RESEARCH COMMUNICATION

Identification and characterization of the gene encoding the human phosphopantetheine adenylyltransferase and dephospho-CoA kinase bifunctional enzyme (CoA synthase)

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The final two enzymes in the CoA biosynthetic pathway, phosphopantetheine adenylyltransferase (PPAT; EC 2.7.7.3) and dephospho-CoA kinase (DPCK; EC 2.7.1.24), are separate proteins in prokaryotes, but exist as a bifunctional enzyme in pig liver. In the present study we have obtained sequence information from purified pig-liver enzyme, and identified the corresponding cDNA in a number of species. The human gene localizes to chromosome 17q12-21 and contains regions with sequence similarity to the monofunctional *Escherichia coli* DPCK and PPAT. The recombinant 564-amino-acid human protein confirmed the associated transferase and kinase activities, and gave similar kinetic properties to the wild-type pig enzyme.

Key words: bifunctional enzyme, CoA synthase, coenzyme A, dephospho-CoA kinase.

INTRODUCTION

CoA is the principal acyl carrier in all living systems, and is widely required in synthetic and degradative metabolic pathways [1]. The five-step pathway for CoA biosynthesis from pantothenic acid, cysteine and ATP is common to all organisms [2]. The genes encoding these enzymes have been identified in bacteria (*coaA* to *coaE*; [3–7]), but the corresponding genes in higher organisms are less well characterized, with pantothenate kinase being the only eukaryotic sequence yet described [8].

The final two steps of the pathway are catalysed by phosphopantetheine adenylyltransferase (PPAT; E.C. 2.7.7.3) and dephospho-CoA (dPCoA) kinase (DPCK; EC 2.7.1.24). The PPAT reaction involves the reversible adenylation of 4′-phosphopantetheine to form 3′-dPCoA and PP_i, and DPCK catalyses phosphorylation of the 3′-hydroxy group of the ribose moiety of dPCoA.

In yeast, the two enzymes are part of a large 350 kDa multienzyme complex [9]. Studies in *Corynebacterium ammoniagenes* suggested that separate enzymes were present [10], and this was subsequently confirmed on identification of the bacterial PPAT/*coaD* [6]. The crystal structure of the *Escherichia coli* PPAT revealed a dimer of trimers, with each subunit displaying a dinucleotide-binding fold [11]. The DPCK/*coaE* gene has also been characterized from *E. coli*, and encodes a 22 kDa monofunctional enzyme [7].

We have shown previously that the pig-liver PPAT and DPCK reactions are catalysed by a bifunctional enzyme, originally designated 'CoA synthase' [12]. This migrated to 60 kDa on SDS/PAGE, and initially appeared to be a homodimer, which was susceptible to proteolytic cleavage without loss of either activity [13].

In the present study, we have isolated and sequenced the cytosolic bifunctional pig-liver enzyme, and have identified the corresponding highly conserved gene in a range of species. The purified recombinant human enzyme behaved as a monomeric 62 kDa protein, and confirmed the associated transferase and kinase activities.

EXPERIMENTAL

Materials

Tosylphenylalanylchloromethane (TPCK)-treated trypsin from bovine pancreas, L-lactate dehydrogenase from rabbit muscle, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, pyruvate kinase from rabbit muscle, hexokinase from baker's yeast, CoA, dPCoA and phosphoenolpyruvate were purchased from Sigma–Aldrich Ireland Ltd (Dublin, Ireland). NAD^+ and NADH were obtained from Roche Diagnostics Ltd (Lewes, East Sussex, U.K.). Sephadex G-25 (fine), Sepharose CL-6B, DEAE-Sepharose and Superdex 200 HR10/30 were from Amersham Biosciences (St Albans, Herts., U.K.). Restriction endonucleases *Bam*HI and *Sal*I were from New England Biolabs (U.K.) Ltd (Hitchin, Herts., U.K.). Moloney-murine-leukaemia virus (MMLV) reverse transcriptase was from Promega (Madison, WI, U.S.A.). *Pfu* Turbo® DNA Polymerase and BL-21(DE3) *E. coli* strain were obtained from Stratagene (Amsterdam, The Netherlands). pCR® 2.1 and INVαF[1] cells were from Invitrogen (San Diego, CA, U.S.A.). The expression vector p^{tac}85 [14] was a gift from Professor P. C. Engel

Abbreviations used: DPCK, dephospho-CoA kinase; dPCoA, dephospho-CoA; DTT, dithiothreitol; EST, expressed sequence tag; MMLV, Moloney-murine-leukaemia virus; PPAT, phosphopantetheine adenylyltransferase; TPCK, tosylphenylalanylchloromethane.

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The nucleotide sequence data reported for the human and pig PPAT/DPCK cDNA will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AY094602 and AY094603 respectively.

(Department of Biochemistry, University College Dublin, Ireland). All other chemicals were of analytical reagent grade.

Enzyme assays

All enzyme assays were performed at 25 °C, and the change in absorbance at 340 nm was monitored. One unit of activity was determined as an amount of enzyme that converts 1 μmol of substrates into corresponding products.

PPAT activity was assayed in the reverse direction using hexokinase and glucose-6-phosphate dehydrogenase to couple ATP production with NAD^+ reduction [15]. The assay mixture (1 ml) consisted of 0.1 mM dPCoA, 2 mM PP_i, 2 mM $MgCl_2$, 1 mM NAD^+, 5 mM glucose, 5 units of hexokinase and 1 unit of glucose-6-phosphate dehydrogenase in 50 mM Tris/HCl buffer, pH 8.0, containing 0.5 mM dithiothreitol (DTT).

DPCK assays followed the production of ADP from dPCoA and ATP using pyruvate kinase and lactate dehydrogenase as coupling enzymes to generate NADH oxidation [16]. The assay mixture (1 ml) consisted of 0.1 mM dPCoA, 2 mM ATP, 2 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate, 0.2 mM NADH, 7 units of pyruvate kinase and 5 units of lactate dehydrogenase in 50 mM Tris/HCl buffer, pH 8.0, containing 0.5 mM DTT.

Purification and sequencing of the wild-type pig-liver bifunctional enzyme

The pig-liver enzyme was purified as described previously [12]. Size-exclusion chromatography on FPLC Superdex 200 HR 10/30 was performed both as a final purification step and to determine the molecular mass of the protein. The elution buffer used was 50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl and 0.5 mM DTT.

The purified 62 kDa material was subjected to N-terminal protein sequencing following electroblotting on to a PVDF membrane. Sequencing was performed using an Applied Biosystems Procise HT protein sequencer (Warrington, Cheshire, U.K.). For limited proteolysis, TPCK-treated trypsin (1 %, w/w) was incubated with the purified protein for 10 min at 25 °C, and the resulting 40 kDa and 22 kDa bands were also transferred to PVDF for N-terminal sequencing. In addition, the 60 kDa band was fully cleaved with cyanogen bromide before sequence analysis.

Cloning of the human gene *ppat/dpck*

The cDNA was amplified from HepG2 cells using reverse transcriptase-PCR. Total RNA was prepared using the guanidinium/phenol-extraction method [17] and first-strand cDNA was synthesized using MMLV reverse transcriptase. The *ppat/dpck* gene was amplified with a forward primer (5′-GGGGATCCATGGCCGTATTCCGGTCGG-3′) incorporating a *Bam*HI restriction site, and a reverse primer (5′-GGGTCGACGTCAGCTCCAGGAGCCAGTCTG-3′) incorporating a *Sal*I restriction site.

The PCR product was inserted into the pCR® 2.1 vector for sequence verification. For protein expression purposes, the product was excised from pCR® 2.1 with *Bam*HI and *Sal*I restriction enzymes, and cloned into the bacterial expression vector $p^{tac}85$.

Expression and purification of recombinant human PPAT/DPCK

E. coli BL21(DE3) cells, containing $p^{tac}85$/*ppat/dpck*, were grown at 37 °C in 4 × 1 litre of Luria–Bertani broth containing 50 μg/ml ampicillin. At a cell density (D_{600}) of 0.6–0.8, isopropyl β-D-thiogalactoside ('IPTG') was added to a final concentration of 1 mM, and growth was allowed to continue at 37 °C for a further 4 h. Cells were harvested, resuspended in 10 mM Tris/HCl buffer, pH 8.0, containing 0.5 mM DTT and 0.5 mM PMSF, sonicated and centrifuged at 45000 ***g*** for 20 min. Purification was performed using separation techniques similar to those used for the wild-type pig enzyme [12]. Briefly, following 30–50 % ammonium sulphate fractionation, ion-exchange chromatography on Q-Sepharose was performed. The enzyme was eluted in a 0–0.6 M linear KCl gradient in 10 mM Tris/HCl buffer, pH 8.0, containing 0.5 mM DTT, and activity-containing fractions were applied to a Procion Red HE-3B Sepharose column, which was developed with a 0–0.6 M KCl gradient in 50 mM Tris/HCl, pH 8.0/0.5 mM DTT. Following sample concentration, a final FPLC Superdex 200 HR 10/30 size exclusion step was carried out, as described above. PPAT/DPCK was stored in 0.1 mM CoA at −20 °C. Before use, CoA was removed from aliquots by Sephadex G-25 (fine) chromatography.

RESULTS

PPAT and DPCK activities were co-purified from pig-liver cytosol as a single protein, as reported previously [12]. Size-exclusion chromatography on FPLC Superdex 200 at the final

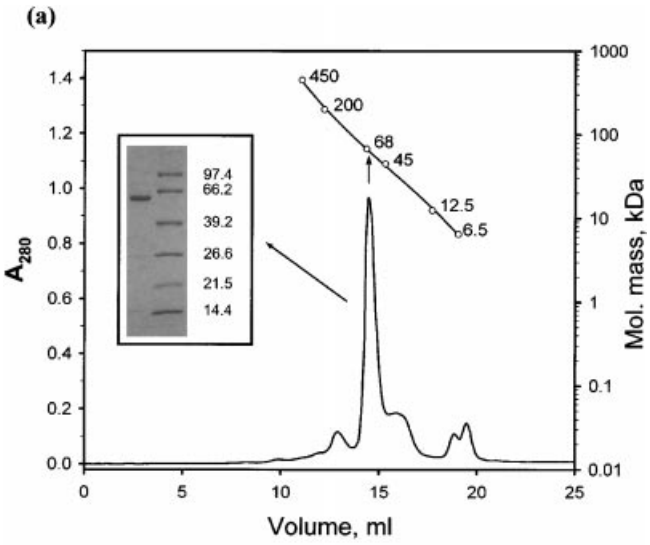

Figure 1 Elution profile from FPLC Superdex 200 HR 10/30 size-exclusion chromatography of the pig-liver PPAT/DPCK (a), and N-terminal sequence analysis of pig-liver PPAT/DPCK enzyme fragments (b)

(**a**) The peak eluted at 62 kDa, as determined by column calibration with known molecular-mass markers, and contained both PPAT and DPCK activities. The inset shows an SDS/polyacrylamide gel of the peak fraction showing a single 62 kDa band. (**b**) N-terminal sequence analysis of pig-liver PPAT/DPCK fragments was performed following either limited proteolysis by trypsin or complete cyanogen bromide cleavage. 'X', unidentified amino acid residue.

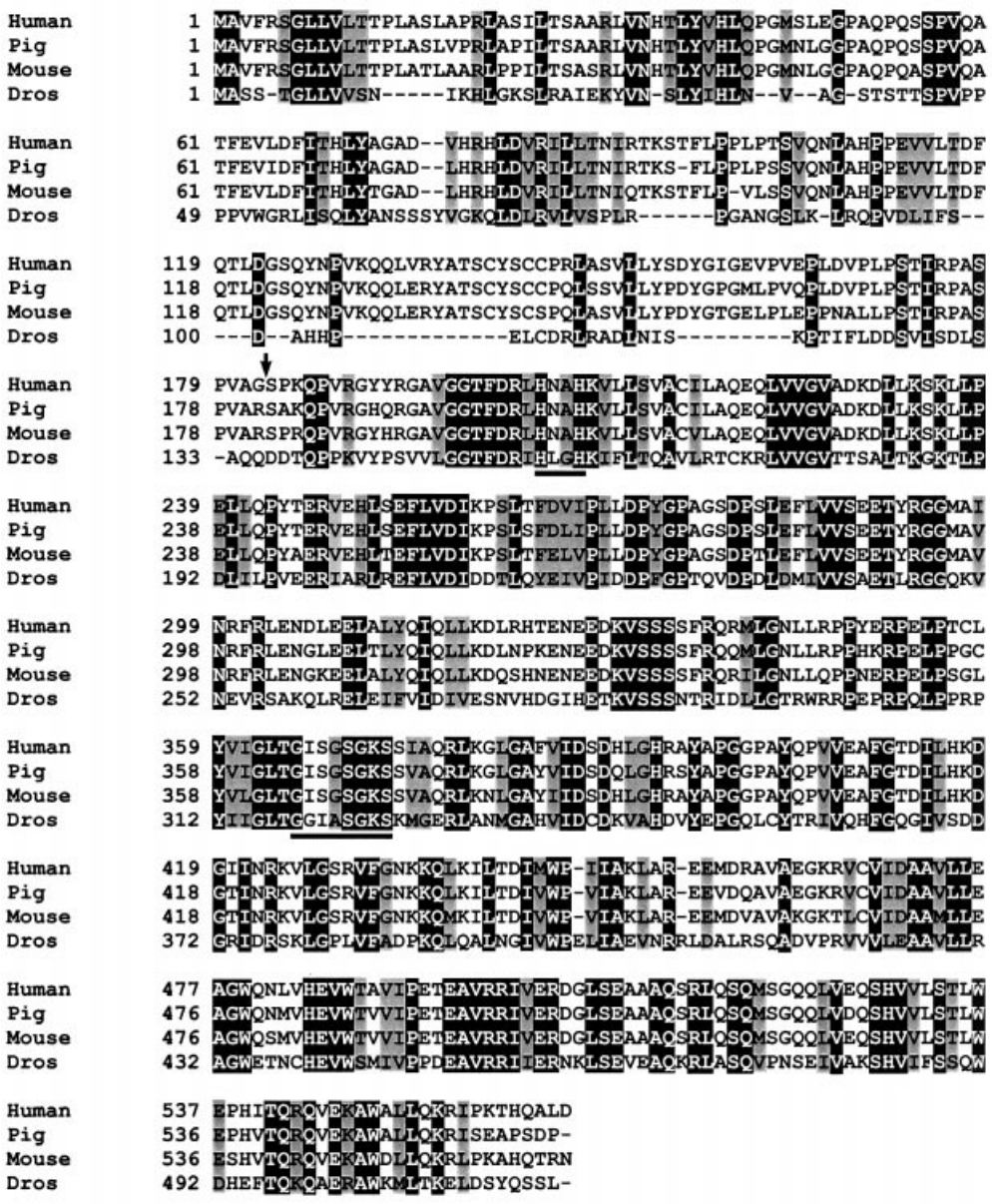

Figure 2 Alignment of the bifunctional PPAT/DPCK sequences from *Homo sapiens* (GenBank® accession no. AY094602), *Sus scrofa* (AY094603), *Mus musculus* (gi:12836393) and *Drosophila melanogaster* (gi:10728128)

Similar residues are shown on a grey-shaded background; identical residues are shown in reversed-out lettering on a black background. The peptide sequences obtained following CNBr treatment of the pig enzyme (Figure 1b) correspond to the following positions: peptide 1, amino acids (aa) 296–315; peptide 2, aa 519–536; peptide 3, aa 482–500; peptide 4, aa 46–64; peptide 5, aa 160–177; and peptide 6, aa 370–355. The site for trypsin nicking of the pig enzyme is indicated by an arrow. The HXXH motif characteristic of nucleotidyl transferases and the Walker A-type motif common to kinases are underlined. Dros, *Drosophila*.

stage of purification revealed that, in the presence of 150 mM NaCl, pig-liver PPAT/DPCK eluted as a peak with molecular mass of 62 kDa (Figure 1a). This indicates that the protein behaves as a monomer, and not a homodimer, as originally suggested [13]. N-terminal sequence analysis of the purified protein revealed a blocked N-terminus, and internal sequencing was performed following either limited trypsin proteolysis or cyanogen bromide cleavage (Figure 1b). The sequences obtained were used for gene database BLAST search analysis [18]. Hits were obtained with a number of pig expressed sequence tag (EST) sequences (gi:6842495, gi:8274195, gi:14305703, gi:13660202, gi:13659612 and gi:11076507). The results allowed the corresponding cDNA to be assembled from the pig EST sequences, and subsequently in a number of other species. ClustalW alignment [19] of the pig, human and mouse sequences are shown in Figure 2, and are highly conserved, with greater than 96% sequence identity. The *Drosophila* sequence has approx. 35% identity with the mammalian sequences.

The human *ppat/dpck* gene is located on chromosome 17q12-21, consists of 10 exons, and incorporates a coding region designated previously as a hypothetical nucleotide-binding protein (gi:894177). However, the correct ATG start codon is

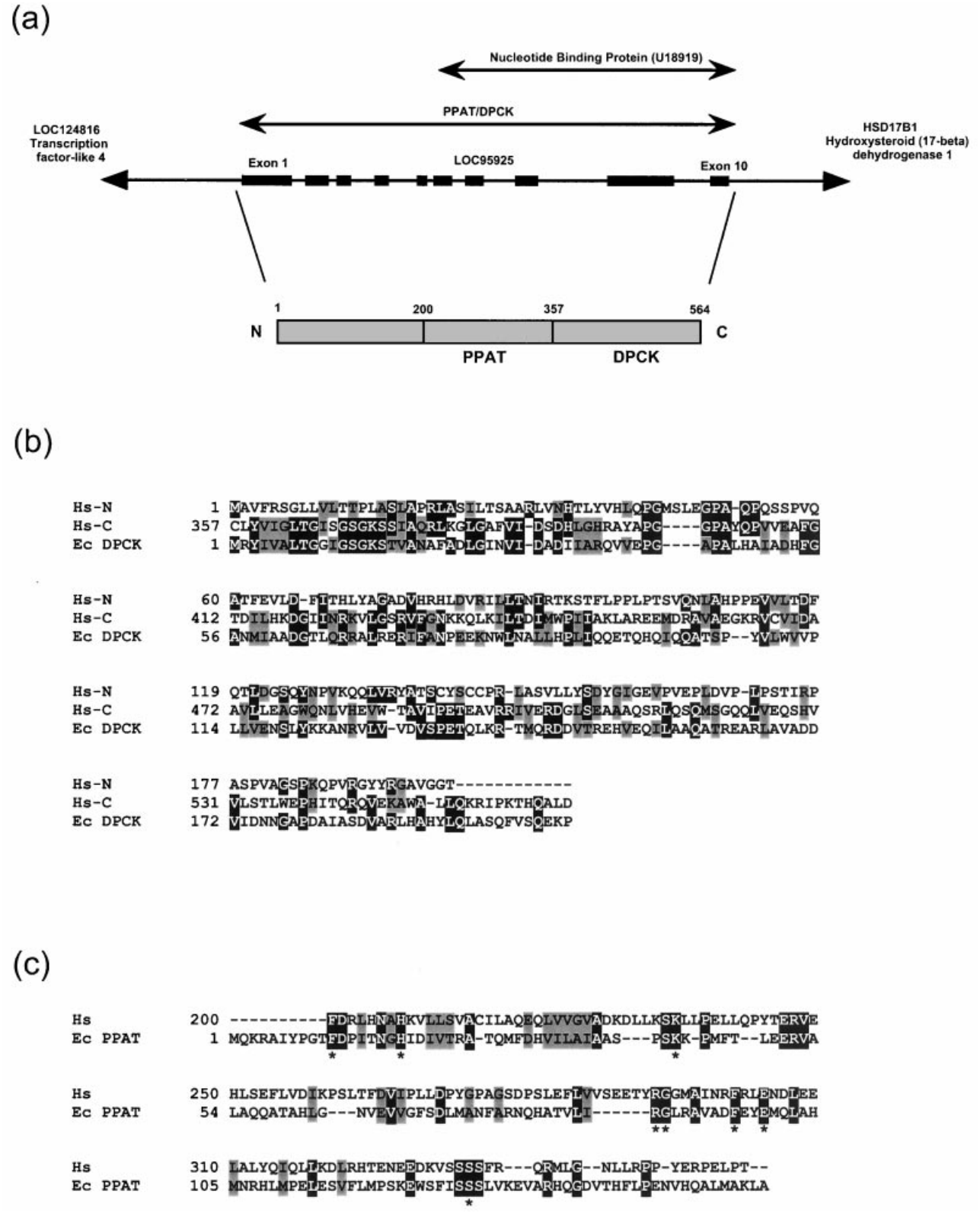

Figure 3 Bioinformatic determination of functional domains of human PPAT/DPCK

(**a**) Gene structure and localization on chromosome 17q12-21, with predicted protein domains below. (**b**) Sequence alignment between the N-terminal domain (Hs-N; amino acids 1–199) and the C-terminal domain (Hs-C; amino acids 357–564) of human PPAT/DPCK and the *E. coli* DPCK (Ec DPCK) sequence [7]. (**c**) Alignment of the middle region of PPAT/DPCK (amino acids 200–356) with the *E. coli* PPAT (Ec PPAT) sequence [6]. In (**b**) and (**c**), similar residues are shown on a grey-shaded background; identical residues are shown in reversed-out lettering on a black background. Conserved residues also identified as invariant active site residues in prokaryotes [11] are indicated with asterisks in (**c**).

Table 1 Kinetic characteristics of the human recombinant and the pig-liver wild-type enzymes

Enzyme assays were carried out at 25 °C in 50 mM Tris/HCl buffer, pH 8.0, containing 0.5 mM DTT. The K_m values for dPCoA in both the PPAT (reverse) and DPCK reactions were measured at a constant concentration of PP_i (2 mM) and ATP (1 mM), respectively. The K_m values for both PP_i and ATP were obtained at a constant concentration of dPCoA (0.1 mM). Data shown in parentheses are for the wild-type pig liver enzyme.

	PPAT (reverse reaction)		DPCK	
Substrate	K_m (μM)	V_{max} (units/mg)	K_m (μM)	V_{max} (units/mg)
dPCoA	14.7 ± 1.2 (11.1 ± 2.2)	7.75 ± 0.15 (8.13 ± 0.86)	5.2 ± 1.5 (4.1 ± 1.4)	5.11 ± 0.15 (4.37 ± 0.61)
PP_i	272 ± 65 (190 ± 40)	7.5 ± 0.3	–	–
ATP	–	–	192 ± 14 (330 ± 100)	5.18 ± 0.04

upstream from this region, and the final transcribed coding region is 1692 bp, giving rise to a 564-amino-acid polypeptide chain (Figure 3a).

Human EST data from different tissues reveal that the gene is widely expressed, with cDNA sequence sources including placenta, fetal liver, spleen, ovary, stomach, kidney, bone marrow, testis, fibroblast, retina, salivary gland, brain, colon, lung, adrenal gland, heart, B cells, NT2 neuronal precursor cells, uterus and prostate. At least 294 current human EST sequences can now be annotated as PPAT/DPCK, in addition to many sequences from other species.

The C-terminal 208-amino-acid region of the gene is similar to that of the *E. coli* DPCK, with 24 % identity (Figure 3b). This was noted previously following sequencing of the *coaE* gene [7], but the particular alignment shown had used a human sequence that was partially out of frame. Interestingly, there is also internal homology within the human protein sequence, with an N-terminal region aligning with the C-terminal one (Figure 3b).

A central domain of 157 amino acids shows weak sequence similarity (18 % identity) with the bacterial PPAT (Figure 3c), although the *E. coli coaD* gene has not been reported to have any mammalian counterparts. This PPAT region contains the His-Xaa-Xaa-His motif (where 'Xaa' represents 'any amino acid') found in the nucleotidyl transferase superfamily, which is thought to be involved in phosphodiester cleavage [20]. The DPCK C-terminal region contains a conserved Walker A-type kinase motif (GISGSGKS) involved in ATP binding [21].

Proteolytic nicking of the pig enzyme by trypsin to yield 40 kDa and 22 kDa fragments has been reported, which does not effect either activity or the proposed tertiary structure [13]. The sequence analysis of the resulting 40 kDa band resulting from SDS/PAGE (the 22 kDa band N-terminal was blocked) shows that this nicking occurs after position Arg^{181}, suggesting that an exposed region exists between the N-terminal region and the PPAT domain.

The human PPAT/DPCK cDNA was cloned from HepG2 cells and the sequence was verified (submitted to GenBank®; accession number AY094602). Expression and purification of the recombinant human protein was performed, and activity assays confirmed that the human gene encodes the bifunctional enzyme PPAT/DPCK. Size-exclusion chromatography on an FPLC Superdex 200 column showed that the human recombinant protein behaved like the pig-liver wild-type enzyme, eluting as a single peak of 62 kDa, suggesting a monomeric native structure (the predicted molecular mass from the open reading frame is 62329 Da).

Analysis of kinetic parameters of the human recombinant enzyme and the pig-liver wild-type enzyme was performed for the PPAT (reverse) reaction and for the DPCK reaction. The results of kinetic analysis are shown in Table 1 (parameters for the pig-liver wild-type enzyme are given in parentheses), and indicate that the kinetic properties of the human recombinant PPAT/DPCK are very similar to those of the pig enzyme, as was anticipated from the high level of sequence similarity.

DISCUSSION

Current knowledge of the CoA biosynthetic enzymes in higher organisms has not kept pace with the identification and characterization of the corresponding genes in bacteria. Previous biochemical evidence suggested that the mammalian PPAT and DPCK enzymes exist as a bifunctional enzyme [12,22], where monofunctional counterparts are found in bacteria. We have now identified the gene encoding the bifunctional enzyme, and have verified the activities to be associated with the 564-amino-acid recombinant human protein.

There is no evidence for any CoA transport system in multicellular organisms, and it is assumed that each cell has the ability to generate an independent pool of CoA using the biosynthetic pathway. This is borne out by the fact that the expressed sequence for the bifunctional PPAT/DPCK is found in a wide range of tissues, and annotation of this gene will now aid in the designation of a large number of previously 'unknown' sequences in all higher organisms.

It is possible that monofunctional enzymes may also exist in higher organisms, particularly in view of mitochondrial subfractionation studies, which suggest differences in activity distribution between PPAT and DPCK [23]. However, no other genes were identified on BLAST analysis of the human genome in the present study.

Following alignment analysis with the known monofunctional *E. coli* sequences, the protein can be essentially divided into three potential domains. The C-terminal domain shows significant sequence similarity to the *E. coli coaE* gene, and is predicted to contain the DPCK activity. The N-terminal and C-terminal domains demonstrate some intramolecular sequence similarity (18 % identity), suggesting that a gene-duplication event followed by a gene-fusion event may have occurred to give rise to part of the bifunctional enzyme. There is also sequence similarity (albeit weaker) between the 159-amino-acid *E. coli* PPAT protein and the central region (amino acids 200–356) of the human PPAT/DPCK enzyme. Within prokaryotic PPAT sequences, 14 residues have been identified as invariant [11], and the crystal structure demonstrated that a number of these are involved in dPCoA binding. Using *E. coli* PPAT numbering, eight of these residues (Phe^{11}, His^{18}, Lys^{42}, Arg^{88}, Gly^{89}, Phe^{96}, Glu^{99} and Ser^{129}) are also conserved in the human sequence (Figure 3c). It is likely that this region of the human protein forms a Rossmann fold, similar to the bacterial PPAT and to class 1 aminoacyl-tRNA synthetases.

A crystal structure of the monofunctional DPCK from *Haemophilus influenzae* has recently been obtained [24], which included an ATP-binding P-loop and a CoA-binding site at the interface of three domains. It is also likely that the comparable C-terminal domain of the bifunctional enzyme adopts a similar structure. It is more difficult to predict the overall structure, which may show internal dimerization between the similar N- and C-terminal regions. Crystallization studies, currently in progress, may also shed light on the total number of ATP- and dPCoA-binding sites, and the possibility of dPCoA channelling between the two activities.

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