecular Forms of 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Expressed in Rat Skeletal Muscle*

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The rat cDNA for the muscle-type (M) isoform of 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase (FBPase-2) contains two putative translation initiation sites. To determine whether the M isoform expressed in rat skeletal muscle corresponds to the short (PFK2M-sf) or the long (PFK2M-lf) isoform, we have expressed them in Escherichia coli. A third construction was also expressed in which the second ATG codon was deleted (PFK2M-lfAATG) to ensure that initiation started at the first ATG. The properties of these recombinant proteins were compared with those of the PFK-2/FBPase-2 present in rat skeletal muscle and liver. The recombinant proteins displayed PFK-2 and FBPase-2 activities and the M, values of the subunits measured by SDS-polyacrylamide gel electrophoresis were compatible with the calculated ones. The purified recombinant If form contained not only the expected If band (54,500 M) but also the sf band (52,000 M), indicating that the expression system could synthesize the long and the short isoforms from the same mRNA. The kinetic properties of the recombinant sf form were not different from those of the rat muscle enzyme. By contrast, IfAATG PFK-2 displayed a higher K\textsubscript{m} for its substrates and a lower V\textsubscript{max}. Immunoblotting with an antibody directed against the long isoform revealed a 54,500 M, band both in the If and the IfAATG recombinant, but no band in rat skeletal muscle extracts. In these extracts, one band of 52,000 and a minor one of 54,500 M, were detected by an anti PFK-2/FBPase-2 antibody. The 54,500 M, band was recognized by an antibody directed against the L isoform, suggesting that a small amount of the latter is expressed in skeletal muscle. Thus, the M isoform differs from the L isoform by replacement of the first 32 amino acids of the L isoform by an unrelated nonapeptide.

At least four isozymes of the bifunctional enzyme 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase (FBPase-2) are present in mammalian tissues (1-4). They are referred to as L- (liver), M- (muscle), H- (heart), and T- (testis) type according to their tissue prevalence. They differ in molecular mass, in kinetic and immunological properties, and in response to phosphorylation by protein kinases (4, 5). They contain two identical subunits, each of which bears the two catalytic sites. The amino acid sequences of these isoforms have been established from the corresponding cDNAs (4, 6-8). The rat L and M isoforms have in common an identical sequence of 438 amino acids. They differ at the N-terminal end which, in the L isoform, contains 32 residues including Ser-32. Phosphorylation of the latter by the cyclic AMP-dependent protein kinase inactivates PFK-2 and activates FBPase-2. In the M isoform, the N-terminal end is unrelated to the unique part of the L isoform. It does not contain Ser-32, and thus the M isoform is not a substrate for cAMP-dependent protein kinase. These two isoforms are encoded by the same gene. They arise from different promoters with splicing of either the first (M isoform) or the second (L isoform) exon with the 13 other common exons (9).

The first exon encoding the unique 5' sequence of the rat M-type mRNA contains two in-frame ATG codons (Fig. 1), which are putative translation initiation sites, and for which Kozak (10) has proposed the following consensus sequence, 5'-GCC/GCCA/GCCATGG-3'. In this respect, the second ATG (Fig. 1) is in a more favorable context for initiation than the first one. However, the participation of the first translation initiation site has not been ruled out, since the N-terminal amino acid sequence of rat muscle PFK-2/FBPase-2 could not be established (11). Therefore, two isoforms of the M isoform could exist, a long one starting at the first in-frame ATG with a calculated molecular mass of 53,971 Da, and a second one, 21 amino acids shorter, starting at the second in-frame ATG with a calculated molecular mass of 51,880 Da. It is very unlikely that translation starts also at the third in-frame ATG corresponding to Met-41, because the sequence of one of the "tryptic" peptides obtained from PFK-2/FBPase-2 purified from rat skeletal muscle (11) started at Thr-29 of the sequence shown in Fig. 1. SDS-PAGE analysis of purified preparations of PFK-2/FBPase-2 from pigeon skeletal muscle displayed a major band of 53,000 M, and a minor constituent of 54,000 M, (3). The latter had the properties of the L isoform (3). Immunoblots of rat skeletal muscle PFK-2/FBPase-2 preparations partially
purified by DEAE-cellulose showed two bands, a major one of 54,000 M₉, which was not phosphorylated by calf thymus-dependent protein kinase, and a minor one of 55,000 M₉, which was a substrate of cAMP-dependent protein kinase and could correspond to the L isozyme (12). Purified preparations of PFK-2/FBPase-2 from rat muscle contained a single 54,000 M₉ band (11). This band corresponds to the calculated molecular mass (50,974 Da) of the low form of the M isozyme, but is not very different from the L isozyme, which has a molecular mass of 54,570 Da.

This study was undertaken to determine whether the M isozyme expressed in rat skeletal muscle corresponds to the short or to the long isoform and whether some L isozyme could be present in skeletal muscle. The cDNAs corresponding to the two isoforms (fl and sf) of the M isozyme were cloned in an expression vector, expressed in Escherichia coli, and the biochemical and immunological properties of these two isoforms were compared with those of the enzyme purified from rat skeletal muscle. In a third construction, the second ATG was removed by site-directed mutagenesis to ensure that initiation started at the first ATG codon, and the properties of this obligatory long isoform (flaATG) expressed in E. coli were studied. Finally, antibodies were raised against two synthetic peptides, one corresponding to the N-terminal portion unique to the L isozyme (13), and one corresponding to a sequence located between the two putative ATG codons of the M isozyme (Fig. 1). These two antibodies were used to detect the L isozyme and the long isoform of the M isozyme by immunoblotting in various purified preparations.

**EXPERIMENTAL PROCEDURES**

**Materials**

Biochemicals and restriction enzymes were purchased from Boehringer. T7 DNA polymerase, T4 DNA ligase, [³²P]ATP (3000 Ci/mmol), and γ-[³²P]ATP (3000 Ci/mmol) were from Amersham International plc. T4 polynucleotide kinase was from New England BioLabs. The pPLcmu299 expression plasmid was a gift from Dr. E. Remaut (Laboratory of Molecular Biology, State University of Ghent, Belgium). The pBlueScript(KS)II+ and XL1-Blue E. coli host were from Pharmacia LKB Biotechnology Inc. All other materials were reagent grade.

**DNA Manipulations**

Standard DNA manipulations were carried out as described (14). Single-stranded phagemid DNA was extracted from phage particles purified from overnight incubation of a culture supernatant with M13K07 at a helper phage/host ratio of 10, when the A₂₆₀ of the culture was 0.5 (15). The cloning host for a-competition analysis and isolating single-stranded phagemid DNA, XL1-Blue, contains the lacZΔM15 on a fertility (F') episome (selectable on minimal medium) encoding pili formation. Sequencing was made using reagents from U. S. Biochemicals, except that Sequenase was replaced by T7 DNA polymerase. Oligonucleotide-directed mutagenesis (16) was performed using the Amersham kit and according to the manufacturer's instructions.

**Construction of the pPL-PFK2M Expression Plasmids**

As a consequence of the cloning procedure, the cDNA corresponding to the M isozyme of PFK-2/FBPase-2 was obtained as two EcoRI fragments, 5c2 (5' end of the cDNA) and 5c1 (7), each of which had been cloned in pBR322 plasmid. These two fragments were then assembled to create the phagemid pBlueScript(KS)II+/PFK2M as follows: (Fig. 2). A 1346-bp EcoRI-SspI fragment was excised from p5c1, thus removing 65 bp of the 5'-untranslated region, and inserted in pBlueScript(KS)II+ cleaved with EcoRI and SmaI. The 5c2 cDNA fragment was then inserted after restriction of the phagemid with EcoRI and checked for correct orientation by restriction analysis. A single-stranded (+)-form of this phagemid was prepared (15). It was used as a template for mutagenesis (16) to create a restriction site suitable for cloning the coding sequence immediately downstream of the translation initiation site of the expression vector. Three constructions were made. The first one corresponded to the short form (PFK2M-sf) and the second to the long form (PFK2M-lf) of the M isozyme, in which the translation began at the second or the first in-frame ATG codon, respectively. The mutagenic oligonucleotides chosen to replace the second ATG by a BstBI site and the first ATG by a SstI site had the following sequence.

- BstBI site 5'... GGA AAA TTA GCG TTC GAA GAA AAA GCC... 3'
- Wild type 5'... GGA AAA TTA GCG ATG GAA GAA AAA GCC... 3'
- (2nd ATG codon)
- SstI site 5'... GGT TTC TAT TTG AGG CCT CCC ACC G... 3'
- Wild type 5'... GGT TTC AT TTG ATG CAA CCC ACC G... 3'
- (1st ATG codon)

**Anti-sense oligonucleotides were used for the site-directed mutagenesis reaction. The mutants were screened by digestion with the appropriate restriction enzyme and sequenced by the Sanger's dideoxy chain termination method (17) on alkali-denatured double-stranded phagemid DNA (14). The procedure for the construction of the expression vectors, schematized in Fig. 3, was as follows. The BstBI mutant was digested with BstBI, its 5' ends were filled-in with DNA polymerase I (Klenow) in the presence of dCTP only, and the 5'-protruding C was removed by the mung bean nuclease (14). The phagemid was then digested with BamHI and inserted into the plasmid pBR322. The first recombinant phagemid, PFK2M-lfΔATG, was obtained by deleting the second ATG from the BstBI site. The three constructs were introduced in an expression vector, pPL, using the inducible leftward promoter of coliphage λ (18). pPLeum239 (19) was digested with Ncol, generating 5' ends which were filled-in with DNA polymerase I (Klenow) in the presence of the four dNTPs. Subsequent digestion with BamHI led to the creation of the compatible ends with the cDNAs. Ligation of the cDNAs into the Ncol/BamHI vector yielded three constructs, pPL/PFK2M-sf, pPL/PFK2M-lf, and pPL/PFK2M-lfΔATG, which contained the cDNAs linked directly to the translation initiation codon of pVL.

**Expression of the Recombinant Proteins**

The pPL plasmids do not contain the cI gene coding for the repressor. Transcription from the P₇ promoter is repressed by the
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Fig. 2. Construction of a recombinant phagemid containing the full-length cDNA coding for the M isozyme of PFK-2/FBPase-2. A 308-bp EcoRI fragment and a 1346-bp EcoRI/SspI fragment obtained from the M isozyme PFK-2/FBPase-2 cDNA clone RL2K-5c were cloned in the phagemid pBluescript (KS)II+ to reconstitute the full-length cDNA.

The purification of PFK-2/FBPase-2 was adapted from described procedures (3, 22, 23) to be completed within 2 days. The starting material consisted of 3 liters of culture corresponding to 0.1-0.2 unit of PFK-2/FBPase-2. The bacteria were collected by centrifugation and resuspended in a buffer containing 100 mM KC1, 50 mM Hepes at pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mg of phenylmethylsulfonyl fluoride/ml, and 2 µg of aprotinin/ml. The bacteria were lysed in a motor-driven French pressure cell at an internal cell pressure of 10,000 p.s.i. and the resulting lysate was purified by the following three steps.

Precipitation by Polyethylene Glycol (PEG): Solid PEG 6000 was added to reach a concentration of 5% (w/v) and the solution was stirred for 30 min and centrifuged at 10,000 × g for 10 min. The supernatant was collected, its pH was adjusted to 7.0, and the concentration of PEG was increased to 20% (w/v). After stirring for 30 min, the 5-20% protein fraction was collected by centrifugation (30,000 × g, 30 min). The pellet was resuspended in a buffer containing 25 mM Hepes, pH 7.5, 150 mM KC1, 1 mM potassium phosphate, 0.1 mM phenylmethylsulfonyl fluoride, 15 mM β-mercaptoethanol, and 0.01% of Brij 35 (buffer A).

Chromatography on Q-Sepharose: The column (22 ml of bed volume) was loaded and then washed with 1 liter of a buffer containing 25 mM Hepes at pH 7.5, 200 mM KC1, 1 mM dithiothreitol, 0.01% Brij 35, and 0.05 mM fructose 6-phosphate. PFK-2/FBPase-2 was then specifically eluted in the same buffer enriched with 1 mM MgATP, 1 mM Mg-citrate, 1 mM potassium phosphate. The KC1 concentration of the eluting buffer was decreased to 0.175 M. A typical elution profile is shown in Fig. 4B. PFK-2/FBPase-2 was purified according to Ref. 23 from rat hind leg muscles and liver. The steps of this purification were the same as above.

Peptide Synthesis and Antibodies

Two decapeptides, GELTQTRLQK, corresponding to the N-terminal region unique for the L isozyme, and PTGQTRLQK, corresponding to the unique N-terminal region of the long isoform (lf) of the M isozyme (see Fig. 1) were prepared as described (13) by solid phase on a Beckman 990 B automatic peptide synthesizer. The synthetic peptides were used to raise antibodies in rabbits as described (13). These anti-L and anti-Mlf antibodies were used after partial purification by precipitation with 45% ammonium sulfate. They
**Fig. 3. Construction of the expression vectors PFK2 M-sf and -II.** These vectors were constructed from pBluescript (KSII + /PFK2M (Fig. 2). A BstBI site and a Stul site were created in the vicinity of the second or the first ATG codon by oligonucleotide-directed mutagenesis, yielding pBluescript(KSII + /PFKZM (BstBI) and pBluescript(KSII + /PFKBM (Stul), respectively. The rest of the procedure is explained in the text. For the construction of pPL/PFKZM-ΔATG, in which the second ATG codon has been deleted, the oligonucleotide 5'GAGGCTTTTTCTTCCGCTAATTTTCCAATG-3', was annealed with pBluescript(KSII + /PFKZM (Stul) single-stranded DNA and the mutagenesis reaction was performed as described under “Experimental Procedures.” The strategy used to construct the corresponding expression vector was the same as the one described here for pPL/PFK2M-ΔII.

recognized specifically their cognate synthetic antigen without cross-reaction in an enzyme-linked immunosorbent assay test. Another antibody, raised against a sequence (EEKASKTRA) of the short isoform, failed to react satisfactorily in immunobots, although it did recognize its cognate antigen in an enzyme-linked immunosorbent assay test (13).

**Protein Electrophoresis and Immunoblotting**

Proteins were separated by SDS-PAGE (10%) (24). The gel was electroblotted onto a Immobilon polyvinylidine difluoride membrane. The antigens were detected by specific polyclonal antibodies. These included the anti-L and anti-M antibodies and MCL-2, an antibody which recognizes epitopes common to the L and M isozymes (25). Bound antibodies were detected by a peroxidase-antiperoxidase complex (PAP from Prosan) revealed by the ECL Western blotting detection system (Amersham).

**Measurement of Enzyme Activities**

PKF-2 activity was measured by the rate of formation of Fru-2,6-P₂. The assay of PKF-2 activity was adapted from methods described previously (26, 27), as follows. In a total volume of 0.2 ml, 0-50-μl samples of the enzyme preparation were incubated at 30 °C for up to 15 min in the presence of 2 mM fructose 6-phosphate, 5 mM MgATP, 5 mM potassium phosphate, 1 mM dithiothreitol, 100 mM KC1, 20 mM KF, 1 mg of albumin/ml, and 50 mM Tris-HCl at pH 8.5 (optimal pH). The reaction was stopped by the addition of 0.2 ml of 50 mM NaOH and by heating at 80 °C for 5 min. The amount of Fru-2,6-P₂ formed was then measured as described (28).

FBPase-2 activity was measured by the formation of [32P]P, from [2-32P]Fru-2,6-P₂, which was synthesized as described (29). The samples (0-50 μl) of the enzyme preparation were incubated at 30 °C for 15 min in a total volume of 0.2 ml containing 5 μM [2-32P]Fru-2,6-P₂, 0.2 mM EDTA, 5 mM MgCl₂, 5 mM potassium phosphate, 1 mM dithiothreitol, 100 mM KC1, 1 mg/ml albumin, and 50 mM Hepes at pH 7.0. The reaction was stopped by the addition of 0.2 ml of 0.1 M NaOH and by heating for 5 min at 80 °C. [32P]P, was then separated from the radioactive substrate by chromatography on columns of Dowex AG 1-X8 (chloride form, 1 ml of bed volume) as described.
buffer containing the ligands and the PFK-2 enriched fractions collected from Q-Sepharose and applied to a column of Q-Sepharose. Fractions contained 6 ml. In response to the absence of enzyme.

Elution profile of recombinant M isozyme PFK-2/FBPase-2 from Q-Sepharose (A) and Blue Sepharose (B). In A, PEG extracts prepared from 1 liter of culture expressing If were applied to a column of Q-Sepharose. Fractions contained 6 ml. In B, the PFK-2 enriched fractions collected from Q-Sepharose and corresponding to 1 liter of culture expressing If were loaded on the Blue Sepharose column. The arrow indicates the application of the elution buffer containing the ligands and 0.175 M KC1 (see "Experimental Procedures").

For all the enzymes studied, the linearity of the reaction as a function of the time of incubation and of the concentration of enzyme was checked. One unit of enzyme activity corresponds to the formation of 1 µmol of product/min under the assay conditions. For the measurement of Vmax, Km, and K, the concentrations of ligands were varied accordingly and the constants were calculated by nonlinear least squares fitting to an hyperbola using a computer program ("Ultrafit") from Biosoft.

Other Methods

Purification of PFK-2/FBPase-2—The three recombinant forms of the M isozyme and PFK-2/FBPase-2 from rat liver and skeletal muscle were purified by PEG fractionation followed by Q-Sepharose and Blue Sepharose chromatography, with a final recovery of 24 ± 5% (mean ± S.E. for 6 preparations). The behavior of the three recombinant forms was similar. PFK-2 activity eluted from the Q-Sepharose column at 0.3 M KC1 and from the Blue Sepharose column at 0.175 M KC1 in the presence of the ligands (Fig. 4). PFK-2 activity from rat liver eluted from Q-Sepharose at 0.35 M KC1 and from the Blue Sepharose at 0.4 M KC1 in the presence of the ligands (not shown). PFK-2 from rat skeletal muscle eluted from Q-Sepharose as two overlapping peaks, one at 0.3 M KC1, like the recombinant M forms, and one at 0.35 M KC1, like liver PFK-2/FBPase-2. The two peaks were loaded on Blue Sepharose and the activity of PFK-2 which eluted as a single peak at 0.175 M KC1 in the presence of the ligands was collected. The L isozyme present in the muscle extract was therefore theoretically discarded, since this concentration of salt did not favor the elution of the L isozyme.

M, of Purified PFK-2/FBPase-2—SDS-PAGE analysis was performed on the recombinant M forms, purified as described above, and on the liver and muscle enzymes eluted from Blue Sepharose. As shown in Fig. 5, the recombinant If yielded one band of 52,000 M, as expected from the calculated mass of 51,880 Da, while the obligatory long form IfATG yielded one band of 54,500 M, as expected from the calculated mass of 53,971 Da. The recombinant If yielded not only the expected band of 54,500 M, but also the band of 52,000 M, (lane 5). This suggests that, in the recombinant E. coli, translation of the long isoform started not only from the first ATG, which belongs to the vector, but also from the second ATG, in the insert. The liver enzyme preparation showed the 55,000 M, band expected from the 54,570 Da of the L isozyme (lane 1). As to the muscle enzyme preparation (lane 2), it showed a band of 52,000 M,, consistent with the idea that it is the short M isoform which is expressed in this tissue.

Cova lent Labeling of PFK-2/FBPase-2—The FBPase-2 domain of PFK-2/FBPase-2 incorporates radioactive [32P]Pi, from the substrate, [2-32P]Fru-2,6-P2, in a ping-pong reaction (32). Since the labeling is specific, it can be used to detect the phosphoryl enzyme intermediate by autoradiography after SDS-PAGE. Labeling of PFK-2/FBPase-2 present in the recombinant bacterial lysates (Fig. 6) showed a 52,000 M,

Protein was measured (30) with γ-globulin as a standard. The SDS-PAGE gels were stained with silver nitrate (31). The molecular masses were calculated by a computer program ("The DNA Inspector II" from Textco).

RESULTS

Preparation of Bacterial Lysates—Different techniques of lysis, such as three freeze-thaw cycles in a dry ice/ethanol bath, sonication (three times 10 s, with 20-s intervals on ice), or the French pressure cell were tested. Total protein concentration and PFK-2 activity varied in the same proportion for a given lysis procedure. We routinely used the French pressure cell because it gave the highest specific PFK-2 activity (1–2 milliunit/mg of protein), it lysed more than 90% of the cells and it yielded about 10 mg of total protein from 50 ml of a culture induced for 2.5 h, while protein recovery was 5 to 10-fold less after sonication, and 100-fold less after freeze-thawing.

Cova lent Labeling of PFK-2/FBPase-2—The FBPase-2 domain of PFK-2/FBPase-2 incorporates radioactive [32P]Pi, from the substrate, [2-32P]Fru-2,6-P2, in a ping-pong reaction (32). Since the labeling is specific, it can be used to detect the phosphoryl enzyme intermediate by autoradiography after SDS-PAGE. Labeling of PFK-2/FBPase-2 present in the recombinant bacterial lysates (Fig. 6) showed a 52,000 M,
band for sf (lane 4) and a 54,500 Mᵦ band for lfΔATG (lane 6). The recombinant lf showed a major band of 54,500 Mᵦ and a minor band of 52,000 Mᵦ (lane 5). This confirms the data with protein staining. PFK-2/FBPase-2 labeled in a PEG extract from liver showed a major band of 54,500 Mᵦ, as expected for the L isozyme, and a minor band of 52,000 Mᵦ (lane 1). This suggests that the liver contains some M isozyme which could correspond to the short isoform. Labeling of a PEG extract of muscle (lane 2) showed a major band of 52,000 Mᵦ, again consistent with expression of the short isoform. Prolonged exposure of the autoradiography revealed a faint band at 54,500 Mᵦ corresponding to the short isoform. The antibodies used were LF, which is specific for the L isozyme, and anti-Mlf, which is specific for the long form of the M isozyme. As to the minor 54,500 Mᵦ band, it could correspond to the long form of the M isozyme or to the L isozyme that had eluted from the Blue Sepharose.

**Immunological Characterization**—We have used the technique of immunoblotting to assign the bands seen on SDS gels to a specific isoform. The antibodies used were MCL-2, which cross-reacts with the L and M PFK-2/FBPase-2 isozymes, anti-L, which is specific for the L isozyme, and anti-Mlf, which is specific for the long form of the M isozyme. Detection was performed on bacterial lysates for the recombinant forms and on PEG extracts or Q-Sepharose fractions from muscle extracts (Fig. 7). As expected, the anti-Mlf antibody (Fig. 7c, lane 2) showed a 52,000 Mᵦ band with sf (lane 3), a 54,500 Mᵦ band with lfAATG (lane 6), and also a minor band of 54,500 Mᵦ with If (lane 6). As expected, the anti-Mlf antibody (Fig. 7c) showed no signal with sf (lane 5), a 54,500 Mᵦ band with lfΔATG (lane 7), and only the 54,500 Mᵦ band with lf (lane 6). Thus, the method was adequate to specifically detect the long (54,500 Mᵦ) isoform of the M isozyme. PEG extracts from liver showed the same 54,500 Mᵦ band when incubated with the MCL-2 (Fig. 7a, lane 1) or with anti-L (Fig. 7b, lane 1) antibody, consistent with the presence of the L isozyme. No signal was obtained with the anti-Mlf antibody (Fig. 7c, lane 1). The 52,000 Mᵦ band seen in these extracts by Fru-2,6-P₂ labeling (Fig. 6, lane 1) could not be detected by immunoblotting. PEG extracts from skeletal muscle showed a major band of 52,000 Mᵦ, and a minor band of 54,500 Mᵦ, with the MCL-2 antibody (Fig. 7a, lane 2). As to the major band (52,000 Mᵦ) it was the main component of the first peak of Q-Sepharose, as expected for the M isozyme (Fig. 7a, lane 3), but was also present in the second peak (lane 4), consistent with the overlapping character of these peaks. No band was revealed with the anti-Mlf antibody in the Q-Sepharose fractions (Fig. 7c, lanes 3 and 4), confirming that the long isoform of the M isozyme is absent in PEG extracts of rat muscle. Therefore, the major 52,000 Mᵦ band present in muscle extracts is the short isoform of the M isozyme. As to the minor 54,500 Mᵦ band, it could be assigned to the L isozyme because it was recognized by the anti-L antibody (Fig. 7b, lane 2), but not by the anti-Mlf antibody (Fig. 7c, lane 2) and because it was more abundant in the second than in the first peak of Q-Sepharose (Fig. 7, lanes 3 and 4).

**Biochemical Properties**—The kinetic properties of the purified PFK-2/FBPase-2 are given in Table I. Except for the lfΔATG, the kinetic parameters of PFK-2 and FBPase-2 did not differ between the isoforms and were similar to those reported previously for rat skeletal muscle PFK-2/FBPase-2 (11). Therefore, the heterologous expression of the short form of the M isozyme results in a protein that displays all the biochemical features of the original enzyme. The interpretation of the results obtained with lf is ambiguous since the purified preparation of lf contained a large amount of sf (Fig. 5, lane 5). Thus, it is expected that lf resembles sf rather than lfΔATG. The obligatory long isoform (lfΔATG) contained slightly less PFK-2 activity than sf. This resulted from a decrease in V₅0 and an increase in the Kₘ values for Fru-6-P and MgATP. It is not known whether this lower PFK-2 activity is due to the N-terminal region or to the deletion of the second ATG and thus of a methionine residue.

**DISCUSSION**

Our previous cloning and characterization of the M-type cDNA showed that translation of the M isozyme could start at two AUG codons separated by 20 amino acids (7). In order
TABLE 1

Kinetic properties of the M isozymes of PFK-2/FBPase-2

|      | Vmax Apparent | Kapp for Frx-6-P | Apparent | Kapp for | Apparent | Kapp for citrate |
|------|---------------|------------------|----------|----------|----------|-----------------|
| WT   | 10.9 ± 0.8    | 46.7             | 151      | 144      | 77        | 47              |
| sf   | 8.9 ± 0.8     | 35.7             | 125      | 117      | 87        | 35              |
| if   | 5.8 ± 0.5     | 59 ± 12          | 52       | 114      | 34 ± 17   |                 |
| ΔATG | 2.4 ± 0.1     | 179 ± 21         | 417 ± 13 |          | 87 ± 72   |                 |

|      | Vmax Apparent | Kapp for Frx-6-P,2 | Apparent | Kapp for Frx-6-P |
|------|---------------|-------------------|----------|------------------|
| WT   | 6.5 ± 0.5     | 0.92 ± 0.07       | 5        |
| sf   | 16.6 ± 0.9    | 1.16 ± 0.03       | 5        |
| if   | 10.0 ± 0.1    | 0.86 ± 0.04       | 3        |
| ΔATG | 5.7 ± 1.1     | 0.71 ± 0.08       | 6        |

*Indicates values that are statistically different from all the other values.

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