PROTEIN EXPRESSION AND PURIFICATION **13,** 251–258 (1998)
ARTICLE NO. PT980896

Cloning, Expression, Purification, and Characterization of the 6-Phosphogluconate Dehydrogenase from Sheep Liver[1]

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Received February 16, 1998, and in revised form March 16, 1998

The mRNA encoding the 51-kDa subunit of 6-phosphogluconate dehydrogenase (6PGDH) from sheep liver was reverse-transcribed and amplified. The resulting cDNA was reamplified in N-terminal and C-terminal segments and spliced to generate a full-length clone, and an internal cDNA fragment was also amplified. The full-length clone containing the complete coding sequence of the 6PGDH cDNA was sequenced and found to contain two mutations and two deletions in the internal region and two mutations outside of the internal region, an A to G point mutation at position 1407 that resulted in the amino acid change Gln 445 to Arg and a silent mutation at position 1426. The internal clone was sequenced and shown to be free of any mutations; therefore the internal piece was used to replace the same region in the full-length clone to correct the mutations in this region. The mutation at position 1407 which was outside of the internal region was corrected using site-directed mutagenesis. The cDNA with the correct codon was then subcloned into the bacterial expression vector pQE-30 and overproduced in *Escherichia coli* strain M15. A protein with a subunit molecular weight of 51,000 was expressed at a level of about 4.5% of the total soluble protein in M15 as judged by SDS/PAGE. Cloning into pQE-30 adds six histidines and a short linker to the N-terminus of the enzyme. The recombinant 6PGDH with His-tag was purified using the Ni-NTA affinity column supplied by Qiagen. The purification procedure resulted in a homogeneous protein by SDS/PAGE with 22.4-fold purification with an overall yield of 61%. The recombinant enzyme exhibits kinetic parameters within error identical to those measured for native sheep liver enzyme. © 1998 Academic Press

Sheep liver 6-phosphogluconate dehydrogenase [6-phosphogluconate; NADP[4] oxidoreductase (decarboxylating), EC 1.1.1.44] is a homodimer with a subunit molecular weight of 51,000. The enzyme catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO_2 with concomitant reduction of NADP to NADPH. 6-Phosphogluconate dehydrogenase has been characterized from the standpoint of the kinetic (1–6) and acid base chemical mechanisms (6, 7–11). The crystal structure of the sheep liver enzyme, including high-resolution structures of the E:NADPH and E:6PG binary complexes, has been solved (12).

Previously, several attempts to isolate a cDNA clone of the sheep liver 6PGDH from a λ cDNA library were unsuccessful. The cDNA sequence was obtained, how-

[1] This work was supported by grants from The National Institutes of Health to P.F.C. (GM 50872) and P.R.S. (DE 11375). P.R.S. was originally Co-I on GM 50872.

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[4] Abbreviations used: Amp, ampicillin; Kan, kanamycin monosulfate; ATP-ribose, 2′-monophosphoadenosine 5′-diphosphoribose; DEPC, diethylpyrocarbonate; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid; NADP, nicotinamide adenine dinucleotide phosphate (the plus sign is omitted for convenience); NADPH, reduced NADP; PAGE, polyacrylamide gel electrophoresis; pBS, phagemid pBluescript; 6PG, 6-phosphogluconate; 6PGDH, 6-phosphogluconate dehydrogenase; SDS, sodium dodecyl sulfate; TE, Tris–EDTA; IPTG, isopropyl-β-D-thiogalactopyranoside.

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ever, using PCR amplification to generate a family of overlapping cDNA clones encoding a mature protein of 482 amino acids (13). Unfortunately, a complete clone was not obtained and the partial overlapping clones were lost (M. Adams, personal communication), requiring the recloning of the entire gene.

In these studies, the cDNA from sheep liver was obtained by RT-PCR and cloned into pBluescript phagemid followed by subcloning into an expression vector pKK223-3. Expression from pKK223-3 in *Escherichia coli* resulted in a copurification of the host 6PGDH with the sheep liver recombinant enzyme. The inability to separate the two enzymes made the expression vector unsuitable for future site-directed mutagenesis studies of the sheep liver 6PGDH. Therefore, the cDNA was subcloned into the pGEX-4T-1 vector to produce a glutathione-*S*-transferase (GST) fusion protein. Cleavage of fusion protein to yield an active recombinant 6PGDH using thrombin was not possible. Consequently, the sheep liver 6PGDH cDNA was cloned into the vector pQE-30, which adds a 6-histidine tag to the N-terminus of 6PGDH. The His-tagged recombinant enzyme has now been expressed, purified, and characterized. The possibility that a heterodimer of the His-tagged sheep liver enzyme and the *E. coli* enzyme was obtained is ruled out by two criteria: (i) a single band is observed via nondenaturing PAGE and (ii) mutant enzymes have been constructed replacing the active site general base with Ala that are structurally like wild type, but inactive. Site-directed mutagenesis of key amino acid residues in 6PGDH using this expression system will further our understanding of the enzyme mechanism from the standpoint of structure and function.

MATERIALS AND METHODS

Chemicals and reagents. Deoxynucleotide triphosphates were from Perkin–Elmer, while *Taq* Plus DNA polymerase and *Pfu* were from Stratagene. Triazol reagent, RNase H, Superscript II reverse transcriptase, and human pancreatic RNase inhibitor were from Gibco BRL. The fmol^R DNA cycle sequencing kit, oligo(dT) primer, T_4 DNA ligase, T_4 kinase, restriction endonucleases, IPTG, protein molecular mass markers, and *E. coli* strain JM109 were from Promega. Protein concentrations were determined according to Bradford (14) using the Bio-Rad protein assay kit with bovine serum albumin as a standard. The oligo(dT) cellulose, protease inhibitors, ampicillin, kanamycin, NADPH, and Hepes buffer were from Sigma. The prokaryotic expression vector pKK223-3 was purchased from Pharmacia Biotech, and pBS was from Stratagene. The DNA molecular weight ladder was purchased from New England Biolabs. DEAE-Spherilose was from Isco and the red A agarose was from Amicon. Mutagenesis and sequencing primers were purchased from either Biosynthesis or Promega. Site-directed mutagenesis was performed using the MORPH Site-Specific Plasmid DNA Mutagenesis Kit purchased from 5 Prime → 3 Prime, Inc. GST Purification Modules, which include the pGEX-4T-1 vector and Glutathione Sepharose 4B, were from Pharmacia Biotech, and the QIAexpress System, which contained the pQE-30 vector, *E. coli* strain M15, and the Ni-NTA matrix, was from Qiagen. All other chemicals used were of the highest quality available and were used without further purification.

Bacterial strains and plasmids. The bacterial strains JM109 (15) and M15[pREP4] (16) were used as a host for expression. Plasmid pKK223-3, pGEX-4T-1, and pQE-30 were used as expression vectors, and phagemid pBluescript was used as the cloning vector.

Isolation of RNA. Total RNA was extracted from 0.3–0.5 g of liver tissue snap-frozen in liquid nitrogen. Liver tissue was placed in 10 ml of Triazol reagent and polytronned until a homogeneous mixture was obtained. Total RNA was isolated as suggested by the manufacturer's protocol. Polyadenylated RNA [poly(A) RNA] was obtained by resuspending 0.5–1 mg of total RNA in an equal volume of 2× column loading buffer (20 mM Tris, 2 mM EDTA, 1 mM NaCl), heating the solution for 5 min at 65°C, and then loading the solution onto a 1-ml oligo(dT) cellulose column. The eluant was collected, reheated to 65°C for another 5 min, and reloaded onto the oligo(dT) cellulose column. The column was then washed with loading buffer until the absorbance of the eluant at 260 nm was zero. The polyadenylated RNA was eluted with 3 bed volumes of column elution buffer (10 mM Tris, 1 mM EDTA). The dead volume of the column was discarded (0.5 ml), and the rest of the eluant was collected. RNA was precipitated at −20°C using 0.1 vol of 3 M sodium acetate, pH 5.3, and 2.5 vol of absolute ethanol. The collected pellet was resuspended in DEPC-treated distilled water. Usually 1 mg of total RNA yielded 25–50 μg of poly(A) RNA.

cDNA synthesis. First-strand cDNA synthesis was primed with oligo(dT) and synthesized at 37°C for 15 min, 42°C for 15 min, and 50°C for 30 min using 200 units of reverse transcriptase per milligram of mRNA. The reaction was terminated by heating the tube to 94°C for 10 min. The cDNA was then amplified using PCR. The sequence of the forward primer (FP.1) is 5′ GCCATGGCCCAAGCTGACATTGC 3′, homologous to nucleotides 68–90 of the coding strand of the sheep liver cDNA, and contains the initiation codon. The sequence of the reverse primer (RP.1) was 5′ TCCTGGTCTGCAGAGTTGTGG 3′, homologous to nucleotides 1539–1560 of the noncoding stand of the cDNA, and contains a single point mutation to create a *Pst*I restriction enzyme site (underlined) 28 bp downstream

from the stop codon. PCRs were performed with 0.5 mg of first strand cDNA; 10 nM concentrations of each of the primers; 0.5 mM concentrations of deoxynucleotide triphosphates; 2.5 mM $MgCl_2$; 10 mM Tris–HCl, pH 8.3; and 2.5 units of *Taq Plus* DNA Polymerase. The samples were subjected to 25 cycles of denaturation at 94°C for 2 min, annealing at 65°C for 2 min, and extension at 73°C for 2 min. The identity of the PCR fragment was confirmed using synthetic oligonucleotides and Southern blot analysis. Attempts to further amplify the initial full-length PCR product were unsuccessful, and thus the coding region of the cDNA was amplified in fragments. The likely reason for the inability to amplify the initial PCR product is the sequence of the primers which were very close to the 5′- and 3′-ends of the coding region of the cDNA. As a result, the primers did not bind with high avidity to the PCR product, resulting in a low yield of the full-length cDNA and a mixture of PCR products in the second round of amplification.

Cloning of amplified cDNA into pBluescript. PCR was used to amplify two overlapping pieces of the 6PGDH coding sequence, one containing the N-terminal half and another containing the C-terminal half. The first fragment was amplified using primers FP.1 (previously used for cDNA synthesis) and RP.2, 5′ ACGCATCGTAGAAAGAGAGAGCAG 3′ corresponding to nucleotides 1367–1344 of the noncoding strand of the cDNA, and primers to amplify the second fragment were FP.2, 5′ TGGTGTCCAAGCTGAAGAAGCCA 3′, corresponding to nucleotides 252–275 of the coding strand and RP.1 (previously used for cDNA synthesis), giving PCR products containing the N-terminus and C-terminus, respectively (Fig. 1). PCRs were performed under conditions identical to those described for cDNA synthesis except that the primer concentration was increased to 1 μM and the number of cycles of ampilification was reduced to 15. Both fragments were subcloned into pBluescript using the TA cloning technique and designated pBS.1 and pBS.2 (17). A complete cDNA clone of 1.51 kb in length was constructed using a unique *Sca*I restriction endonuclease site located at position 1235 within the overlapping region of the fragments in pBS.1 and pBS.2 to generate a phagemid with the complete cDNA sequence, designated pBS.FL. The entire insert was sequenced using the *fmol* DNA Cycle Sequencing System and was found to have several mutations relative to the published sequence of sheep liver 6PGDH cDNA (13). Mutations included a C to G mutation at position 1075, an A to G mutation at position 1114, an A to G mutation at position 1407, a C to G silent mutation at position 1426, and G and T deletions at positions 1045 and 1154, respectively. To correct mutations, an internal PCR product corresponding to nucleotides 252–1344 within the coding region of 6PGDH was generated using primers RP.1 and RP.2 and sheep liver cDNA as a template. The internal PCR product was subcloned into pBluescript at a *Sma*I site, sequenced, and named pBS.IF. The internal PCR product had a sequence identical to the previously published sequence (13). A 900-bp fragment, obtained by digestion of the plasmid pBS.IF with *Sfi*I and *Nsi*I restriction endonucleases, was spliced into the corresponding region in the full-length cDNA clone (pBS.FL) digested with the same enzymes. The A to G mutation at nucleotide 1407 outside of the replaced region was corrected using site-directed mutagenesis. The resulting phagemid is designated pBS.PGDH (Fig. 1). The phagemid was transformed into *E. coli* strain JM109 using an EC100 electroporator (EC Apparatus Corp.) and the recombinant cells were stored at −70°C in LB/Amp medium containing 15% glycerol.

Subcloning of the sheep liver 6PGDH cDNA into expression vectors pKK223-3, pGEX-4T-1, and pQE-30. Although the expression vector that works for this enzyme is pQE-30, it is also important to document attempts with other systems. The pKK223-3 vector gives very good inducible expression, and pKK223-3 gave excellent expression, but we were unable to separate the *E. coli* enzyme from the sheep liver recombinant. As a result, the cDNA was subcloned into the pGEX-4T-1 vector which gives a GST–6PGDH fusion protein that can be isolated using a glutathione affinity column. The pGEX-4T-1 vector also gave very good expression, but we were unable, using a variety of conditions including partial denaturation, to cleave the GST away from 6PGDH. The cDNA was finally subcloned into the pQE-30 vector, which adds a 6-His-tag onto the N-terminus of the recombinant protein, and allows isolation using a Ni column. Again, excellent expression was obtained, and the recombinant protein could be readily separated from the wild-type *E. coli* enzyme. Information for subcloning into all of the expression vectors is provided below.

The 6PGDH gene in plasmid pBS.PGDH was amplified using primer pairs 5′ CGCCGCCGCCATCGAATTC**ATG**GCCCAAGCTG 3′(FP.3), which creates an *Eco*RI restriction endonuclease site (underlined) in front of the initiation codon (boldface), and the RP.1 primer. The resulting 1.49-kb DNA fragment containing the cDNA for 6PGDH was ligated into the expression vector pKK223-3 at the corresponding restriction sites.

The cDNA was also subcloned into expression vector pGEX-4T-1. The 6PGDH gene in plasmid pBS.PGDH was amplified using the forward primer 5′ CGCCGCCGCCATCGAATTC**ATG**GCCCAAGCTG 3′ (FP.3) and

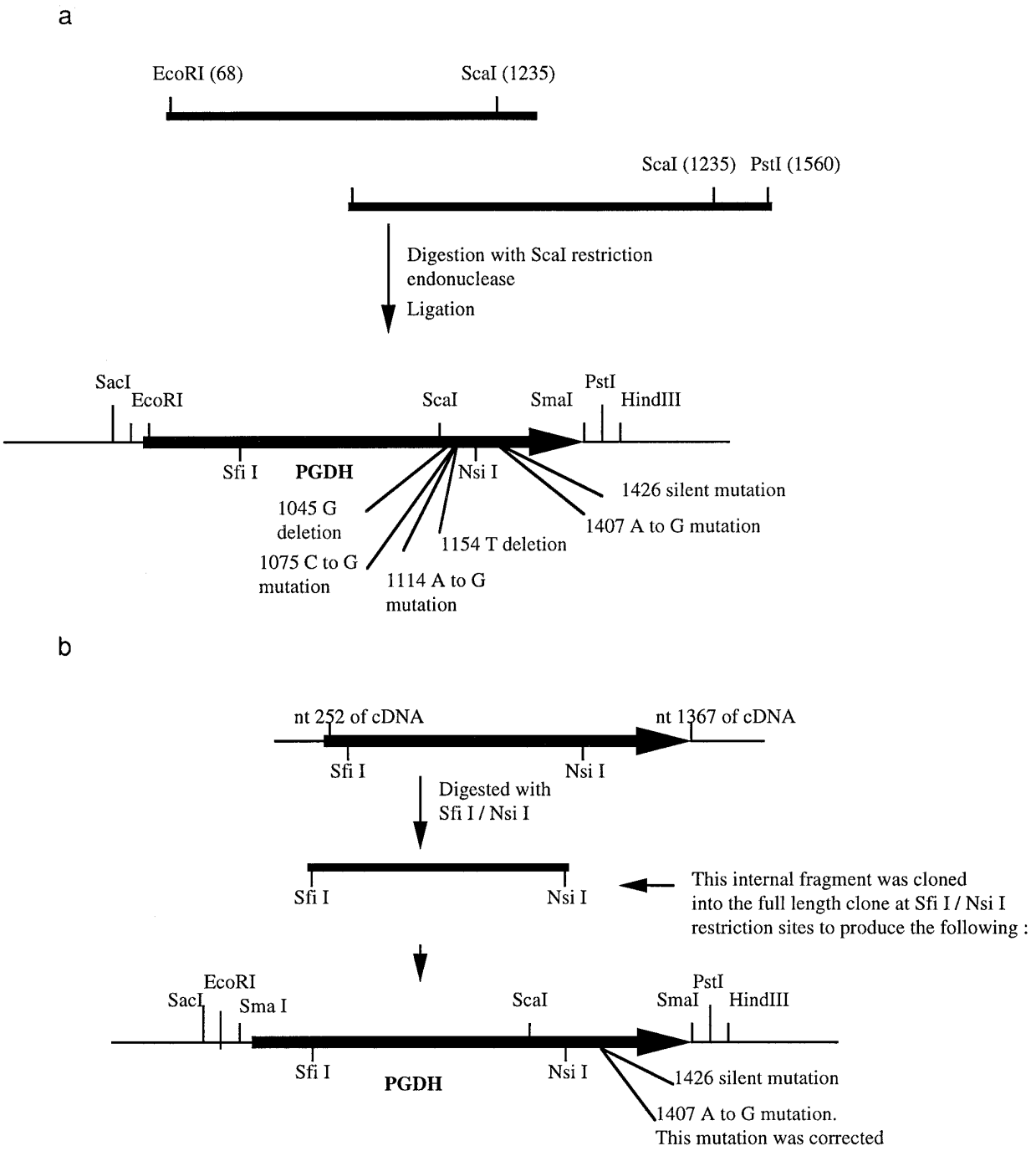

FIG. 1. Schematic representation of sheep liver 6PGDH cloned into the pBluescript vector at a *Sma*I restriction endonuclease site. (a) Cloning of the full-length fragment and locations of the deletions and mutations. (b) Cloning of the internal fragment and the subcloning of this fragment into the full-length clone to correct the anomalies located this region. (The mutation at position 1407 was corrected using site-directed mutagenesis.)

the reverse primer 5′AATGTCCTGGTCTCTCGAGTT-GTGGCTTG 3′, which creates an *Xho*I site (underlined) at the end of the coding sequence. The resulting 1.49-kb DNA fragment containing the cDNA for 6PGDH was ligated into the pGEX-4T-1 vector at the corresponding restriction sites.

Subcloning into the pQE-30 vector from the plasmid pBS.PGDH made use of primer pair 5′ CGC-

CGCCGCCATCGCATGCATGGCCCAAG 3′, which creates an *Sph*I restriction endonuclease site (underlined) at the beginning of the coding sequence and the RP.1 primer. The resulting 1.49-kb cDNA fragment containing the DNA for 6PGDH was subcloned into the pQE-30 vector at the corresponding restriction sites.

Competent cells of *E. coli* strain JM109 or M15 were transformed using an EC100 electroporator according to the manufacturer's specifications or simple transformation using the Qiagen protocol. Frozen stocks of strains harboring plasmids were stored in LB/Amp or LB/Amp/Kan containing 15% glycerol at −70°C. Synthetic oligonucleotide primers of 18–20 bases in length were used to sequence the entire cloned gene. The recombinant plasmids resulting from subcloning into pKK223-3, pGEX-4T-1, and pQE-30 were designated pPGDH.LC2, pPGDH.LC3, and pPGDH.LC4.

Site-directed mutagenesis. Site-directed mutagenesis was performed according to the manufacturer's procedure using 5′TCCAGGCTCAGCGCGACTAC 3′ as the mutagenic primer, where the underlined A corrects the G mutation at position 1407.

Growth conditions. The growth conditions for pPGDH.LC2 and pPGDH.LC3 were as previously described (18) with the exception that 4 liters of medium were used for the culture. The strain pPGDH.LC4 was grown in 1 liter of LB/Amp/Kan medium until an absorbance of 0.7 was reached at 600 nm, and IPTG was then added to a final concentration of 1 mM.

Protein purification. The pPGDH.LC2 bacterial cells were sonicated with a Misonix Inc. Model XL ultrasonic liquid processor in 200-ml batches. Each batch was sonicated on ice continuously for 4 min. Sonicates were centrifuged at 8,000*g* for 20 min to remove cell debris. Streptomycin sulfate was added to the supernatant to a final concentration of 1% and the solution was centrifuged as before. The supernatant was recovered, solid ammonium sulfate was added to 40% saturation, and the solution was centrifuged at 12,000*g* for 25 min. The supernatant was recovered and solid ammonium sulfate was added to 80% saturation. The protein was then pelleted at 12,000*g* for 25 min. The protein pellet was resuspended in 10 ml of TE buffer, pH 7.0, containing 10 mM β-mercaptoethanol, 10 mM triethanolamine, and 1× protease inhibitor cocktail [protease inhibitor cocktail (150×) contains 2 mg/ml aprotinin in H_2O, 1 mg/ml leupeptin in H_2O, and 1 mg/ml pepstatin A in methanol], and dialyzed overnight against the same buffer. The enzyme was loaded onto a 2.5 × 30 cm DEAE–Spherilose column preequilibrated with TE buffer. The column was washed with TE buffer and the enzyme was eluted from the column with a linear gradient (0–400 mM) of NaCl in TE buffer. The eluant from the DEAE–Spherilose column was directly applied to a 1.5 × 15 cm red A agarose column preequilibrated with 20 mM Hepes, pH 7.0. The column was washed with Hepes buffer containing 300 mM NaCl until the absorbance at 280 nm for eluant was zero. The enzyme was eluted from the column with Hepes buffer containing 500 mM NaCl. Fractions containing 6PGDH activity were pooled. Solid ammonium sulfate was added to 75% saturation and the enzyme stored at 4°C.

Bacterial cells containing pPGDH.LC3 were sonicated as above and centrifuged to remove cell debris. The supernatant containing the 6PGDH–GST fusion protein was added to a 2-ml slurry of glutathione–Sepharose 4B previously equilibrated with 1× PBS and incubated with gentle agitation at room temperature for 30 min. The mixture was then centrifuged at 500 rpm for 5 min and the supernatant was discarded. The matrix was washed with 20 ml of 1× PBS. The fusion protein was eluted using 25 mM glutathione, 100 mM Tris–HCl (pH 7.5), and 120 mM NaCl. All attempts to cleave 6PGDH from the fusion protein using thrombin protease failed (19).

The pPGDH.LC4 bacterial cells were sonicated as above and centrifuged to remove cell debris. The His-tagged 6PGDH was purified using column chromatographic procedure supplied by Qiagen. The His-tagged 6PGDH was eluted with a 20-ml gradient of 0.1–0.4 M imidazole in wash buffer (pH 7.5). Solid ammonium sulfate was added to 75% saturation and the enzyme was stored at 4°C.

For enzyme assays, a small aliquot of the stored protein was centrifuged to precipitate the protein. The supernatant was discarded and the protein pellet was resuspended in 20 mM Hepes, pH 7.0, containing 20% glycerol and 1 mM β-mercaptoethanol.

Initial velocity studies. All data were collected using a Beckman DU 640 spectrophotometer. The appearance of NADPH ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) at 340 nm was monitored. All assays were run at 25°C, and the temperature was maintained using a Neslab RTE-111 circulating water bath. Initial velocity pattern were obtained in 100 mM Hepes, pH 7.0, 20 μM ATP-ribose using variable concentrations of 6PG (20–200 μM) and NADP (20–200 μM) (6). Initial velocity data were fitted using the appropriate rate equation and a Basic version of the program developed by Cleland (20). Data conformed to a sequential initial velocity pattern and were fitted using

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB), \quad (1)$$

where v and V represent initial and maximum velocities, A and B represent reactant concentrations, K_a and K_b are Michaelis constants for A and B, and K_{ia} is the inhibition constant for A.

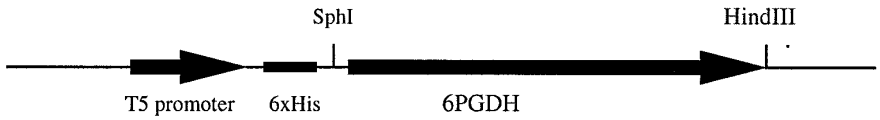

FIG. 2. Schematic representation of the restriction map of sheep liver 6PGDH subcloned into pQE-30 expression vector at *Sph*I/*Hin*dIII restriction sites.

RESULTS AND DISCUSSION

Construction of plasmids pBS.PGDH, pPGDH.LC2, pPGDH.LC3, and pPGDH.LC4. The 3.2-kb cloning vector pBluescript contains T3 and T7 bacteriophage promoters, allowing efficient *in vitro* synthesis of strand-specific RNA and 10 unique restriction sites in the multiple cloning region and allows blue/white color screening of recombinant colonies. The 1.51-kb DNA fragment containing the coding sequence of 6PGDH was cloned into pBS phagemid vector at a *Sma*I restriction endonuclease site, resulting in plasmid pBS.PGDH. The sequence of the 1.5-kb insert in pBS.PGDH was identical to that published by Somers *et al.* (13) and corroborated by the crystal structure of 6PGDH (12).

Two oligonucleotides were used to amplify the 1.49-kb cDNA contained in phagemid pBS.PGDH. The amplified fragment was digested with *Eco*RI/*Pst*I, *Eco*RI/*Xho*I, or *Sph*I/*Hin*dIII restriction endonucleases and subcloned into pKK223-3, pGEX-4T-1, or pQE-30 expression vectors at the corresponding sites, resulting in plasmids pPGDH.LC2, pPGDH.LC3, and pPGDH.LC4, respectively. The restriction map for pPGDH.LC4 is shown in Fig. 2.

Expression of 6-phosphogluconate dehydrogenase in E. coli. *E. coli* cells (JM109, M15) transformed with plasmid pPGDH.LC2, pPGDH.LC3, or pPGDH.LC4 were tested for enzyme activity after sonication and centrifugation, and the presence of the expressed protein was further verified using SDS/PAGE. A protein band with a molecular weight similar to that of 6PGDH isolated from sheep liver was detected in the *E. coli* strain containing pPGDH.LC2 (data not shown) and pPGDH.LC4; see Fig. 3. In the case of pPGDH.LC3, a protein with a molecular mass equal to that predicted for the GST fusion protein was observed (data not shown).

Enzyme activity was measured in the crude extract of the recombinant strains and the control *E. coli* strains. Enzyme activity was detected in *E. coli* containing all three expression vectors, and was greater than the activity of the *E. coli* 6PGDH detected in control strains.

Protein purification. Sheep liver 6PGDH was previously purified using cellulose phosphate ion-exchange and NADP-agarose affinity column chromatographies (21). In the present study the recombinant enzyme pPGDH.LC2 was purified using the procedure outlined under Materials and Methods. The final enzyme preparation is 80% pure based on Coomassie blue-stained SDS polyacrylamide gel. The apparent subunit molecular weight of the recombinant protein is 51,000, identical to that of the wild-type enzyme (data not shown).

Recombinant protein isolated from plasmid pPGDH.LC3 was purified according to the Pharmacia protocol (19). Attempts to cleave the recombinant enzyme from GST even at different concentration of thrombin protease was unsuccessful. It is possible that the 77-kDa fusion protein is folded in such a way that the thrombin protease site is inaccessible, but even the use of 0.5–2 M urea to partially unfold the fusion protein to allow cleavage was unsuccessful.

The recombinant enzyme derived from pPGDH.LC4 was purified in one step using the Ni-NTA affinity column. High affinity of Ni-NTA for the His-tagged 6PGDH resulted in efficient purification of 6PGDH recombinant enzyme from all contaminants (Fig. 3). The total fold purification is 22.4, with an overall yield of 61%, resulting in an apparently homogeneous preparation (Table 1).

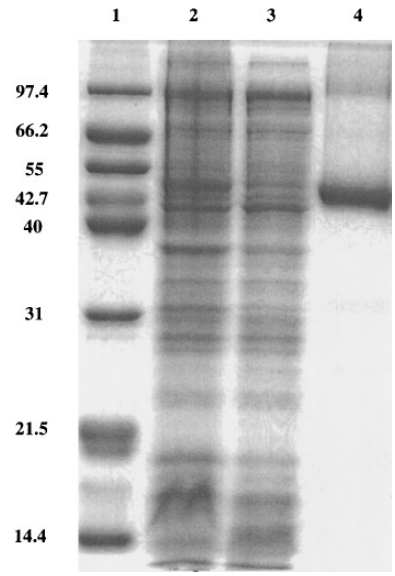

FIG. 3. SDS/PAGE of the purification steps for 6PGDH from the pPGDH.LC4 strain. Lane 1, molecular mass standards; lane 2, sonicate; lane 3, crude extract; lane 4, fraction from the Ni-NTA affinity column.

TABLE 1

Purification of Recombinant 6-Phosphogluconate Dehydrogenase from the pPGDH.LC4 Strain[a]

	Total protein (mg)	Total units[b] (μmol/min)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	44.7	9.2	0.21	0	100
Ni-NTA	1.2	5.6	4.7	22.4	61

[a] Purification was performed on 5.4 g of wet cell paste.
[b] Corrected for *E. coli* 6PGDH.

Initial velocity studies. Purified recombinant 6PGDHs were analyzed kinetically in parallel with the native enzyme. Kinetic parameters obtained for the recombinant proteins isolated from the pPGDH.LC2 and pPGDH.LC4 plasmid-containing strains are identical within error to those of the native enzyme. Interestingly, GST-fused 6PGDH obtained from the pPGDH.LC3 plasmid-containing strain also gave kinetic parameters that were within error identical to those of the native enzyme. It would thus appear that the N-terminus is away from the active site. Based on the three-dimensional structure of the enzyme:6PG complex (12), the N-terminal alanine is about 35 Å away from the N-terminus on the opposite side of the protein. Kinetic parameters for the recombinant enzyme isolated from pPGDH.LC4 are summarized in Table 2.

Conclusions. A construct which directs the expression of the sheep liver 6-phosphogluconate dehydrogenase gene in *E. coli* at a high level has been produced. A purification scheme has been developed for the cloned enzyme. The recombinant enzyme has kinetic parameters identical to those of the native enzyme. We are in the process of mutating key amino acid residues to study the role of these amino acids in substrate binding and catalysis.

TABLE 2

Values of Kinetic Parameters for Recombinant and Native 6-Phosphogluconate Dehydrogenase

	Recombinant	Native[a]
K_{6PG} (μM)	36 ± 15	19 ± 10
K_{NADP} (μM)	2 ± 1	5 ± 1
V/E_t (s^{-1})	3.5 ± 0.1[b]	2.0 ± 0.4
$V/K_{6PG}/E$ ($M^{-1}s^{-1}$)	$(1.0 \pm 0.4) \times 10^5$	$(1.05 \pm 0.5) \times 10^5$
$V/K_{NADP}/E_t$ ($M^{-1}s^{-1}$)	$(1.75 \pm 0.6) \times 10^6$	$(4 \pm 2) \times 10^5$

[a] Ref. 6. Although there appears to be a greater V/E_t value for the recombinant enzyme, the published study made use of enzyme from Sigma that was about 50% pure based on SDS/PAGE.

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