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Expression of Mammalian Liver Glycolytic/Gluconeogenic Enzymes in *Escherichia coli:* Recovery of Active Enzyme Is Strain and Temperature Dependent

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A number of mammalian enzymes have been expressed in *Escherichia coli* using the T7 RNA polymerase system, but the production of large amounts of these proteins has been limited by the low percentage of active enzyme that is found in the soluble fraction. In this report the effect of induction temperature was tested on the recovery of four rat liver enzymes, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, fructose-2,6-bisphosphatase, glucokinase, and fructose-1,6-bisphosphatase. We also tested the effect using a host cell strain that contains a plasmid encoding T7 lysozyme, an inhibitor of T7 RNA polymerase. Large amounts of the first three enzymes accumulated in the cells after 4 h of induction at 37°C, but only about 1–2% of the total expressed proteins were recovered in a soluble, active form. When the induction was carried out at 22°C for 48 h with the pLysS strain, 20- to 30-fold higher amounts of the active expressed enzymes were recovered in the soluble fraction, even though the total accumulation and the rate of synthesis of these proteins were reduced. The optimal concentration of isopropyl-1-thio-β-D-galactopyranoside required for induction was the same at both temperatures. On the other hand, the recovery of active fructose-1,6-bisphosphatase, a heat-stable enzyme, was 66% at 37°C and was essentially unchanged at an induction temperature of 22°C. Lowered induction temperature would appear to be of utility for enhanced recovery of active mammalian enzymes which are insoluble in *E. coli* cytosol at 37°C. Temperature stability of the protein may also influence the recovery of active enzyme. © 1990 Academic Press, Inc.

Liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2-K/Fru-2,6-P_2ase),[1] fructose-1,6-bisphosphatase (Fru-1,6-P_2ase), and glucokinase are important regulatory enzymes in the glycolytic/gluconeogenic pathway (1,2). Our knowledge of their molecular structure and function has been greatly aided by the cloning of their cDNAs and expression of the proteins in various cell types (3). The T7 RNA polymerase expression system has been used to express these three enzymes (4–6) as well as many other eukaryotic proteins (7), but a major problem has been the limited yield of active, expressed proteins (4–6,8). Typically, the yield of active, soluble protein is less than 1 mg/liter of culture. These three enzymes, along with the separate bisphosphatase domain (Fru-2,6-P_2ase) of 6PF-2-K/Fru-2,6-P_2ase (8), were used as model systems to investigate ways to maximize the yield of soluble, active enzyme. This report describes the effects of temperature and different *Escherichia coli* strains on the overexpression of 6PF-2-K/Fru-2,6-P_2ase, Fru-2,6-P_2ase, and glucokinase using the T7 system, which improve the yield of active enzyme to 10–13 mg/liter and which may be of general utility for proteins that tend to aggregate in *E. coli* cytosol at 37°C. In addition, a new rapid purification scheme for *E. coli*-expressed 6PF-2-K/Fru-2,6-P_2ase is reported.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes and bacteriophage T4 DNA ligase were obtained from New England Biolabs. Phosphocellulose was from Whatman. L-[^{35}S]Methionine (1061 Ci/mmol) was from DuPont. Egg white lysozyme, isopropyl-1-thio-β-D-galactopyranoside (IPTG), DNase I, polyethylene glycol 8000, Fru 6-P, and Fru 2,6-P_2, were obtained from Sigma. Bacterial

[1] Abbreviations used: 6PF-2-K/Fru-2,6-P_2ase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Fru-1,6-P_2ase, fructose-1,6-bisphosphatase; Fru-2,6-P_2ase, fructose-2,6-bisphosphatase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Fru 6-P, fructose 6-phosphate; Fru 2,6-P_2, fructose 2,6-bisphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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strains BL21(DE3) and BL21(DE3)pLysS were kindly provided by Dr. William Studier (Brookhaven National Lab). Color development reagent for immunoblotting was obtained from Bio-Rad.

Protein expression. The proteins were expressed by using the phage T7 expression system described by Studier and colleagues (7,9). *E. coli* BL21(DE3) and BL21(DE3)pLysS strains were transformed with pET/6PF-2-K/Fru-2,6-P_2ase (4), pET/glucokinase (5), pET/Fru-2,6-P_2ase (8), or pET/Fru-1,6-P_2ase (6) expression plasmids and were streaked on LB/ampicillin or LB/ampicillin plus chloramphenicol plates, respectively. Single colonies were picked from each plate and grown overnight. Five milliliters of overnight culture was added to 1 liter of M9 medium containing 100 μg/ml ampicillin and 30 μg/ml chloramphenicol for the BL21(DE3)pLysS strain and 100 μg/ml ampicillin for the BL21(DE3) strain. Cultures were grown at 37°C until the $A_{600\ nm}$ reached 0.7. Each strain was then divided into two flasks and grown at 37°C and at 22°C for 30 min. IPTG was then added to a final concentration of 0.4 mM, and the cultures grown at 37°C for 4 h or at 22°C for 48 h. Cell growth was monitored by measuring $A_{600\ nm}$ from the time of inoculation to the end of the induction. Cells were diluted when the absorbance was greater than 0.6.

Estimation of total induced protein. Cells (100 μl) were taken from each strain at the two different temperatures and harvested by centrifugation just before induction and at various times after induction. The cell pellets were dissolved in SDS–PAGE loading buffer, boiled for 5 min, and subjected to SDS–PAGE, and the gels were stained with Coomassie blue. The amount of protein was estimated from the stained protein bands using the purified enzymes as standards.

Estimation of soluble enzyme activity and total soluble protein. Cells (10 ml) were taken at various times, centrifuged, washed in buffer B (20 mM 2 *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, pH 7.5/100 mM KCl/1 mM dithiothreitol/2 mM EDTA/5 mM potassium phosphate/0.5 mM phenylmethanesulfonyl fluoride/2.5 μg leupeptin/ml), and pelleted again, and the pellet was suspended in 0.5 ml of buffer B containing 0.5 μg/ml lysozyme. The cells were frozen and thawed three times, treated with 5 mg of DNase I in the presence of 5 mM $MgSO_4$ at 4°C for 1 h, and centrifuged (12,000*g* for 5 min). The supernates were assayed for soluble 6PF-2-K/Fru-2,6-P_2ase and Fru-2,6-P_2ase by the E-P assay (10) and for glucokinase (11) and Fru-1,6-P_2ase (12) by spectrophotometry. Total soluble protein was estimated by absorption at 280 nm and/or by Lowry protein determination (13).

Rate of [^{35}S]methionine incorporation. Cells (100 μl), taken at various times, were incubated with [^{35}S]methionine (20 μCi/ml; 1 μCi = 37 kBq) for 3 min at 37°C before harvesting and treatment with SDS–PAGE loading buffer. The gels were exposed for 6 h and the rate of incorporation of [^{35}S]methionine was estimated by autoradiography and scanning densitometry and/or by cutting out the bands and determining incorporation directly by liquid scintillation counting.

Enzyme assays. 6PF-2-K and Fru-2,6-P_2ase activities were assayed as previously described (4).

Determination of plasmid copy number. Just before induction by IPTG and at the end of the induction period, the cells from 1-ml aliquots of the growing cultures were harvested by centrifugation and lysed by the addition of 50 mM NaOH/1% SDS/5 mM EDTA/8% glycerol. The heated lysate (100°C for 2 min) was applied to a 1% agarose gel and the plasmid copy number was determined after staining of the DNA with ethidium bromide.

Immunoblot analysis. Immunoblot analyses were performed essentially as described by Towbin *et al.* (14) with a 1:500 dilution of the antiserum as described previously (8).

Other methods. [2-^{32}P]Fru 2,6-P_2 was prepared enzymatically from [γ-^{32}P] ATP and Fru 6-P as described (15).

RESULTS

The Effect of Temperature on Expression of 6PF-2-K/Fru-2,6-P_2ase

Induction of protein using the T7 RNA polymerase system is usually carried out at 37°C. Figure 1A shows the time course of induction by IPTG of 6PF-2-K/Fru-2,6-P_2ase in the BL21(DE3) strain of *E. coli* at this temperature. Induction was seen as early as 30 min and the amount of enzyme protein increased steadily, reaching 45 mg/liter of culture at 4 h. However, only a very small fraction of 6PF-2-K/Fru-2,6-P_2ase was active; only 0.6% at 2 h and 0.8% at 4 h of the total amount of expressed protein.

One reason for the low recovery of soluble, active enzyme may be that the rate of protein production is too rapid for proper folding in the milieu of the host cell and/or that the protein is inactivated at 37°C. Both possibilities could be tested by inducing protein expression at a lower temperature. Figure 1B shows the induction by IPTG of 6PF-2-K/Fru-2,6-P_2ase expression in the same strain of *E. coli* at 22°C. There was an increase in the total amount of bifunctional enzyme protein that reached a maximum which was substantially less than that obtained at 37°C (20 mg/liter vs 45 mg/liter). This level remained unchanged from 5 to 45 h. Despite a lower level of total protein expression, the fraction of soluble, active enzyme continued to increase, reaching a

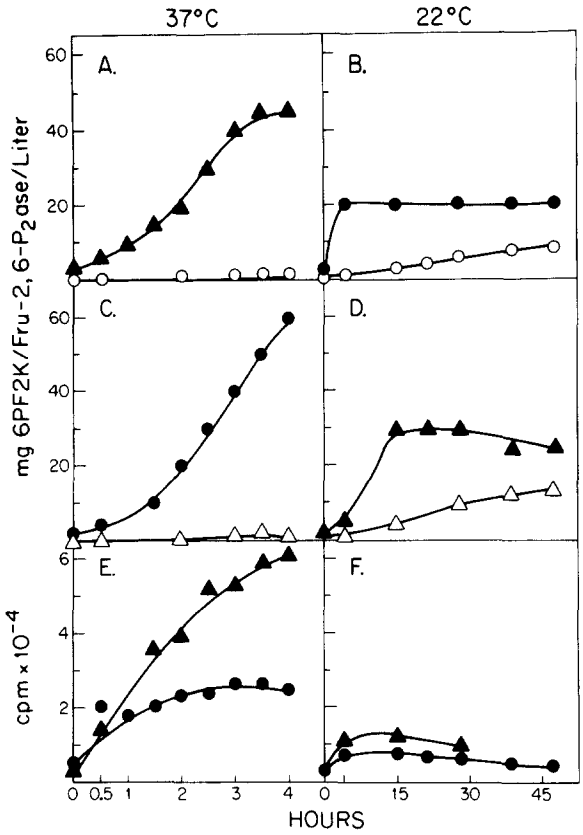

FIG. 1. Time course of expression and synthesis of rat liver 6PF-2-K/Fru-2,6-P_2ase in *E. coli.* Either BL21(DE3) or BL21(DE3)pLysS was transformed with pET/6PF-2-K/Fru-2,6-P_2ase expression plasmid and the protein was induced by the addition of 0.4 mM IPTG as described under Experimental Procedures. Total protein was estimated from Coomassie blue-stained SDS–PAGE gels using purified enzyme as a standard. Active enzyme was determined by E-P assay. (A) Induction in BL21(DE3) at 37°C: ▲, total 6PF-2-K/Fru-2,6-P_2ase protein; ○, active 6PF-2-K/Fru-2,6-P_2ase protein. (B) Induction in BL21(DE3) at 22°C: ●, total 6PF-2-K/Fru-2,6-P_2ase; ○ enzymatically active 6PF-2-K/Fru-2,6-P_2ase. (C) Induction in BL21(DE3)pLysS at 37°C: ●, total 6PF-2-K/Fru-2,6-P_2ase; △, active 6PF-2-K/Fru-2,6-P_2ase. (D) Induction in BL21(DE3)pLysS at 22°C: ▲, total 6PF-2-K/Fru-2,6-P_2ase; △, active 6PF-2-K/Fru-2,6-P_2ase. (E) [^{35}S]Methionine incorporation into 6PF-2-K/Fru-2,6-P_2ase during induction in BL21(DE3) ●, or BL21(DE3)pLysS ▲, at 37°C. (F) [^{35}S]Methionine incorporation rate into 6PF-2-K/Fru-2,6-P_2ase during induction in BL21(DE3) ●, or BL(DE3)pLysS ▲, at 22°C. The values in E and F represent the total counts incorporated during a 3-min pulse given at the indicated times. This experiment was repeated three times with essentially identical results.

level (7 mg/liter) at 40 h that was 14-fold higher than that obtained at 37°C (0.5 mg/liter).

Another possible way to slow the rate of protein production is to decrease the rate of transcription. The BL21(DE3)pLysS strain carries a plasmid (pLysS) encoding T7 lysozyme (7,9) which is a natural inhibitor of T7 RNA polymerase (16). Inhibition of the polymerase might be expected to inhibit protein production at early induction times. Figures 1C and 1D show the induction of 6PF-2-K/Fru-2,6-P_2ase in this strain at 37 and 22°C, respectively. There was very little difference between this strain and the BL21(DE3) strain in the total amount of protein produced. The only differences were a lag in the time, 15 h, taken to reach maximum levels of protein induction at 22°C rather than 3 h observed with the BL21(DE3) strain, and an even greater amount of active enzyme (13 mg/liter) in BL21(DE3)pLysS than in BL21(DE3) (7 mg/liter). In both strains and at both temperatures, the protein produced migrated with the same mass (55 kDa) and Western blotting revealed that it was immunologically identical to rat liver 6PF-2-K/Fru-2,6-P_2ase (data not shown). Consistent with the lack of difference in total protein produced between the strains, the plasmid copy number was also essentially identical for the two strains before and after induction (data not shown).

The increased recovery of active enzyme at 22°C may be due to a slower rate of synthesis of 6PF-2-K/Fru-2,6-P_2ase at this temperature. In order to estimate the rate of synthesis, cells were pulse-labeled with [^{35}S]methionine at various times after induction with IPTG. The rate of incorporation into the enzyme at 37°C increased in both strains, reaching rates at 4 h that were 10- and 20-fold greater than that in the noninduced state in the BL21(DE3) and BL21(DE3)pLysS strains, respectively (Fig. 1E). Except for β-lactamase, which is also induced (7,9), no other peptides showed increased incorporation (data not shown; see Ref. (9)). The rate of [^{35}S]methionine incorporation was also stimulated at 22°C in both strains (Fig. 1F), but the increase was much less than that seen at 37°C and the rate appeared to decline slightly after 15 h. At 37°C, 6PF-2-K/Fru-2,6-P_2ase synthesis represents 85–90% of all newly synthesized protein (4). In contrast to 37°C, [^{35}S]methionine incorporation into the bifunctional enzyme at 22°C accounts for only about 25% of the newly synthesized proteins (data not shown).

Effect of Temperature on the Recovery of Soluble 6PF-2-K/Fru-2,6-P_2ase

The above results show that lowered rates of protein production are related to the increased recovery of active 6PF-2-K/Fru-2,6-P_2ase. The increased amount of active enzyme recovered at 22°C could be a reflection of an increase in the fraction of soluble 6PF-2-K/Fru-2,6-P_2ase; i.e., soluble enzyme is equivalent to active enzyme. In order to test this hypothesis, the fraction of active enzyme, determined by direct enzyme assay, was compared with the amount of soluble 6PF-2-K/Fru-2,6-P_2ase estimated by SDS–PAGE and immunoblotting of soluble protein fractions extracted from *E. coli.* The amount of soluble, immunoreactive 6PF-2-K/Fru-2,6-P_2ase produced was 3- to 5-fold higher at 22°C than that

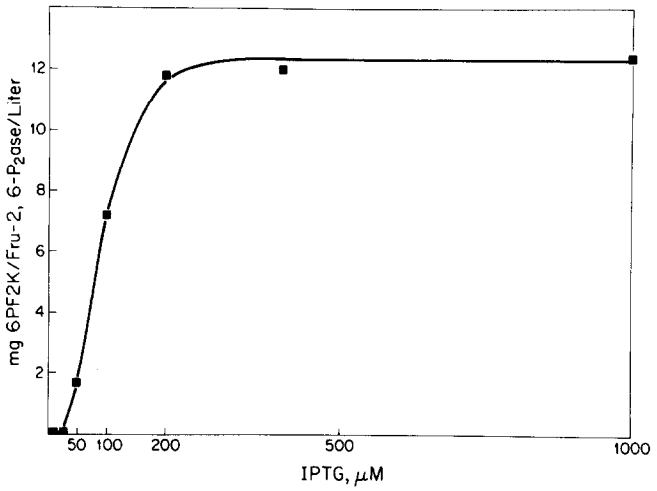

FIG. 2. Effect of IPTG concentration on the recovery of active 6PF-2-K/Fru-2,6-P_2ase. Induction was performed with the BL21(DE3)pLysS strain at 22°C as described under Experimental Procedures.

at 37°C (data not shown), while 20- to 30-fold more activity was seen at 22°C compared to that at 37°C. This strongly suggests that much of the immunoreactive "soluble" 6PF-2-K/Fru-2,6-P_2ase detected in extracts prepared from cells induced at 37°C represents either improperly folded enzyme and/or inactivated protein.

Effect of IPTG Concentration on the Recovery of Soluble 6PF-2-K/Fru-2,6-P_2ase

In the previous experiments, 0.4 mM IPTG was used in the induction step since we had shown that this was a maximally effective concentration for expression of 6PF-2-K/Fru-2,6-P_2ase in *E. coli* at 37°C (4–6,8). Figure 2 shows that the recovery of active 6PF-2-K/Fru-2,6-P_2ase at 22°C was also dependent on the concentration of inducer added. The concentration of IPTG giving half-maximal recovery of active protein was 0.09 mM and the effect was maximal at 0.2 mM and did not decline at high inducer concentration. This is essentially similar to the IPTG concentration dependence seen at 37°C (A. Tauler, unpublished results). These results suggest that both the level of inducer and the temperature are important in the recovery of biologically active proteins from *E. coli,* but that enhanced rates of protein production brought about by increasing inducer concentrations do not inhibit recovery of active protein.

Purification and Properties of E. coli-Expressed 6PF-2-K/Fru-2,6-P_2ase

Since expression of the bifunctional enzyme in BL21(DE3)pLysS reaches a high percentage of the total soluble protein, it should be possible to purify it more easily from this source than from rat liver where it is only 0.01% of the total soluble protein. A rapid, high yield purification scheme that utilizes a single affinity chromatography step to yield homogeneous enzyme was developed. One liter of BL21(DE3)pLysS carrying the pET/bifunctional enzyme expression plasmid (4) was grown in M9 medium at 37°C until the $A_{600\ nm}$ reached ~0.7. The temperature was then lowered to 22°C for 30 min and IPTG added to a final concentration of 0.4 mM. Cells were harvested after 40 h and cell extract was obtained as described previously (4,9). The extract was subjected to a 5–18% PEG precipitation, and the 18% PEG pellet was dissolved in 100 ml buffer A (20 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, pH 7.5, 10 mM KCl, 1 mM dithiothreitol 0.5 mM phenylmethanesulfonyl fluoride, 0.1 mM EDTA, and 2 μg/ml leupeptin) and loaded onto a 30-ml phosphocellulose column (Fig. 3, arrow 1). The column was first washed with 600 ml of buffer A, and then 6PF-2-K/Fru-2,6-P_2ase was eluted as a sharp peak with 2 mM Fru 6-P (Fig. 3, arrow 2). Both 6PF-2-K and Fru-2,6-P_2ase activities coeluted upon addition of 2 mM Fru 6-P to the col-

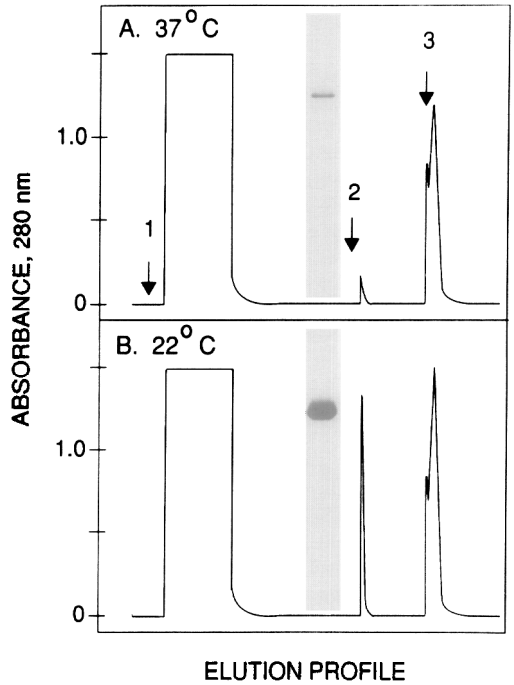

FIG. 3. Elution of *E. coli* expressed rat liver 6PF-2-K/Fru-2,6-P_2ase from phosphocellulose. (A) Enzyme purification from *E. coli* extracts when induction temperature was 37°C. (B) Enzyme purification from *E. coli* extracts when induction temperature was 22°C. Arrow 1 indicates the application of sample; arrow 2 indicates the addition of 2 mM Fru 6-P; and arrow 3 indicates the addition of 500 mM KCl. The tracing represents absorbance at 280 mm. Both 6PF-2-K and Fru-2,6-P_2ase activities eluted upon addition of 2 mM Fru 6-P (arrow 2). The insets represent SDS–gel electrophoresis of the protein peaks obtained after Fru 6-P addition.

umn. After elution of the enzyme, buffer A containing 500 mM KCl (Fig. 3, arrow 3) was added to elute protein remaining on the column. Only 5–10% of the total bifunctional enzyme activities were eluted with high salt while 90–95% eluted with 2 mM Fru 6-P. Figure 3 also shows that greater amounts of enzyme were obtained from *E. coli* induced at 22°C than from that induced at 37°C (see also insets, Fig. 3). The eluted enzyme was precipitated with 75% $(NH_4)_2SO_4$, dissolved in buffer A containing 50 mM KCl and 20% (V/V) glycerol, and dialyzed overnight against 500 ml of the same buffer with four buffer changes. The enzyme was homogeneous by the criterion of SDS–gel electrophoresis (see insets, Fig. 3), and migrated with a molecular size of 55 kDa. The yield of enzyme is 74% of that applied to the column (Table 1) and the procedure can be completed in one day. In contrast to the high yield of induced enzyme from *E. coli*, the yield of enzyme is only about 15% when purified from rat liver (17), only about 7 mg of enzyme can be obtained from 1 kg of rat liver, and the preparation takes about 1 week to complete. Table 2 shows that the properties of *E. coli*-expressed bifunctional enzyme were essentially identical to those of the native rat liver enzyme.

Effect of Temperature on the Recovery of Other Rat Liver Enzymes

The results above suggest that reduction of the temperature at which 6PF-2-K/Fru-2,6-P_2ase was induced was the single most important factor for increasing the amount of active enzyme. It was of interest to determine whether this effect was restricted to the rat hepatic bifunctional enzyme or whether it was a more general phenomenon. If the latter were the case, it would be expected that the yield of active protein would be increased for other enzymes at the lower induction temperature as well. The induction by ITPG of three other rat liver enzymes was compared at the two temperatures. The results are summarized in Table 3, along with those for 6PF-2-K/Fru-2,6-P_2ase. The bisphosphatase (Fru-2,6-P_2ase) domain of hepatic 6PF-2-K/Fru-2,6-P_2ase and glucokinase exhibited an induction pattern similar to that of the bifunctional enzyme. Induction produced more total protein at 37°C than at 22°C, but more active enzyme was recovered at 22°C. Also, the yield of active protein was greater from the BL21(DE3)pLysS strain than that from the BL21(DE3) strain. As in the case with 6PF-2-K/Fru-2,6-P_2ase, the plasmid copy number was the same for BL21(DE3) and the BL21(DE3)pLysS strains containing the glucokinase expression plasmid both before and after induction (data not shown). The pattern of enzyme synthesis, using [^{35}S]methionine pulse-labeling, also was the same for glucokinase as it was for 6PF-2-K/Fru-2,6-P_2ase (data not shown). In contrast, expression of Fru-1,6-P_2ase was very different from that of the other three enzymes (Table 3). More total enzyme protein was induced at 37°C than at 22°C but the fraction that was soluble and active was the same at both temperatures. Also, the BL21(DE3)pLysS strain produced less protein, both total and soluble, than did the BL21(DE3) strain.

TABLE 1

Purification of Wild-Type 6PF-2-K/Fru-2,6-P_2ase from *E. coli* Induced at 22°C

Stage	Protein (mg)	Kinase (mU)	Specific activity (mU/mg)	Purification (fold)	Yield (%)
Extract	305	513	1.7	1	100
PEG 5–18%	181	508	2.8	1.6	99
P-cellulose	10.3	378	37	22	74

Note. 6PF-2-K/Fru-2,6-P_2ase was induced in *E. coli* at 22°C as described in the text. Enzyme was monitored by measuring 6PF-2-K activity.

TABLE 2

Comparison of Kinetic Properties of Rat Liver and *E. coli*-Expressed Rat Liver 6PF-2-K/Fru-2,6-P_2ase

Kinetic parameter	Rat liver 6PF-2-K/ Fru-2,6-P_2ase	*E.coli*-expressed 6-PF-2-K/ Fru-2,6-P_2ase
6-PF-2-K		
V_{max} (mU/mg)		
$-P_i$	31.0	30.0
+5mM P_i	31.0	30.0
K_m ATP (mM)	0.10	0.15
K_m Fru 6-P (mM)		
$-P_i$	0.40	0.7
+5 mM P_i	0.015	0.02
Fru-2,6-P_2ase		
V_{max} (mU/mg)		
+5 mM P_i	40.0	50.0
K_m Fru 2,6-P_2 (nM)		
$-P_i$	10.0	15.0
+5 mM P_i	300.0	300.0
Phosphoenzyme formation (mol P/mol)	0.2	0.15

DISCUSSION

The T7 RNA polymerase expression system has been used to express a number of eukaryotic proteins, but, as in the case of other *E. coli* expression systems, the ex-

TABLE 3

Effect of Induction Temperature on Protein Expression in *E. coli*

Protein	Temperature (°C)	Strain	Total protein produced (mg/liter)	Active protein recovered (mg/liter)	% Active enzyme
6PF-2-K/Fru-2,6-P_2ase	22	BL21(DE3)	20	7.0	35
	22	BL21(DE3)pLysS	25	11–13	50
	37	BL21(DE3)	45	0.5	1
	37	BL21(DE3)pLysS	60	1.0	2
Fru-2,6-P_2ase	22	BL21(DE3)	20	10–12	50
	37	BL21(DE3)	40	0.5–1.0	2
Glucokinase	22	BL21(DE3)	20	2.0	10
	22	BL21(DE3)pLysS	30	10–12	40
	37	BL21(DE3)	10	0.4	4
	37	BL21(DE3)pLysS	25	0.6	2.5
Fru-1,6-P_2ase	22	BL21(DE3)	12	8	66
	22	BL21(DE3)LysS	8	4	50
	37	BL21(DE3)	24	14	58
	37	BL21(DE3)LysS	12	6	50

Note. In inductions done at 37°C cells were harvested 3 h after addition of IPTG, total protein induced was estimated by Coomassie blue staining of SDS gels as described under Experimental Procedures, and active enzyme was estimated in the soluble fraction. When inductions were done at 22°C cells were harvested 40 h after IPTG induction.

pressed polypeptides frequently accumulate as insoluble aggregates ("inclusion bodies") (18,19). In previous studies using the T7 RNA polymerase system, we were able to produce active rat hepatic 6PF-2-K/Fru-2,6-P_2ase (4) and glucokinase (5) but the yield was very low. The same problem has been reported for rabbit muscle phosphorylase where almost no active protein was found with the T7 RNA polymerase system when the induction was done at 37°C (20). In addition to the present data, several other recent reports also have demonstrated that the fraction of total expressed protein that is active increases as the temperature is lowered (20–22). Proteins produced in *E. coli* where this has been shown to be the case (and the percentage which is soluble or active) include human interferon-β (30–90%) (22), subtilisin (82%) (21), and phosphorylase (90%) (20). For example, Browner *et al.* (20), using the pTAC-TAC bacterial expression vector, have reported the recovery of high levels of active rabbit muscle phosphorylase (20 mg/liter) when induction was done at 22°C. The expression of active phosphorylase was not only temperature sensitive, but also depended on the strain of bacteria employed (20). Based on these findings and those reported here for 6PF-2-K/Fru-2,6-P_2ase, Fru-2,6-P_2ase, and glucokinase, it now seems reasonable to predict that many mammalian enzymes can be expressed in active form in *E. coli* simply by lowering the temperature and extending the time of induction.

The results with 6PF-2-K/Fru-2,6-P_2ase, Fru-2,6-P_2ase, and glucokinase suggest that the most important factor in obtaining active protein is the lowered temperature. The effect of lowered temperature involves a decreased rate of production of the protein. It is interesting that at 22°C the amount of total protein peaks early but the amount of active enzyme continues to increase. It could be that the amount of total protein reflects a steady state between synthesis and degradation and that proper folding of the bifunctional enzyme to yield active product requires many hours. However, the time course of induction of native glucokinase at 22°C was different from that for 6PF-2-K/Fru-2,6-P_2ase. The amount of active glucokinase reached a peak by 6 h (data not shown) compared to 40 h for 6PF-2-K/Fru-2,6-P_2ase. This suggests that the transit time for passage through the folding pathway for 6PF-2-K/Fru-2,6-P_2ase is much longer than that for glucokinase. It is likely that the time course of folding of soluble polypeptides expressed in *E. coli* will vary depending on the particular folding pathway and protein conformation and the possible role of ligands and/or chaperones in the folding pathway (23). The finding of only 3- to 5-fold more immunoreactive, soluble 6PF-2-K/Fru-2,6-P_2ase at 22°C compared to at 37°C, while the amount of active protein was 20–30 times higher, suggests that the increase in active protein is not entirely due to differences in solubility but that temperature stability may also play a role. An alternative explanation may be that there is a temperature-dependent accumulation of denatured folding intermediates which appear in the soluble fraction at 37°C. It would appear that for 6PF-2-K/Fru-2,6-P_2ase expression the terms "soluble" and "active" are not synonymous.

An important finding of this study was the greater recovery of both active 6PF-2-K/Fru-2,6-P_2ase and glucokinase at 22°C when BL21(DE3)pLysS was used rather than the BL21(DE3) strain. However, while there was a lag in the production of total 6PF-2-K/Fru-2,6-P_2ase in the pLysS containing strain (compare Figs. 1B and 1D), there was little change in the total amount of enzyme accumulation in the cells or in the rate of synthesis, which was low in both strains at 22°C. The plasmid copy number was identical in the two strains containing the appropriate expression plasmids. Although the greater yield of active enzymes from BL21(DE3)pLysS may be attributed, at least in part, to the diminished early rate of protein accumulation, the complete explanation remains unknown. The finding has the practical advantage of allowing production of these enzymes at high levels (10–13 mg/liter).

There was no effect of lower induction temperature to enhance recovery of active Fru-1,6-P_2ase. This suggests that enhanced recovery of active enzyme with lowered induction temperature is not a universal finding for all mammalian proteins but will apply only to proteins that inherently tend to aggregate or become inactivated in *E. coli* cytosol at 37°C. Rat liver Fru-1,6-P_2ase is a heat-stable enzyme (24) and this fact may relate to its ability to fold properly in *E. coli* cytosol at 37°C. On the other hand, rat liver 6PF-2-K/Fru-2,6-P_2ase is not stable at 37°C. Incubation of the purified enzyme at 37°C caused enzyme inactivation with a half-life of about 3 h while no inactivation was observed at 22°C (data not shown). Glucokinase is also a heat-labile enzyme (25). Therefore, the intrinsic stability of the protein to temperature may also play a role in its recovery in an active form.

The reason for protein aggregation in *E. coli* and how the temperature and/or the inducer level influences this process remain to be elucidated. The current consensus is that protein aggregation occurs from incorrect, but specific, association of partially folded intermediates in the folding pathway (26). The results reported here are consistent with there being a crucial step(s) for proper folding of 6PF-2-K/Fru-2,6-P_2ase, the Fru-2,6-P_2ase domain, and glucokinase which is adversely affected by growth at 37°C when these enzymes are synthesized at a high rate.

The ability to overexpress glucokinase, Fru-1,6-P_2ase, Fru-2,6-P_2ase, and 6PF-2-K/Fru-2,6-P_2ase in *E. coli* has the practical advantage of allowing for production of quantities of these enzymes which will permit their crystallization and the analysis of wild-type and mutant proteins by biophysical methods.

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