Cloning, sequencing and expression of rat liver 3-phosphoglycerate dehydrogenase

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Rat liver D-3-phosphoglycerate dehydrogenase was purified to homogeneity and digested with trypsin, and the sequences of two peptides were determined. This sequence information was used to screen a rat hepatoma cDNA library. Among 11 positive clones, two covered the whole coding sequence. The deduced amino acid sequence (533 residues; M_r 56493) shared closer similarity with *Bacillus subtilis* 3-phosphoglycerate dehydrogenase than with the enzymes from *Escherichia coli*, *Haemophilus influenzae* and *Saccharomyces cerevisiae*. In all cases the similarity was most apparent in the substrate- and NAD^+-binding domains, and low or insignificant in the C-terminal domain. A corresponding 2.1 kb mRNA was present in rat tissues including kidney, brain and testis, whatever the dietary status, and also in livers of animals fed a protein-free, carbohydrate-rich diet, but not in livers of control rats, suggesting transcriptional regulation. The full-length rat 3-phosphoglycerate dehydrogenase was expressed in *E. coli* and purified. The recombinant enzyme and the protein purified from liver displayed hyperbolic kinetics with respect to 3-phosphoglycerate, NAD^+ and NADH, but substrate inhibition by 3-phosphohydroxypyruvate was observed; this inhibition was antagonized by salts. Similar properties were observed with a truncated form of 3-phosphoglycerate dehydrogenase lacking the C-terminal domain, indicating that the latter is not implicated in substrate inhibition or in salt effects. By contrast with the bacterial enzyme, rat 3-phosphoglycerate dehydrogenase did not catalyse the reduction of 2-oxoglutarate, indicating that this enzyme is not involved in human D- or L-hydroxyglutaric aciduria.

INTRODUCTION

D-3-Phosphoglycerate dehydrogenase (EC 1.1.1.95) catalyses the first step in the pathway of serine formation from glycolytic intermediates (reviewed in [1]). This enzyme is widely distributed in organisms and in tissues. In rat liver, its activity depends strongly on nutritional status, being low in animals fed a normal diet and increasing more than 10-fold upon ingestion of a low-protein, carbohydrate-rich diet [2,3]. When measured in the non-physiological direction, the enzyme present in human fibroblasts is inhibited by concentrations of the substrate 3-phosphohydroxypyruvate above 10 μM, and this inhibition is released by salts [4]. Similar properties have been described for bovine liver D-glycerate dehydrogenase, the inhibitory substrate being hydroxypyruvate in this case [5,6], but were not observed with 3-phosphoglycerate dehydrogenases from bacteria [7] or plants [8]. In *Escherichia coli* [9], *Bacillus subtilis* [7] and plants [8], 3-phosphoglycerate dehydrogenase is subject to allosteric control by the terminal product of the pathway, serine, whereas such control does not appear to be present in the animal enzyme [10].

Recently, the possible involvement of 3-phosphoglycerate dehydrogenase in the pathogenesis of two newly described hereditary disorders has attracted our attention. The activity of this enzyme was indeed markedly decreased (to 13 and 22% of the normal value) in fibroblasts from two patients with a decreased concentration of serine in the plasma and cerebrospinal fluid [4]. Furthermore, *E. coli* 3-phosphoglycerate dehydrogenase was shown to catalyse the reduction of 2-oxoglutarate to both the D- and L-isomers of 2-hydroxyglutarate, suggesting that a mutation of the human enzyme may contribute to the neurometabolic diseases D- and L-hydroxyglutaric aciduria [11].

The primary sequences of 3-phosphoglycerate dehydrogenases from *E. coli* [12], *Haemophilus influenzae* (GenBank accession no. L45106; [13]), *Saccharomyces cerevisiae* (GenBank P40054) and *Bacillus subtilis* (GenBank L47648; [14]) are known, as is the three-dimensional structure of the *E. coli* enzyme [15]. Each subunit of the tetrameric protein has three distinct domains: a nucleotide-binding domain (residues 108–294), a substrate-binding domain (residues 7–107 and 295–336) and a regulatory domain, which binds L-serine (residues 337–410). The main contact points between the subunits are at the level of the coenzyme-binding domains and the regulatory domains, indicating the importance of these zones for the tetramerization of the enzyme.

The aim of the present work was to initiate an investigation of the mammalian enzyme at the molecular level, and to study its kinetics and specificity, particularly its ability to catalyse the reduction of 2-oxoglutarate to 2-hydroxyglutarate, and the mechanism by which its activity is controlled by diet.

MATERIALS AND METHODS

Materials

3-Phosphoglycerate, NAD^+, NADH, the random prime DNA labelling kit and *Taq* and *Pwo* DNA polymerases were from Boehringer Mannheim. 3-Phosphohydroxypyruvate was prepared from the tricyclohexylammonium salt of the dimethyl ketal derivative [16] purchased from Sigma. [α-^{32}P]dCTP (approx. 3000 Ci/mmol), [α-^{35}S]dATP (> 1000 Ci/mmol), the Δ Taq Cycle Sequencing kit (USB) and Hybond N® membranes were from Amersham International. The T7 Sequencing® kit was

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The sequence of the *R. norvegicus* cDNA encoding 3-phosphoglycerate dehydrogenase has been deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession no. X97772 RND3PGDEH.

from Pharmacia Biotech Inc., and the IRD41 dye-labelled M13 reverse primer was from LI-COR.

Enzyme purification from rat liver and amino acid sequencing of two tryptic peptides

Male Wistar rats were fed on cornflour for 2 weeks before being killed to increase the expression of the liver enzyme [2]. The livers of 14 rats were homogenized in 4 vol. of 0.25 M sucrose containing 20 mM Hepes, pH 7.5, 100 mM KCl, 1 mM dithiothreitol and 0.25 mM PMSF. The homogenate was spun for 30 min at 9000 ***g*** and 4 °C. A 7–22 % (w/v) poly(ethylene glycol) 8000 fraction was prepared from this extract and dissolved in 200 ml of 10 mM Hepes, pH 7.5, 1 mM dithiothreitol and 1 mM EDTA (buffer A) supplemented with 0.25 mM PMSF. The preparation was clarified by centrifugation and loaded on to a DEAE-Sepharose column (2.5 cm × 22 cm; flow rate 2.5 ml/min). The column was washed with 100 ml of buffer A containing 0.25 mM PMSF and eluted with a linear salt gradient (0–400 mM KCl in 400 ml of buffer A containing 0.25 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml antipain). The enzyme was then diluted 13-fold in buffer A and loaded on to an AMP–Sepharose column (1 cm × 3 cm) equilibrated with buffer A. The enzyme was eluted from the affinity column with 0.125 mM NAD^+ and 0.125 mM 3-phosphoglycerate in buffer A. In order to obtain internal tryptic peptides, the band corresponding to 3-phosphoglycerate dehydrogenase in the purified preparation was cut from a Coomassie Blue-stained SDS/polyacrylamide gel and the protein was concentrated in agarose for trypsin digestion [17]. Peptides were purified by narrow-bore HPLC for microsequencing [17].

Enzyme assays

3-Phosphoglycerate dehydrogenase was assayed spectrophotometrically at 30 °C in a mixture comprising 25 mM Hepes, pH 7.1, 90 μM phosphohydroxypyruvate, 90 μM NADH and 400 mM KCl, unless otherwise indicated. One unit is the amount of enzyme catalysing the reduction of 1 μmol of phosphohydroxypyruvate/min under these assay conditions. For the determination of kinetic constants in the physiological direction, activity was measured in a mixture containing 200 mM Tris, pH 9, 180 mM hydrazine sulphate, 1 mM EDTA and the indicated concentrations of 3-phosphoglycerate and NAD^+.

Amplification of a cDNA fragment by PCR with degenerate oligonucleotides

A library constructed from rat FTO2B hepatoma cells in Uni-Zap® XR (Stratagene), with oligo(dT)-primed cDNAs inserted between *Eco*RI and *Xho*I restriction sites, was kindly provided by V. J. Dupriez (I.C.P. Brussels) [18]. The DNA purified from this library was used as a template to amplify a cDNA fragment with *Taq* DNA polymerase. The sense primer [5′-GC(C/T)G-G(A/C)AC(A/C)GG(A/C)GT(G/C)GA(C/T)AA(C/T)GT-(G/C)GA-3′] corresponded to the first nine amino acids (AGTGVDNVD) of one of the sequenced peptides (see Figure 1); the antisense primer was a 20-mer corresponding to the vector-specific T7 promoter. The amplified product (1450 bp) was purified, cloned in a T vector prepared from pBluescript [19] and sequenced.

cDNA cloning and sequencing

About 300000 plaques from the cDNA library described above were plated and lifted on to Hybond N® membranes. The DNA was cross-linked to the dried filters by UV irradiation. Prehybrization for 1 h and hybridization for 15 h were performed at 65 °C in a solution containing 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution [20], 0.5 % SDS and 20 μg/ml denatured herring sperm DNA. An approx. 1000 bp *Xho*I restriction fragment corresponding to the 3′ end of the cDNA was radiolabelled with [α-^{32}P]dCTP by random priming [20] and used as a probe. The membranes were washed at 65 °C (2× SSC, 3 × 10 min; 2× SSC/0.1 % SDS, 1 × 10 min; 2× SSC, 1 × 10 min; 0.2× SSC, 1 × 10 min), dried and exposed to Kodak XAR-5 film at room temperature for 2–18 h.

Recombinant pBluescript cDNAs were excised *in vivo* from the lambda Uni-ZAP® XR vector as recommended by the manufacturer and purified by the alkaline lysis method [20]. The plasmid containing the longest insert was sequenced completely in both directions by the dideoxy method [21] with the T7 Sequencing® kit, [α-^{35}S]dATP and specific primers from within the cDNA sequence and from flanking regions of the vector. In some cases, the Δ Taq Cycle Sequencing kit® and the IR-dye-labelled M13 reverse primer were used and the products were analysed using an automated laser fluorescence DNA sequencer 4000L from LI-COR. Sequences were analysed using the DNA Strider programme [22]. Multiple sequence alignment was performed using the GenBank program PILEUP.

Northern blots

Total RNA was isolated from various tissues by the guanidinium isothiocyanate/CsCl procedure, subjected to electrophoresis in 1 % agarose/formaldehyde gels and transferred by capillarity to nylon membranes [20]. These were hybridized and washed as described above.

Expression and purification of recombinant 3-phosphoglycerate dehydrogenase

The coding region of the full-length cDNA clone of 3-phosphoglycerate dehydrogenase was amplified by PCR using *Pwo* DNA polymerase, a low-error enzyme, with one primer (GGAATTC-CATATG GCCTTCGCAAATCTG) containing the start codon (underlined) inserted in a *Nde*I site, and a second primer (CGGGATCCTTCAGAAGCAGAACTGGAA) containing the stop codon (underlined) flanked by a *Bam*HI site. The amplified fragment was cloned into pBluescript restricted with *Eco*RV, excised from this vector with *Nde*I and *Bam*HI and inserted into the expression vector pET3a [23] to produce the recombinant plasmid pET3PGDH. For the preparation of the shortened form of the enzyme, the second primer used in the amplification (GGTGTGGGATCCTCAGGCACTGGTAAG) was designed to replace Phe-325 by a stop codon (underlined). To produce the recombinant enzymes, BL21(DE3)p*Lys*S cells transformed with the expression plasmids were aerobically grown in 1 litre of LB medium containing 0.2 % glucose and 50 μg/ml ampicillin at 37 °C up to an A_{600} of 0.6 unit. The culture was then quenched for 30 min on ice before addition of 0.4 mM isopropyl β-D-thiogalactoside to induce expression of the cloned enzyme. Culture was continued for another 18 h at 18 °C. Cells were harvested by centrifugation (12000 ***g***, 30 min, 4 °C) and extracted as described in [24]. The recombinant enzyme was purified by chromatography on DEAE-Sepharose and AMP–Sepharose, as described for the native liver enzyme. The active fractions were diluted with 1 vol. of glycerol and stored at −20 °C, under which conditions the enzyme was stable for several months.

Protein was determined by the procedure of Bradford [25], with bovine γ-globulin as a standard.

RESULTS AND DISCUSSION

Purification of rat liver 3-phosphoglycerate dehydrogenase and screening of the cDNA library

Rat liver 3-phosphoglycerate dehydrogenase was purified to homogeneity from the livers of rats that had been fed for 2 weeks with a protein-free, high-carbohydrate diet, by a procedure derived from that used by Lund et al. [26]. The purified preparation had a specific activity of 35 units/mg of protein at 30 °C and contained a single polypeptide chain of M_r 57000 in SDS/polyacrylamide gels (results not shown). The protein was concentrated and digested with trypsin. Tryptic peptides were purified, two of which gave the following sequences: AGTGVDNVDLEAATR (peptide 1) and ALESGECAGAALDVFTEEPP (peptide 2). PCR-amplification with a primer derived from peptide 1, a primer corresponding to the T7 promoter and DNA from a hepatoma cDNA library yielded an approx. 1450 bp fragment, which was cloned and found by sequencing to encode a protein homologous to *E. coli* 3-phosphoglycerate dehydrogenase.

This ~ 1450 bp fragment was used to screen the hepatoma cDNA library. The 11 positive clones that were obtained had inserts of between 0.63 and 1.78 kb that were differently truncated at their 5′ ends. The largest clone was sequenced over its entire length on both strands.

Nucleotide sequence and deduced amino acid sequence

The nucleotide and predicted amino acid sequences are shown in Figure 1. The proposed ATG start codon lies within a suitable consensus sequence for translation initiation by eukaryotic ribosomes [27]. It is preceded by an in-frame TAA stop codon and opens a reading frame encoding a protein of 533 amino acids with a predicted M_r of 56493 and a pI of 6.26. These values are in agreement with the behaviour of the liver enzyme in SDS/PAGE (57000 M_r) and in chromatography on ion exchangers. The enzyme is indeed retained by DEAE-Sepharose at pH 7.5 and by SP-Sepharose at pH 6.0 (results not shown). The next ATG codon is situated about 285 bp downstream. Its use would result in a peptide with a much lower M_r than observed (approx. 45000). The open reading frame is preceded by 68 bp of 5′-untranslated region and followed by a 3′-untranslated sequence of 113 bp containing an 18 bp poly(A) tail. The putative, somewhat atypical, poly(A) addition signal AGTAAA starts 18 bp upstream of the poly(A) tail.

The amino acid sequences of the two peptides resulting from digestion with trypsin (Figure 1) match the sequence of the recombinant enzyme, with the exception of two glutamine residues that were detected as glutamic acid during sequencing of the second peptide, presumably as a result of deamidation. These data indicate that the cloned cDNA encodes the subunit of the liver D-3-phosphoglycerate dehydrogenase. Partial sequences obtained from the other, incomplete, cDNA clones were consistent with that shown in Figure 1, indicating that the enzyme is expressed as a single molecular form in FTO2B cells.

Comparison with related enzymes from other species

The predicted sequence of the rat liver enzyme has been aligned with 3-phosphoglycerate dehydrogenases from *E. coli*, *H. influenzae*, *S. cerevisiae* and *B. subtilis* [12–14] (Figure 2). The Figure indicates the positions of different domains in the *E. coli* enzyme, as well as amino acids implicated in catalysis. GenBank also contains the sequences of mouse and human partial cDNAs encoding peptides displaying > 90% identity with rat liver 3-phosphoglycerate dehydrogenase (not shown).

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GCCTTCAGTTTCCTGTACTAAGTGCTTCTGCCCACCAGAGCAACCGATTCTAAGGCCTGG        60
CTCTAGCAATGGCCTTCGCAAATCTGCGCAAAATACTCATCAGTGATAGCCTCGACCCCT       120
         M  A  F  A  N  L  R  K  I  L  I  S  D  S  L  D  P    17
GCTGCCGGAAGATCCTGCAAGATGGAGGGCTGCAGGTGGTGGAGAAGCAGAACTTGAGCA       180
C  C  R  K  I  L  Q  D  G  G  L  Q  V  V  E  K  Q  N  L  S    37
AGGAGGAGCTGATAGCCGAACTCCAGGACTGTGAAGGCCTTATCGTCCGGTCAGCTACTA       240
K  E  E  L  I  A  E  L  Q  D  C  E  G  L  I  V  R  S  A  T    57
AGGTCACTGCTGATGTCATCAATGCAGCAGAGAAGCTCCAGGTGGTGGGCAGGGCTGGTA       300
K  V  T  A  D  V  I  N  A  A  E  K  L  Q  V  V  G  R  A  G    77
CAGGCGTGGACAATGTGGATCTGGAGGCTGCCACAAGGAAGGGCGTCCTCGTCATGAACA       360
T  G  V  D  N  V  D  L  E  A  A  T  R  K  G  V  L  V  M  N    97
CCCCCAATGGAAATAGCCTCAGTGCTGCGGAACTCACCTGTGGGATGCTCATGTGCCTGG       420
T  P  N  G  N  S  L  S  A  A  E  L  T  C  G  M  L  M  C  L   117
CCAGGCAGATCCCCCAGGCGACGGCTTCGATGAAAGATGGCAAATGGGACCGGAAGAAGT       480
A  R  Q  I  P  Q  A  T  A  S  M  K  D  G  K  W  D  R  K  K   137
TCATGGGGACAGAGCTGAACGGGAAGACACTGGGAATTCTTGGCCTGGGCAGAATTGGAA       540
F  M  G  T  E  L  N  G  K  T  L  G  I  L  G  L  G  R  I  G   157
GAGAGGTGGCCGCCCGAATGCAGGCCTTTGGAATGAAGACTGTAGGCTATGACCCCATCA       600
R  E  V  A  A  R  M  Q  A  F  G  M  K  T  V  G  Y  D  P  I   177
TTTCTCCAGAAGTCGCTGCCTCCTTTGGTGTTCAGCAGCTGCCGCTAGAGGAGATCTGGC       660
I  S  P  E  V  A  A  S  F  G  V  Q  Q  L  P  L  E  E  I  W   197
CTCTCTGTGATTTCATCACTGTCCATACCCCGCTCCTGCCCTCCACTACAGGCTTGCTCA       720
P  L  C  D  F  I  T  V  H  T  P  L  L  P  S  T  T  G  I  L   217
ATGACAGCACCTTTGCCCAGTGCAAGAAAGGCGTGCGGGTGGTGAACTGTGCTCGAGGAG       780
N  D  S  T  F  A  Q  C  K  K  G  V  R  V  V  N  C  A  R  G   237
GCATTGTGGATGAAGGTGCCCTGCTCCGTGCCCTGCAGTCTGGTCAGTGTGCTGGTGCTG       840
G  I  V  D  E  G  A  L  L  R  A  L  O  S  G  Q  C  A  G  A   257
CACTGGATGTGTTTACAGAAGAGCCACCACGGGACCGGGCCTTAGTGGACCACGAGAACG       900
A  L  D  V  F  T  E  E  P  P  R  D  R  A  L  V  D  H  E  N   277
TCATCAGCTGTCCCCACCTGGGCGCCAGCACCAAGGAGGCCCAGAGCCGCTGTGGGGAGG       960
V  I  S  C  P  H  L  G  A  S  T  K  E  A  Q  S  R  C  G  E   297
AAATCGCAGTCCAGTTTGTGGACATGGTGAAGGGGAAATCTCTAACAGGGGTTGTAAACG      1020
E  I  A  V  Q  F  V  D  M  V  K  G  K  S  L  T  G  V  V  N   317
CCCAGGCTCTTACCAGTGCCTTCTCTCCACACACCAAGCCTTGGATTGGTCTGGCAGAAG      1080
A  Q  A  L  T  S  A  F  S  P  H  T  K  P  W  I  G  L  A  E   337
CATTGGGCACGCTGATGCACGCCTGGGCTGGCTCCCCTAAAGGGACCATCCAGGTGGTGA      1140
A  L  G  T  L  M  H  A  W  A  G  S  P  K  G  T  I  Q  V  V   357
CACAAGGAACATCTCTGAAGAATGCTGGGACCTGCCTGAGCCCTGCGGTCATTGTCGGCC      1200
T  Q  G  T  S  L  K  N  A  G  T  C  L  S  P  A  V  I  V  G   377
TTCTGAGAGAAGCATCAAAACAGGCAGATGTGAACTTGGTGAACGCTAAGCTACTGGTGA      1260
L  L  R  E  A  S  K  Q  A  D  V  N  L  V  N  A  K  L  L  V   397
AAGAGGCTGGCCTCAATGTCACCACCTCCCACAGTCCTGGTGTCCCAGGAGAGCAGGGCA      1320
K  E  A  G  L  N  V  T  T  S  H  S  P  G  V  P  G  E  Q  G   417
TCGGGGAATGCCTCCTGACTGTGGCCTTGGCAGGTGCCCCCTACCAAGCTGTGGGCTTGG      1380
I  G  E  C  L  L  T  V  A  L  A  G  A  P  Y  Q  A  V  G  L   437
TCCAGGGCACCACACCAATGTTGCAGATGCTCAACGGAGCTGTCTTCAGGCCAGAGGTGC      1440
V  Q  G  T  T  P  M  L  Q  M  L  N  G  A  V  F  R  P  E  V   457
CTCTACGCAGGGGCCAGCCCCTGCTCCTGTTCCGGGCTCAGCCCTCCGACCCTGTCATGC      1500
P  L  R  R  G  Q  P  L  L  L  F  R  A  Q  P  S  D  P  V  M   477
TGCCCACTATGATCGGCCTACTGGCAGAGGCGGGGGTACAGCTGCTGTCCTACCAGACCT      1560
L  P  T  M  I  G  L  L  A  E  A  G  V  Q  L  L  S  Y  Q  T   497
CCAAGGTGTCTGACGGAGACACTTGGCACGTCATGGGCCTCTCCTCCCTACTGCCCAGCC      1620
S  K  V  S  D  G  D  T  W  H  V  M  G  L  S  S  L  L  P  S   517
TGGACGCATGGAAGCAGCATGTTTCTGAGGCTTTCCAGTTCTGCTTCTGACCCAGGGGCT      1680
L  D  A  W  K  Q  H  V  S  E  A  F  Q  F  C  F              533
CAGCGGTCCCAGCCCCTCAGGCTCTTCTGAGGAAACCCGCTCACTGTGACCTGAACTAAT      1740
ATCTAGTAAAGAATCTAACTCCAAAAAAAAAAAAAAAAAA                           1780
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Figure 1 Sequence of the cDNA encoding rat hepatoma 3-phosphoglycerate dehydrogenase and its deduced amino acid sequence

The initiator ATG codon, the stop codon and the putative polyadenylation signal are underlined. The in-frame TAA stop codon upstream of the Met initiator codon is shown in italics. The amino acid sequences corresponding to the two tryptic peptides are underlined. Note that the two predicted glutamine residues of the second peptide were recovered as glutamate by amino acid sequencing.

Remarkably, the rat enzyme shows closer similarity to its *B. subtilis* counterpart than to the three other enzymes. The rat and *B. subtilis* 3-phosphoglycerate dehydrogenases are of about the same length, which is about 120 residues longer than either the *E. coli* or *H. influenzae* enzymes. This difference is mainly due to the presence of a longer C-terminal domain (~ 200 residues, as compared with ~ 75 residues). Furthermore, the nucleotide- and substrate-binding domains of the rat enzyme show more identity with the *B. subtilis* enzyme (46%) than with the other enzymes (32%). For the *E. coli*/*S. cerevisiae* pair, the corresponding value is even higher (53%), indicating that the yeast enzyme is closer to the *E. coli* enzyme than to the mammalian enzyme. The percentage identity is much lower in the regulatory domain than in the other domains, and is 15% for the rat/*B. subtilis* pair, 30% for the *E. coli*/*S. cerevisiae* pair and not significant when the rat enzyme is compared with that from *E. coli*. These observations suggest that there are two different types of 3-phosphoglycerate dehydrogenase that originated from a duplication event occurring before the separation of eukaryotes and prokaryotes.

Figure 2 also shows that the consensus sequence Gly-Xaa-Gly-Xaa-Xaa-Gly-Xaa$_{17}$-Asp (Figure 2), involved in binding the

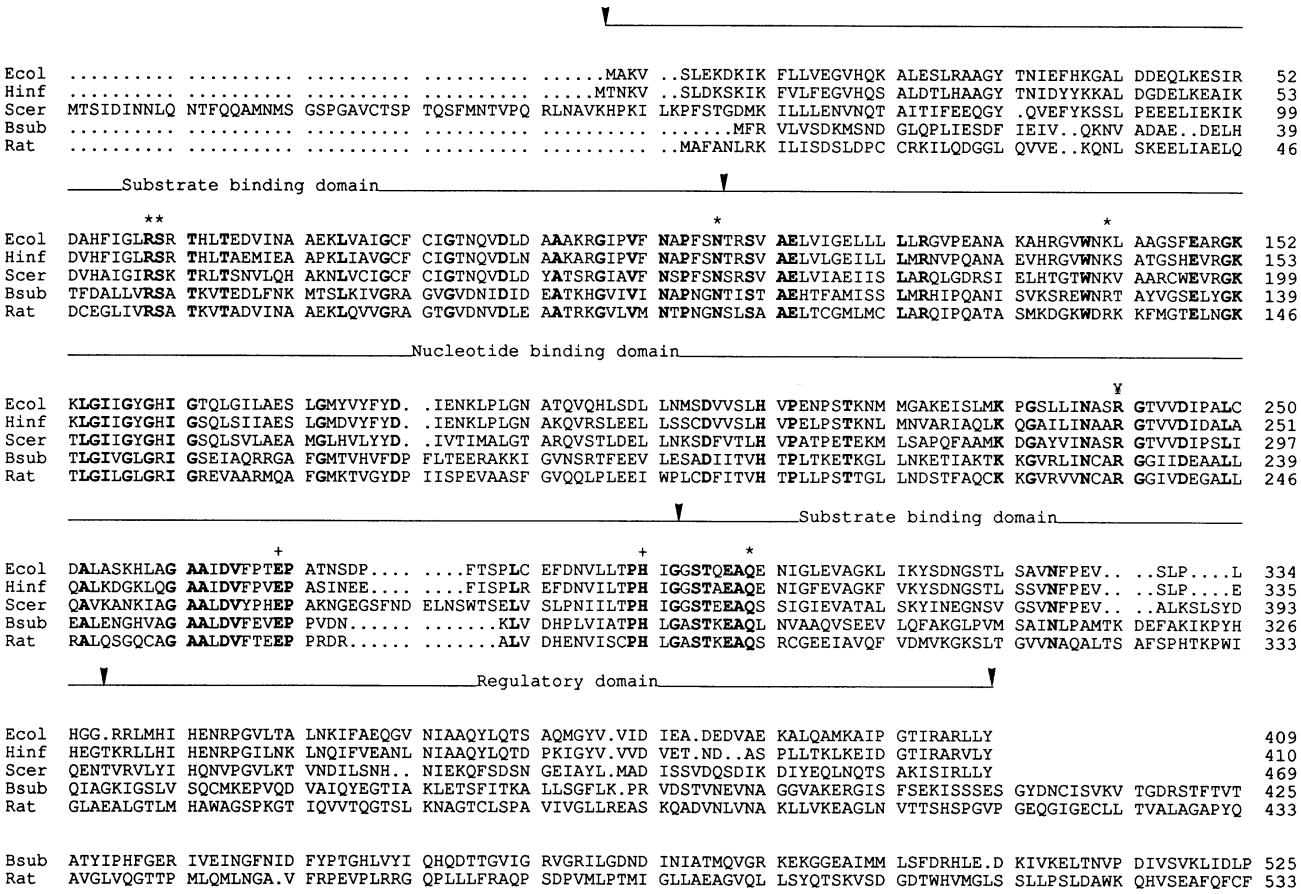

Figure 2 Alignment of the sequences of 3-phosphoglycerate dehydrogenase from various species

The amino acid sequences of the enzyme from *E. coli* (Ecol; [12]), *H. influenzae* (Hinf; GenBank accession no. L45106; [13]), *S. cerevisiae* (Scer; GenBank P40054), *B. subtilis* (Bsub; GenBank L47648; [14]) and rat hepatoma (the present work) are displayed in single-letter code after alignment for maximal identity. Gaps are indicated by dots and strictly conserved amino acids are in bold. Asterisks indicate residues surrounding the catalytic pocket, where hydride transfer occurs. Residues proposed to be involved in proton shuttling (+) and the arginine presumably involved in binding of the carboxylate group of the substrate (¥) [15] are also shown. The domains of the *E. coli* enzyme are shown above the alignment.

adenosine portion of NAD^+ [28], is located in the rat enzyme at residues 152–175. Several residues lining the catalytic pocket in the *E. coli* enzyme are strictly conserved in the mammalian enzyme (Arg-60, Ser-61, Asn-108 and Gln-301) or replaced by functionally similar residues (Lys-141 replaced by Arg-136). By analogy with the *E. coli* enzyme, it is likely that Arg-236 binds the carboxy group of the substrate and that His-292 and Glu-269 function as a proton shuttle [15].

Northern blots

Northern blot analysis of total RNA indicated the presence of a specific ~ 2.1 kb mRNA in livers derived from animals maintained for 3 days on a protein-free, high-carbohydrate diet (Figure 3). The length of this mRNA is consistent with the size of the longest clone that we obtained, allowing for the presence of the poly(A) tail. Strikingly, the mRNA was not detectable in the livers of animals fed on a normal diet, suggesting that control of the expression of 3-phosphoglycerate dehydrogenase is exerted at the level of transcription or by stabilization of the mRNA. A 2.1 kb mRNA was also observed in kidney (Figure 3), testis and brain (results not shown), and in lower amounts in lung (Figure 3), skeletal muscle and heart (results not shown). In these tissues, no effect of dietary manipulation on 3-phosphoglycerate dehydrogenase mRNA was observed. These results are in agreement with the observation that the induction of the enzyme by a low-protein diet is restricted to the liver [29].

Expression of recombinant rat 3-phosphoglycerate deydrogenase

The isolated cDNA was expressed in *E. coli* to verify that it encoded a protein with similar properties to the enzyme purified from liver. Addition of isopropyl thiogalactoside to BL21-(DE)p*Lys*S cells carrying the expression plasmid resulted in the induction of 3-phosphoglycerate dehydrogenase, the activity of which reached about 8 units/mg of protein after 18 h at 18 °C (as compared with 0.01 unit/mg of protein in control cells). The recombinant protein was purified by chromatography on DEAE-Sepharose, and was nearly homogeneous after this single step, corresponding to a polypeptide with the expected size (M_r 57000 by SDS/PAGE; results not shown). After AMP–Sepharose chromatography, the preparation had a specific activity of 35 units/mg of protein. From 1 litre of culture, 4700 units of purified enzyme was obtained, corresponding to a 50% yield. Upon gel filtration on Sephacryl S-200, the enzyme was eluted with an M_r of approx. 220000 (results not shown), indicating that it behaves as a tetramer, like the enzyme from *E. coli* [30], rabbit

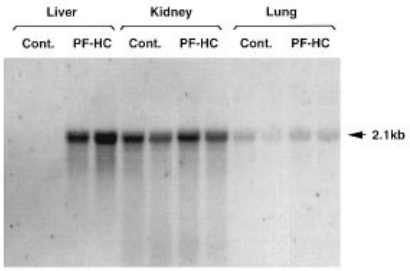

Figure 3 Northern blot analysis of mRNA from liver, kidney and lung from rats fed either a normal diet or a protein-free, carbohydrate-rich diet

Rats either were fed laboratory pellets (Cont.) or were maintained for 3 days on a protein-free, high-carbohydrate diet (PF-HC). Total RNA was extracted and subjected to electrophoresis (15 μg/lane). For each condition, samples from two different animals are shown.

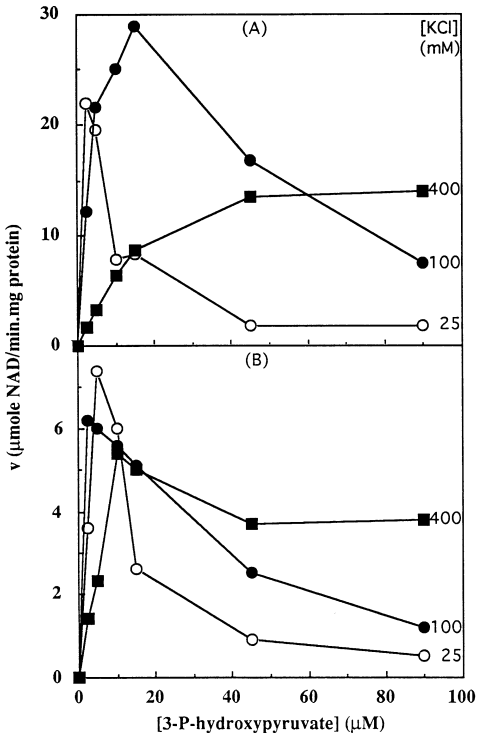

Figure 4 Effects of 3-phosphohydroxypyruvate and KCl on the activity of the recombinant full-length (A) and truncated (B) forms of liver 3-phosphoglycerate dehydrogenase

The enzyme was assayed with the indicated concentrations of phosphohydroxypyruvate (3-P-hydroxypyruvate) and KCl, in the presence of 90 μM NADH.

liver [26] and chicken liver [31]. The recombinant protein purified on DEAE-Sepharose was used in the kinetic studies.

Kinetic properties

The kinetic properties of the recombinant and native enzymes were investigated. When measured in the physiological direction, both enzymes showed K_m values of about 100 μM for 3-phosphoglycerate and 27 μM for NAD^+. In the opposite direction, the enzyme displayed a K_m of 20–25 μM for NADH, and was inhibited by elevated concentrations of 3-phosphohydroxypyruvate (Figure 4A). As previously reported by Jaeken et al. [4], this inhibition was released by 100–400 mM KCl. The data obtained in the present study at low substrate concentrations indicate that the salt displaced the saturation curve to the right, inhibiting enzyme activity at low concentrations of substrate and stimulating activity at elevated concentrations. Similar effects were obtained with other salts. When measured with 90 μM 3-phosphohydroxypyruvate, the effect was biphasic, with an optimum at a concentration close to 400 mM for univalent salts and 80 mM for bivalent salts (results not shown).

We have also tested the effects of serine, which is known to inhibit the activity of the 3-phosphoglycerate dehydrogenases from *E. coli* [9], *B. subtilis* [7] and plants [8], and of the other 19 standard amino acids at concentrations up to 5 mM, on the activity both in the physiological direction and in the reverse direction; none of these amino acids affected the enzymic activity. Since the enzyme from rat appears to be closer to the *B. subtilis* enzyme than to the other enzymes, we took the precaution of testing the sensitivity to serine under conditions similar to those described by Saski and Pizer [7], i.e. by preincubating the enzyme with serine in the presence of dithiothreitol. No effect was observed under these conditions.

Properties of a truncated form of rat 3-phosphoglycerate dehydrogenase

Because the inhibition by excess substrate appeared to be specific for the mammalian enzyme, we tested the hypothesis that the C-terminal domain mediates this inhibition. We therefore expressed in *E. coli* a truncated form of 3-phosphoglycerate dehydrogenase lacking the last 209 amino acids. The recombinant protein was purified to a specific activity of 14 units/mg of protein. As expected, its subunit migrated with an M_r of 36000 in SDS/PAGE (results not shown). Gel filtration on Sephacryl S-200 gave an M_r of 80000, indicating that the enzyme behaved as a dimer and confirming the role of the C-terminal domain in the tetramerization of the enzyme [15]. However, the mutant enzyme displayed substrate inhibition by phosphohydroxypyruvate, and this inhibition was sensitive to salt (Figure 4B). These results indicate that the C-terminal domain of the rat enzyme is not involved in the inhibition by excess phosphohydroxypyruvate.

Lack of 2-oxoglutarate reductase activity

Since *E. coli* 3-phosphoglycerate dehydrogenase has been reported to catalyse the reduction of 2-oxoglutarate to L- and D-hydroxyglutarate [11], we tested whether the rat enzyme could catalyse a similar reaction. However, at concentrations between 10 μM and 10 mM, 2-oxoglutarate was not a substrate for rat 3-phosphoglycerate dehydrogenase, whether 400 mM NaCl was present or not. Furthermore, at 10 mM, 2-oxoglutarate did not behave as an inhibitor of the enzyme in either the forward or reverse direction. Similar results were observed with the truncated enzyme. These results therefore argue against the possibility that 3-phosphoglycerate dehydrogenase is implicated in the pathogenesis of D- or L-hydroxyglutaric aciduria [11]. The results indicate also that 3-phosphoglycerate dehydrogenase is not responsible for the L-2-hydroxyglutarate dehydrogenase activity found in rat liver [32].

Conclusion

This paper describes the cloning of the cDNA encoding rat liver 3-phosphoglycerate dehydrogenase and the confirmation of its identity by expression of the encoded protein in bacteria. The availability of this clone now opens up the possibility of studying the molecular basis of human 3-phosphoglycerate dehydrogenase

deficiency and the mechanism by which protein intake regulates the expression of this enzyme in the liver.

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