Expression of Human Liver 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in *Escherichia coli*

ROLE OF N-2 PROLINE IN DEGRADATION OF THE PROTEIN

*(Received for publication, November 17, 1992)*

Alex J. Lange, Lin Li‡, Alberto M. Vargas§, and Simon J. Pilkins¶

From the Department of Physiology and Biophysics, Health Science Center, State University of New York, Stony Brook, New York 11794-8661

Human liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is 96% identical to the rat and bovine liver enzymes, and all of the critical catalytic and substrate binding residues in both the kinase and bisphosphatase domains are conserved in the three enzymes. However, in contrast to rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, which is readily expressed in an *Escherichia coli* T-7 RNA polymerase-based expression system, the human liver bifunctional enzyme could not be expressed in this system. Western blot and slot blot analysis revealed that although both the bifunctional enzyme protein and its mRNA were rapidly induced by the addition of isoprropyl-1-thio-β-D-galactosidase, the protein underwent rapid degradation. Deletion of the N-2 proline residue or its mutation to arginine, the corresponding residue in the rat liver enzyme, revealed that this proline residue was responsible for its rapid degradation. The Pro-2→Arg mutant could be expressed with a high yield (20 mg/liter) in *E. coli*. The results support the hypothesis that a proline residue at N-2 facilitates bifunctional enzyme degradation in *E. coli*. The *E. coli* expressed mutant form was purified to homogeneity by phosphocellulose chromatography, and its kinetic properties were compared with those of the rat liver enzyme. The kinetic properties of the two enzymes were identical except for the presence of substrate (fructose 6-phosphate) inhibition of the human liver enzyme but not of the rat liver enzyme. The ability to express and purify large amounts of human liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase will permit structure/function and x-ray crystal structure studies of the enzyme and ultimately its targeting for drug therapy.

Fructose 2,6-bisphosphate (Fru-2,6-P<sub>2</sub>) is a regulatory me-

* This work was supported by National Institutes of Health Grant DK2835405 (to S. J. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Shanghai Inst. of Biochemistry, Academia Sinica, 320 Yue-Yang Rd., Shanghai 200031, China.

§ Present address: Dept. Bioquimica y B. Molecular, Universidad de Granada, Facultad de Ciencias, 18071 Granada, Spain.

¶ To whom correspondence should be addressed.

1 The abbreviations used are: Fru-2,6-P<sub>2</sub>, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; 6PF-2-K/Fru-2,6-P<sub>2</sub>ase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Fru-1,6-P<sub>2</sub>ase, fructose-1,6-bisphosphatase; PIGT, isopropyl-1-thio-β-D-galactosidase; PAGE, polyacrylamide gel electrophoresis; TES, N-tri(hydroxymethyl)methyl-2-aminoe-thane sulfonic acid.

tabolite whose steady-state level is an important determinant for glycolysis in mammalian tissues (1). In tissues capable of gluconeogenesis, net glucose synthesis occurs only when the concentration of Fru-2,6-P<sub>2</sub> is low (1). A single bifunctional enzyme, 6PF-2-K/Fru-2,6-P<sub>2</sub>ase, catalyzes both the synthesis and hydrolysis of Fru-2,6-P<sub>2</sub> at two distinct active sites in the NH<sub>2</sub>- and COOH-terminal domains, respectively, of the protein and is responsible for determining the steady-state level of this regulatory metabolite. Isoforms of 6PF-2-K/Fru-2,6-P<sub>2</sub>ase have been isolated from different tissues, including skeletal muscle (2), liver (3, 4), heart (5), and testes (6). A unique neural (brain) isoform of the enzyme has also been isolated recently (7). The liver and skeletal muscle forms of 6PF-2-K/Fru-2,6-P<sub>2</sub>ase are the products of alternate splicing from two distinct promoters of a single gene (2), whereas the heart, testes, and brain forms of the enzyme are the products of three distinct genes (2, 6–8).

Much of the previous work on 6PF-2-K/Fru-2,6-P<sub>2</sub>ase has been carried out on the rat liver enzyme (9–11). Previously we have briefly described a full-length coding cDNA of the human liver form of the enzyme (12), and here we present the prokaryotic expression, purification, and characterization of the kinetic properties of this form of the enzyme. This report shows that the presence of a proline residue at the N-2 position in the native human liver 6PF-2-K/Fru-2,6-P<sub>2</sub>ase led to its rapid degradation in a T-7 RNA polymerase-based *Escherichia coli* expression system, which suggests that a proline at this position facilitates its degradation in *E. coli*. It is also demonstrated that the human 6PF-2-K but not the rat liver 6PF-2-K exhibits inhibition by its substrate, Fru-6-P.

**EXPERIMENTAL PROCEDURES**

Materials—Restriction enzymes and bacteriophage T<sub>1</sub> DNA ligase were from New England Biolabs. RNase-free DNase I was from Promega. Phosphocellulose F11 was from Whatman. ATP, Fru-6-P, and Fru-2,6-P<sub>2</sub> were from Sigma. [35S]thio-dATP was from Amer-sham. [1,23]ATP and GeneScreen nylon filter membranes were from DuPont. Q-Sepharose was from Pharmacia LKB Biotechnology Inc. Horseradish peroxidase-conjugated goat anti-rabbit antibody was from Boehringer Mannheim, and color reagent (4-chloro-1-naphthol) was from Bio-Rad. *E. coli* strain BL21 (DE3) was a generous gift from Dr. Studier at Brookhaven National Laboratory (Upton, NY). Oligonucleotides were synthesized on an Applied Biosystems model 380A synthesizer and purified on OPT™ from this company.

Library Screening, Plaque Purification, and Sequencing—A *agt11* human liver library (a gift from Ganeane Bell, University of Chicago) was screened by hybridization to a rat liver cDNA probe (3). The probe was hybridized at 42 °C in 50% formamide, 5 × SSPE, 0.05% SDS, 10 μg/ml salmon sperm DNA, and 5 × Denhardt’s solution for 12 h. The probe was labeled with [α-32P]dATP by the random primer method (13). Phage plaques were purified, and cDNA inserts were subcloned into the pBS/SK(+) plasmid (14). Sequencing was carried
using the Sanger dideoxy method (15). Cloned purified double-stranded DNA templates were primed with either M13 (forward and reverse) or rat liver 6PF-2-K/ Fru-2,6-Pase (3) primers. The nucleotide sequence is briefly described in Ref. 12. All sequence analysis was done using the GCG (Madison, WI) software package (16). Multiple sequence alignments were done using the GAP program with default parameters, and the SwissProt data base was searched using the FASTA program.

Construction of pET Human Liver 6PF-2-K/Fru-2,6-Pase Vectors—The expression of the human liver 6PF-2-K/Fru-2,6-Pase was accomplished using the T-7 RNA polymerase-based system of Studier and coworkers (17). The 3050-bp human liver 6PF-2-K/ Fru-2,6-Pase cDNA fragment (12) was ligated into the EcoRI site of the pET3A vector (17, 18), and the proper orientation was determined. This construct was then digested with NdeI and SacI and religated with an NdeI-SacI linker, which established the ATG in the proper position for translation in this system. The 1,400-base pair 5' cDNA fragment was then ligated into the pET3A vector containing the 5' coding sequence, which had been digested at the EcoRI site. The orientation of the 1,400-base pair 3' fragment was confirmed by restriction enzyme digestion analysis. This construct, designated pETHL2K, now contained the entire coding region of human liver 6PF-2-K/Fru-2,6-Pase cDNA in the proper position and orientation for expression.

Site-directed Mutagenesis—Mutants at Pro-2 were introduced into the pETHL2K expression construct by the cassette method whereby complementary oligonucleotides were synthesized containing NdeI and SacI restriction sites and terminal cDNA sequences. The NdeI site was placed just upstream of the start codon, ATG, and a naturally occurring SacI site is 24 bases downstream. The sequences for the forward and reverse oligonucleotides for the Pro-2 → Arg (CGA), the proline (CCG), proline (CTG), proline (CCC), and the Pro-2 deletion were 5'-TATG/TCT/CTG/XXX/GAG/ATG/GGA/GAG/CT-3' and 5'-CTC/TCT/CTG/XXX/AGA/CA-3', where XXX represents the arginine codon, one of the three proline codons, or the deletion of the proline codon, respectively.

Induction of 6PF-2-K/Fru-2,6-Pase in E. coli—E. coli strain BL21(DE3) carrying the pET3A plasmid expression construct were grown in LB medium containing ampicillin (100 μg/ml) at 37°C until an A600 of 0.8 was reached. A sample of cell culture was removed for a "minus induction" control, the remaining culture then induced by the addition of IPTG to a concentration of 0.4 mM and grown at 22°C for 4 h unless otherwise indicated. Extracts were prepared by centrifugation of the cultures, resuspension in buffer B plus 1 mg/ml lysozyme, and freezing (dry ice/ethanol) and thawing four times. The extract was made 10 mM in MgSO4. DNano 1 was added to a final concentration of 5 μg/ml, and the extract was placed on ice for 1 h. Cellular debris was removed by centrifugation at 10,000 × g for 10 min, and the supernatants were either assayed or further purified.

Determination of Expressed 6PF-2-K/Fru-2,6-Pase mRNA Levels—The RNA from the BL21(DE3) cells by the method described in Ref. 14, with the following modification. The RNA was treated with RNase-free DNase I at a final concentration of 5 units/μl for 30 min at 37°C. RNA was then phenol extracted and ethanol precipitated. RNA samples were then denatured in 100 mM methyl mercury at room temperature for 10 min and loaded onto a slot-blot apparatus fitted with a nylon membrane filter (GeneScreen). The filter was hybridized with a 1,400-base pair human liver cDNA (12) probe labeled as described above and then washed as described above. The filter was exposed to film for radioautography, filter slots cut out, and mRNA quantitated by scintillation coupling.

Western Blot Analysis—Culture samples were centrifuged, and cell pellets were dissolved in SDS gel buffer, heated for 5 min at 100°C and run on SDS-PAGE (10% acrylamide) with prestained protein standards. The electrophoresed proteins were electroeluted onto cellulose-nitrate membranes at 60 mA for 12 h. Membranes were incubated in 100 ml of 3% powdered milk/immunobuffer (20 mM Tris, 150 mM NaCl, pH 7.5) at 37°C for 2 h. Primary antibody, anti-6PF2K (19) (diluted 1:1,000) was added directly to the blocking solution and incubated at 37°C for 2 h. Membranes were washed five times for 30 min each with 100 ml of immunobuffer. Membranes were incubated in 100 ml of 3% powdered milk/immunobuffer for 2 h at 37°C and then rinsed as above. Color was developed by immersing the membrane in 100 ml of immunobuffer containing 20% methanol, 60 μg of color-developing reagent (4-chloro-1-naphthol) in 60 μl of 30% H2O2, and 30 μl of 3% H2O2.

Determination of 6PF-2-K/Fru-2,6-Pase Activities—6PF-2-kinase activity was measured at pH 7.8 by quantitation of the production of Fru-2,6-P2, as described previously (20). Fru-2,6-Pase activity was assayed by following the rate of production of 32P, from [2-32P]Fru-2,6-P2 (21, 22).

Protein Determination—Protein concentration was determined with the BCA protein assay reagent from Pierce Chemical Co. using the enhanced protocol and bovine serum albumin as a standard.

RESULTS

Primary Sequence of Mammalian Hepatic 6PF-2-K/Fru-2,6-Pases—The primary sequences of the rat, bovine, and human enzymes are compared in Fig. 1, which shows that these forms are highly homologous. For example, the human and rat liver enzymes have a 96% amino acid identity. All three sequences have a consensus cAMP-dependent phosphorylation site at Ser-32 which includes a serine residue preceded by 3 arginyl residues and a single spacer amino acid (Gly). Recent work has identified a number of important catalytic and substrate-binding residues in the rat liver enzyme (23, 24). For example, the ATP signature sequence β-loop marking the turn between the first β-strand and first α-helix is conserved in all of these structures. Two basic residues, Arg-230 and Arg-238, have been shown to be important ATP-binding residues in the rat liver enzyme (23) and are conserved in the bovine and rat liver forms. The active site sequence (resides 143-180) including the putative base catalyst His-165 (24) is conserved in all three enzymes. Li et al. (23) demonstrated that Arg-135 of the rat liver enzyme is an essential residue for Fru-6-P and Fru-2,6-P2 binding to the kinase domain of the rat liver enzyme, and this residue is also conserved in the bovine and human liver enzymes. The catalytic residues, His-258, Glu-327, and His-392, are a hallmark of all three enzymes. Based on this analysis one would predict that the human liver enzyme would have kinetic and physical properties that are identical to those of the rat liver enzyme. Since the tissue source for the human enzyme is not available for purification of the enzyme, this prediction could only be tested by expressing the human and rat liver enzymes in a bacterial expression system and by comparing their properties.

Lack of Expression of Human Liver 6PF-2-K/Fru-2,6-Pase in the pET-3A T-7 RNA Polymerase-Based Expression System—The coding region of the human liver cDNA of 6PF-2-K/Fru-2,6-Pase was cloned into the pET3A expression vector at the NdeI and EcoRI restriction sites as previously described for the rat liver enzyme (9). This construct was transformed into BL21(DE3) cells and expression was induced with IPTG. However, no expression of 6PF-2-K/Fru-2,6-Pase protein was detected even though a variety of growth conditions, induction temperatures (22°C and 37°C), and growth media (MS and LB medium) were used. A number of methods were used to detect the induced protein including Coomassie Blue staining of total E. coli protein on SDS-polyacrylamide gels, 125I-methionine incorporation, and analysis of newly synthesized protein by autoradiography after SDS-PAGE, or by monitoring E-P formation, a very sensitive method for bi-functional enzyme protein which detects nanogram quantities of enzyme (22). In contrast, rat liver 6PF-2-K/Fru-2,6-Pase was readily induced, based on Coomassie Blue staining, to a level of 10 mg/liter after 4 h of induction with IPTG at 22°C (data not shown), which confirms previous results (9).

Expression of the Pro-2 → Arg Mutant of Human Liver 6PF-2-K/Fru-2,6-Pase—The lack of expression of the human liver enzyme was surprising since the sequence of the coding regions of the human and rat cDNAs is highly con-
**Effect of Different Proline Codons on Human Liver Bifunctional Enzyme Expression**—To determine if wild-type human liver enzyme expression was limited by usage of the CCA (wild-type) codon by *E. coli* we constructed expression plasmids containing the other three proline codons (CCG, CCT, and CCC). As seen in Fig. 2A, lanes 7–12, none of these expression constructs produced detectable amounts of protein, demonstrating that regardless of which proline codon was used there was no expression of 6PF-2-K/Fru-2,6-P2ase. Additionally, an expression construct was engineered in which the proline was not seen in Fig. 2A, lanes 7–12, and none of these constructs expressed 6PF-2-K/Fru-2,6-P2ase protein in high amounts (Fig. 2A, lanes 13 and 14). As can be seen in Fig. 2A, lanes 3, 4, and 7–12, no expression of 6PF-2-K/Fru-2,6-Pase was seen in *E. coli* containing a proline at position 2, whereas induction of 6PF-2-K/Fru-2,6-Pase was seen in *E. coli* containing the constructs in which the proline was not present (Fig. 2A, lanes 5, 6, 13, and 14). The results clearly demonstrate that the presence of proline per se prevents the expression of the human enzyme and that codon usage is not a factor.

**Effect of IPTG on 6PF-2-K/Fru-2,6-P2ase mRNA Induction**

| Lane | Description |
|------|-------------|
| 1    | Blpflk     |
| 2    | Blpflk     |
| 3    | Blpflk     |
| 4    | Blpflk     |
| 5    | Blpflk     |
| 6    | Blpflk     |
| 7    | Blpflk     |
| 8    | Blpflk     |
| 9    | Blpflk     |
| 10   | Blpflk     |
| 11   | Blpflk     |
| 12   | Blpflk     |
| 13   | Blpflk     |
| 14   | Blpflk     |

*Note: The table above is a representation of the data presented in the text. The exact values and conditions are not shown.*
for Wild-type 6PF-2-K/Fru-2,6-P_2ase and Its Pro-2

for Wild-type 6PF-2-K/Fru-2,6-P_2ase and Its Pro-2 → Arg Mutants—To determine whether the lack of 6PF-2-K/Fru-2,6-P_2ase expression was caused by a defect in transcription, the induction of messenger RNA specific for 6PF-2-K/Fru-2,6-P_2ase was measured in E. coli cultures transformed with expression plasmids containing the coding sequence for the wild-type bifunctional enzyme and its Pro-2 → Arg mutants. As seen in Fig. 3, induction of specific mRNA was seen in all samples. The fold induction was minimally 12-fold, and there was no correlation between induction of the specific mRNA and expression of the protein. Based on this result, it was concluded that the mRNA levels were sufficient for expression and that transcription was not compromised by the presence of a proline codon at the N-2.

Effect of the N-2 Proline on the Degradation of 6PF-2-K/Fru-2,6-P_2ase in E. coli Cytosol—A rapid rate of protein degradation would explain why expression of the wild-type enzyme was not detected by Coomassie Blue staining of total E. coli proteins after SDS-PAGE. To detect early induction of the protein and its degradation products, Western blots of total E. coli extracts were utilized, and the human liver enzyme was detected with a high titer polyclonal antiserum to the rat liver enzyme (19). Although the wild-type human liver 6PF-2-K/Fru-2,6-P_2ase was not detectable by Coomassie Blue staining, it was easily visualized by Western blot analysis, which has much more sensitivity. As can be seen in the time course in Fig. 2B, not only was there an intact bifunctional enzyme subunit (55 kDa) detected with the specific antiserum after IPTG induction, but a major 30-kDa degradation product was also observed at early induction times. Over this relatively short time course (4 h) the 30-kDa peptide and other degradation products appeared in an increasing proportion compared with the intact 55-kDa peptide. This finding indicates that degradation of the wild-type protein in E. coli cytosol is taking place at a rapid rate. After 1–2 h degradation outstrips synthesis, and accumulation of the undegraded protein does not occur. Consistent with this hypothesis is the appearance of the 30-kDa degradation product (see arrow) which is visible in Fig. 2A upon Coomassie Blue staining of total E. coli protein only in the induced (+IPTG) lanes in which proline residues are present at N-2 (lanes 4, 8, 10, and 12). Intact bifunctional enzyme protein accumulates only in the induced lanes in which proline is not present at N-2. It is concluded that wild-type human liver 6PF-2-K/Fru-2,6-P_2ase is not expressed in E. coli because it is rapidly degraded due to the presence of proline at N-2.

**Purification of Human Liver 6PF-2-K/Fru-2,6-P_2ase—**The expressed human liver 6PF-2-K/Fru-2,6-P_2ase Pro-2 → Arg mutant was purified to homogeneity from E. coli cell extracts by sequential column chromatography on Q-Sepharose and phosphocellulose, with slight modifications of the previously described method for the rat liver enzyme (26). After freezing and thawing E. coli extracts, a crude extract from 2 liters of E. coli (BL21(DE3)) culture in buffer B (20 mM Tris-Cl, 2 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) plus 50 mM KCl, pH 7.8, was applied to a Q-Sepharose column (2 × 25 cm) that had been equilibrated in the same buffer. The column was then washed with 5 column volumes of this buffer and eluted with a linear KCl gradient (0.05–1.0 M KCl) in buffer B. Active fractions were pooled, precipitated with (NH_4)_2SO_4 to a saturation of 75%, and resuspended in buffer B plus 10 mM KCl, pH 7.5. After extensive dialysis in this buffer, this sample was then applied at a rate of 30 ml/h to a phosphocellulose column (2
Human Liver 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase

The column was then washed with 5 column volumes of buffer, and the enzyme was eluted in the same buffer supplemented with 2 mM Fru-2,6-P₂. Table I shows the purification scheme. Since the enzyme was the major protein present in the crude soluble fraction, it only required a 51-fold purification to achieve a homogeneous preparation, and the yield was 41%. SDS-PAGE and Coomassie Blue staining of the human liver form and the pure rat liver form of the enzyme is shown in Fig. 4 and revealed that the enzyme was homogeneous and had the same molecular mass (55 kDa) as the rat liver enzyme.

Kinetic Properties of Human Liver 6PF-2-K/Fru-2,6-P₂ase—The kinetic properties of the rat and human liver Pro-2→Arg mutant forms of 6PF-2-K/Fru-2,6-P₂ase are shown in Table II. All of the kinetic parameters of the human liver enzyme, including Vₘₐₓ, Kₘ for Fru-6-P, Kₘ for ATP of the 6PF-2-K reaction, and Vₘₐₓ, Kₘ for Fru-2,6-P, Kᵢ for Fru-6-P, and E-P formation of the bisphosphatase, were very similar and in good agreement with the literature values for the rat liver form (27). Fig. 5 and Table II also show that the affinity for Fru-6-P of both enzymes was increased by inorganic phosphate. In the absence of phosphate the Kᵢ for Fru-

![Table I](image)

**Table I**

| Protein          | 6PF-2-K activity (milliunits/mg) | Specific activity (milliunits/mg) | Fold purification | Yield |
|------------------|----------------------------------|----------------------------------|-------------------|-------|
| Extract          | 855                              | 760                              | 0.9               | 1     |
| Q-Sepharose       | 116                              | 592                              | 5.1               | 6     |
| Phosphocellulose  | 7                                | 310                              | 46.0              | 51    |

The human liver Pro2Arg mutant was purified after extraction from 2 liters of E. coli culture by sequential column chromatography on Q-Sepharose and phosphocellulose as described under "Results." The enzyme was monitored by E-P formation as described under "Experimental Procedures." 6PF-2-K and Fru-2,6-P₂ase activities were determined as described under "Experimental Procedures." Where indicated enzyme assays were done in the presence of 5 mM KPi. Kᵢ for Pi is defined as the concentration giving half-maximal activation of 6PF-2-K at a concentration of Fru-6-P equal to the Kᵢ value. Values represent the average ± S.E. for three to five determinations.

**Table II**

Comparison of kinetic properties of human liver Pro2Arg of bifunctional enzyme with those of the rat liver enzyme expressed in E. coli

![Fig. 4](image)

**Fig. 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of human liver Pro-2→Arg mutant and rat liver wild-type 6PF-2-K/Fru-2,6-P₂ase. Human liver Pro-2→Arg mutant and rat liver wild-type enzyme were purified from the expressed E. coli cells as described under "Experimental Procedures." 3 µg each of the enzyme was subjected to 12% SDS-PAGE, and the gel was stained with Coomassie Blue. The protein standards were a mixture of 5 µg each of bovine albumin (67 kDa), egg albumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and bovine milk α-lactalbumin (14 kDa).

![Fig. 5](image)

**Fig. 5.** Fru-6-P saturation curves of the kinase activity of human liver Pro-2→Arg and rat liver wild-type enzymes. The 6PF-2-K/Fru-2,6-P₂ase activities of the human liver Pro-2→Arg mutant (solid circles) and the rat wild-type (open circles) enzymes were measured in the absence (panel A) and presence (panel B) of 5 mM KPi, by following the production of Fru-2,6-P₂ as described under "Experimental Procedures."
6-P was about 1 mM for both enzymes, and the addition of 5 mM inorganic phosphate decreased the $K_m$ values for Fru-6-P to about 20 μM. The concentration dependence of the effect of phosphate was the same for both the human and rat liver enzyme (0.022 ± 0.012 mM and 0.036 ± 0.010 mM, respectively). The only significant difference in the properties of the two enzymes, as summarized in Table II, was that the $K_m$ for ATP for the human liver Pro-2 → Arg mutant was 3-fold higher than that of the rat liver enzyme.

However, a striking difference between the rat and the human Pro-2 → Arg mutant forms was observed when the Fru-6-P concentration dependence of the kinase reaction was examined in more detail. As shown in Fig. 5A, the human form, but not the rat liver enzyme, exhibited substrate inhibition by Fru-6-P. Inhibition of 41% was observed at a Fru-6-P concentration of 2 mM in the presence of 5 mM KPi. Substrate inhibition increased to 50% in the absence of inorganic phosphate when the substrate concentration was increased to 50 mM (Fig. 5B). In the absence and presence of inorganic phosphate the rat liver form exhibited normal Michaelis-Menten saturation kinetics, and no substrate inhibition was observed. As shown in Fig. 5A both the human and rat liver forms exhibited identical dependence on substrate concentration in the presence of inorganic phosphate at low Fru-6-P concentrations (0-0.3 mM), with substrate inhibition manifest only at concentrations of Fru-6-P above 0.3 mM. This result suggests that Fru-6-P is binding to the E-ADP complex (see "Discussion") and/or that the reaction mechanisms of the rat and human liver 6PF-2-K/Fru-2,6-Pase are different.

**DISCUSSION**

A comparison of the kinetic properties of human liver 6PF-2-K/Fru-2,6-Pase Pro-2 → Arg mutant with those of rat liver 6PF-2-K/Fru-2,6-Pase revealed that these enzymes are very similar as would be predicted from their high degree of amino acid sequence identity (96%). The $K_m$ for ATP of the kinase reaction was the only kinetic parameter that was different when the rat and human liver forms were compared. However, the most striking difference between the two enzymes was the finding that the human, but not the liver form, was subject to substrate inhibition of the kinase reaction by Fru-6-P. Substrate inhibition is observed in the 0.10 mM range of Fru-6-P concentrations, which represents physiological concentrations of the sugar phosphate in liver cytosol (19). The mechanism of substrate inhibition of the human enzyme is unknown. One possibility is that there is another Fru-6-P binding site and/or additional Fru-6-P binding determinants in the human enzyme. This would appear unlikely based on the high degree of homology between the two forms including conservation of all known catalytic and substrate binding site residues in the kinase domain. For example, the most important Fru-6-P binding residue in the liver 6PF-2-K domain, Arg-195, is conserved in the human enzyme (Fig. 1). Also, Arg-225, another residue involved in Fru-6-P binding based on its reactivity with phenylglyoxal (28), is similarly conserved. Detailed kinetic analysis, i.e. product inhibition studies, have not yet been done for the human liver enzyme, and therefore no direct evidence exists vis-a-vis its reaction mechanism. However, based on the assumption that the reaction mechanism for the human liver 6PF-2-K is the same as the rat liver enzyme, that is an ordered Bi Bi scheme (29), substrate inhibition can be explained as in Mechanism 1. In this mechanism, the second substrate, B (Fru-6-P) cannot only interact with the E-A (E-ATP) complex but also interacts with the E-Q (E-ADP) complex, which results in substrate inhibition. Work is in progress to obtain evidence for this mechanism by a combination of kinetic analysis and site-directed mutagenesis. The latter approach can be used to define the amino acid residue(s) responsible for the putative E-ADP·Fru-6-P complex. The observed kinetic differences between the rat and human enzymes are confined to the kinase reaction, since all of the kinetic properties of the bisphosphatase were identical for the two enzymes, which is consistent with the conservation of the catalytic triad (His-258, Glu-327, His-392) (11, 30) and all substrate/product binding residues (Arg-257, Arg-307, Arg-352, Lys-356, and Arg-360) (31–32). Interestingly, the rat liver bisphosphatase exhibits strong substrate inhibition which is also observed for the human liver bisphosphatase domain. A difference in the amino acid sequence exists at N-2 between the human and rat forms. The human bifunctional enzyme has a proline in place of an arginine at this position. Since the wild-type human liver 6PF-2-K/Fru-2,6-Pase was not expressed in the pET system while the rat liver enzyme was expressed to high levels, the N-2 residue of the human sequence was mutated to an arginine to recapitulate the rat sequence at N-2. With this change, high levels of expression were seen for the human liver 6PF-2-K/Fru-2,6-Pase. We have demonstrated that the lack of expression was because of an increased rate of protein degradation of the human liver 6PF-2-K/Fru-2,6-Pase when N-2 was occupied by a proline residue and not because of a lack of transcription of the mRNA. The targeting of human liver 6PF-2-K/Fru-2,6-Pase for degradation in E. coli by a proline at N-2 may be analogous to how certain amino acid residues at the N-end, or N-1, target proteins for degradation in a number of different cell types, including E. coli (33), yeast (34), reticulocytes (35), and some mouse cell lines (36). The N-end rule (33, 37) describes how the residues Arg, Lys, Leu, Phe, Tyr, or Trp at N-1 confer half-lives of 2 min or less to proteins in bacteria, whereas all other amino acid residues at this position confer half-lives of greater than 10 h. Interestingly, the status of proline in the E. coli N-end rule is unknown (33). It is possible that this N-2 amino acid, by virtue of its structural characteristics and geometry in relation to other residues, acts as a primary destabilizing residue much as do other destabilizing E. coli protein NH$_2$-terminal residues, e.g. Arg, Lys, Phe, Leu, Trp, and Tyr (33). The ATP-dependent protease clp is required for protein degradation by N-end rule substrates (37). A search (16) of 1510 E. coli amino acid sequences in the SwissProt data base revealed that the sequence Met-X-Pro occurred 35 times but never at the NH$_2$-terminus of any protein. This observation supports the hypothesis that proline may not occur at N-2 in E. coli because of its destabilizing effect.

The results of this study suggest that the N-2 proline of the human liver 6PF-2-K/Fru-2,6-Pase acts as a degron in the E. coli expression system. The minimal geometry for degron formation of the human liver 6PF-2-K/Fru-2,6-Pase has not been established, nor has a folding defect been completely ruled out. The N-2 degron of the human liver 6PF-2-K/Fru-2,6-Pase may be a substrate for another protease system in E. coli (BL21(DE3)), which is deficient in the ATP-dependent lon protease (17, 19). It is not known whether the presence of proline at N-2 is sufficient in other E. coli proteins to form degrons that could be described by an N-2 rule. The minimal requirements for expression arrest can be studied by introducing this NH$_2$-terminal region into other proteins that are...
expressed at high levels, and such studies are currently in progress.

Acknowledgment—We thank A. Varshavsky for invaluable advice and discussions in the preparation of this manuscript.

REFERENCES

1. El-Maghrabi, M. R., Colosio, A. D., Lange, A. J., Tauler, A., Kurland, I. J., and Pilikis, S. J. (1990) in Fructose-2,6-Bisphosphatase (Pilkis, S. J., ed.) pp. 87-124, CRC Press, Boca Raton, FL.
2. Darville, M. I., Crepin, K. M., Hue, L., and Rousseau, G. G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6543-6547.
3. Colosio, A. D., Lovely, M. O., El-Maghrabi, M. R., and Pilikis, S. J. (1987) Biochem. Biophys. Res. Commun. 152, 1092-1098.
4. Darville, M. I., Crepin, K. M., Van der Venn, J., and Tauler, A., Lin, K., and Pilkis, S. J. (1987) J. Biol. Chem. 262, 17939-17943.
5. Sakata, J., and Uyeda, K. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4951-4955.
6. Sakata, J., Abe, Y., and Uyeda, K. (1991) J. Biol. Chem. 266, 15764-15770.
7. Ventura, F., Ross, J. L., Ambrozio, S., Pilikes, S. J., and Bartrons, R. (1992) J. Biol. Chem. 267, 17943-17948.
8. Lange, A. J., El-Maghrabi, M. R., and Pilikis, S. J. (1991) Arch. Biochem. Biophys. 280, 258-263.
9. Tauler, A., Lange, A. J., El-Maghrabi, M. R., and Pilikis, S. J. (1992) Arch. Biochem. Biophys. 280, 258-263.
10. Tauler, A., Rosenburg, A. H., Colosio, A. D., Studier, W., and Pilikis, S. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6645-6649.
11. Tauler, A., Lin, K., and Pilikis, S. J. (1990) J. Biol. Chem. 265, 15617-15622.
12. Lange, A. J., and Pilikis, S. J. (1990) Nucleic Acids Res. 18, 3652-3655.
13. Feinburg, A. P., and Vogelstein, B. (1984) Anal. Biochem. 137, 266-273.
14. Ausuble, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds.) (1989) Protocols in Molecular Biology, Wiley Interscience, New York.
15. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467.
16. Devereaux, J., Haerberli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387-393.
17. Studier, F. W., and Moffat, B. M. (1986) J. Mol. Biol. 189, 113-130.
18. Studier, F. W., Rosenburg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 69-89.
19. Colosio, A. D., Marker, A. J., Lange, A. J., El-Maghrabi, M. R., Graner, D. K., Tauler, A., Pilikis, J., and Pilikis, S. J. (1988) J. Biol. Chem. 263, 18669-18677.
20. El-Maghrabi, M. R., Zate, T. M., Murray, K. J., and Pilikis, S. J. (1984) J. Biol. Chem. 259, 13096-13103.
21. Pilikis, S. J., El-Maghrabi, M. R., McGrane, M., Pilikis, J., Fox, E., and Claus, T. H. (1982) Mol. Cell. Endocrinol. 20, 243-266.
22. El-Maghrabi, M. R., Correia, J. J., Heil, P. J., Petef, T. M., Cobb, C., and Pilikis, S. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5005-5009.
23. Li, L., Lin, K., Kurland, I. J., Correia, J. J., and Pilikis, S. J. (1992) J. Biol. Chem. 267, 4836-4839.
24. El-Maghrabi, M. R., Pate, J. T., D’Angelo, G., Correia, J. J., Lively, M. O., and Pilikis, S. J. (1987) J. Biol. Chem. 262, 11714-11720.
25. Lively, M. O., El-Maghrabi, M. R., Pilikis, J., D’Angelo, G., Colosio, A. D., Cavata, J., Frazer, B. A., and Pilikis, S. J. (1988) J. Biol. Chem. 263, 839-849.
26. Lin, K., Kurland, I. J., Xu, Z. L., Lange, A. J., Pilikis, J., El-Maghrabi, M. R., and Pilikis, S. J. (1990) in Protein Expression Purification, 1-2, 169-176.
27. Pilikis, S. J., Claus, T. H., Kountz, P. D., and El-Maghrabi, M. R. (1987) J. Biol. Chem. 262, 1-8.
28. Rider, M. H., and Hue, L. (1982) Eur. J. Biochem. 207, 967-972.
29. Kountz, P. D., Freeman, S. C., Cook, A. G., El-Maghrabi, M. R., Knowles, J. R., and Pilikis, S. J. (1988) J. Biol. Chem. 263, 16069-16072.
30. Lin, K., Li, L., Correia, J. J., and Pilikis, S. J. (1992) J. Biol. Chem. 267, 6556-6562.
31. Lin, K., Li, L., Correia, J. J., and Pilikis, S. J. (1992) J. Biol. Chem. 267, 19163-19171.
32. Lin, L., Lin, K., Pilikis, J., Correia, J. J., and Pilikis, S. J. (1992) J. Biol. Chem. 267, 21588-21594.
33. Tohll, J. W., Schild, G. A., and Varshavsky, A. (1991) Science 254, 1374-1377.
34. Bachmair, A., and Varshavsky, A. (1989) Cell 56, 1019-1033.
35. Varshavsky, A., Bachmair, A., Finley, D., Gonda, D., and Wartnig, I. (1988) in Ubiquitin (M. Rechsteiner, ed), pp. 287-324, Plenum Publishing Corp., New York.
36. Townsend, A., Bastin, J., Gould, K., Brownlee, G., Andrew, M., Coupur, B., Boyle, D., Chan, S., and Smith, G. (1988) J. Exp. Med. 168, 1211-1224.
37. Varshavsky, A. (1992) Cell 68, 725-735.

**Human Liver 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase**

**ATP** |
| (Fru-6-P) |
| (Fru-2,6-P) |
| ADP |

| E |
| E-A |
| (E-AB - E-PQ) |
| E-Q |
| —B |
| E |
| E-BQ |
| (Fru-6-P) |

**MECHANISM 1**