Purification, cDNA cloning and heterologous expression of the human mitochondrial NADP$^+$-dependent malic enzyme

Gerhard LOEBER,* Ingrid MAURER-FOGY and Renate SCHWENDENWEIN
Bender & Co, Dr. Boehringergasse 5–11, A-1121 Vienna, Austria

Mitochondrial NADP$^+$-dependent malic enzyme (ME; EC 1.1.1.39) has been purified to homogeneity and characterized kinetically from bovine heart. Partial amino acid sequence information allowed amplification of a specific bovine cDNA, which was used to isolate a full-length human cDNA of this isoform of ME. The cDNA is 1930 bp long and codes for a protein of 604 amino acids. Comparison of the amino acid sequence of this isoform with published sequences of other human ME isoforms shows stretches of homology interrupted by larger regions with significant differences. The human protein has been expressed in *Escherichia coli*, and the recombinant human protein has the same kinetic properties as the corresponding protein purified from bovine heart. Northern blot analysis showed a strong tissue-specific transcription with a predominantly high expression-rate in organs with a low division-rate.

INTRODUCTION

Malic enzyme (ME; EC 1.1.1.39) catalyses the oxidative decarboxylation of malate to pyruvate using either NAD$^+$ or NADP$^+$ as a cofactor. In mammalian tissues, three distinct isoforms have been described: a mitochondrial and a cytosolic NADP$^+$-dependent isoform (EC 1.1.1.40; Frenkel, 1975) and a mitochondrial NAD$^+$-dependent isoform (EC 1.1.1.39; Sauer, 1973). The cytosolic NADP$^+$-ME has been studied extensively. The highest levels are found in liver and adipose tissue, where it is linked to the generation of NADPH required for fatty acid biosynthesis (Frenkel, 1975), but is also present in other tissues and tumour cells (Chang et al., 1991; Loeber et al., 1994). The expression of this isoform is under dietary control and it can be induced with a carbohydrate-rich, fat-free diet (Frenkel 1975) or thyroid hormones (Dozin et al., 1985). Recently, a thyroid-responsive element has been found in the promoter region of the gene (Petty et al., 1990). The mitochondrial NAD$^+$-ME is found mostly in rapidly dividing tissues and tumour cells (Sauer and Dauchy, 1978; Nagel et al., 1980), but has also been reported in placenta (Zolnierowicz et al., 1988) and adrenal gland (Mandella and Sauer, 1975). Probably, this isoform is required to convert amino acid carbon, mostly glutamine and glutamate, into pyruvate (McKeehan, 1982).

Very little is known about mitochondrial NADP$^+$-ME. It has been described to be expressed in heart (Saito and Tomita, 1972; Lin and Davis, 1974), skeletal muscle (Taroni et al., 1988) and brain (Frenkel, 1975). Also, this isoform has been found in adrenal cortex, where Simpson and Estabrook (1969) have postulated that it provides NADPH for the β-hydroxylation reactions of the steroid biosynthesis in the adrenal mitochondria.

To characterize this isoform of ME in more detail, we have purified the mitochondrial NADP$^+$-ME from bovine heart. Using PCR primers corresponding to peptide sequences, we have amplified and cloned a specific cDNA fragment coding for this isoform of ME from a bovine heart cDNA library. This fragment was used to isolate a full-length cDNA clone from a human hippocampus cDNA library. Significant differences were found when comparing the cDNA-derived amino acid sequence of the protein to the other two human ME isoforms and other known plant and animal MEs. Expression of the open reading frame in *Escherichia coli* resulted in a protein with kinetic properties similar to the protein isolated from bovine heart mitochondria. A Northern-blot analysis of RNA from 16 different human tissues with a probe specific for this isoform showed a strong tissue-specific expression, mostly in tissues with a low division-rate such as heart and skeletal muscle or tissues involved in steroid hormone biosynthesis such as ovary and testes.

MATERIALS AND METHODS

Purification of NADP$^+$-dependent ME from bovine heart mitochondria

Bovine heart mitochondria were prepared following the method of Blaire (1967). In brief, 200 g of fresh beef heart were trimmed, cut into small pieces and ground in a meat grinder. The ground tissue was suspended in 200 ml of solution A (0.25 M sucrose, 20 mM KCl and 25 mM Tris/HCl, pH 7.4). The suspension was homogenized in a high-speed blender, clarified by spinning in a Beckman JA10 rotor for 10 min at 6000 *g* and the supernatant was filtered through cheese-cloth. To separate mitochondrial and cytosolic fractions, the filtrate was recentrifuged in the same rotor for 10 min at 14500 *g*. The pellet was resuspended in 30-ml of solution A and recentrifuged for 10 min in a Beckman JA20 rotor at 10000 *g*. The purified mitochondrial pellet was suspended in 25 ml of buffer B (30 mM Tris/HCl, 3 mM $MgCl_2$, 0.2 mM EDTA and 0.2% Triton X-100, pH 7.4), passed through a Dounce homogenizer ten times and sonicated for 2 × 30 s in an MSI150 sonicator. After clarification for 10 min at 120000 *g* in a Beckman JA20 rotor, the supernatant was loaded onto a 2 cm × 13 cm anion-exchange column (TMAE fractogel, Merck 16881K) and eluted with a 0–200 mM KCl gradient in buffer B. This chromatography step removes mitochondrial NAD$^+$-ME as well as contaminating cytosolic NADP$^+$-ME, since the

Abbreviations used: ME, malic enzyme.
* To whom correspondence should be addressed.

Table 1 Amino acid sequence of tryptic peptides from bovine ME

Amino acids are given in the single letter code. X = Amino acid was not identified. Peptide sequences selected to synthesize PCR primers are underlined. *, Identity refers to identical amino acids of bovine mitochondrial ME when compared with the human cytoplasmic NADP$^+$-dependent ME. Amino acid position refers to the position of homologous sequences in the human cytoplasmic ME.

No.	Amino acid sequence	Identity	%	Amino acid position
	** ***			
1	DLMTLOD	5/7	71	74–80
	*** ***** ***** **			
2	QXLPVLLDVGTNNEELLRDP	15/20	75	187–206
	* ** * * ** *			
3	EMFAODHPEVNSLEEVV	8/17	47	352–368
	*** * **********			
4	DMAXFHEHPIIFALSNPTS	16/19	84	393–411
	******** *** *			
5	IFASGSPFPSVTLEDG	12/16	81	429–444
	* ** * * *** *			
6	LVYTPEYDSFTLDSYTXPEEAMNVQXQ	9/27	33	543–569

mitochondrial NADP$^+$-ME elutes at significantly lower KCl-concentrations (Loeber et al., 1994). Fractions containing NADP$^+$-ME activity were pooled and further purified using an NADP$^+$–agarose affinity column. The column was eluted with buffer B containing 5 mM NADP$^+$ and the eluate was loaded on a 5/5 MonoQ column (Pharmacia). An 80 ml KCl gradient (30–200 mM KCl) in buffer B yielded 65 μg of pure NADP$^+$-ME which could be used directly for amino acid sequencing.

Tryptic digest and h.p.l.c. characterization of the fragments

Purified NADP$^+$-ME (40 μg) from beef heart mitochondria were dissolved in 208 μl of a 1 % NH_4-bicarbonate solution and digested with 4 μl of a 0.2 mg/ml trypsin solution (Boehringer-Mannheim, sequencing grade) for 4 h at 37 °C. After addition of another 4 μl of trypsin, the incubation was continued overnight. Resulting peptides were separated with reverse-phase h.p.l.c. using a Merck Supersphere column (125-4, 100 Å pore-diameter, 4 μm particle-diameter) at a column temperature of 30 °C. Bound peptides were eluted with 70 ml of a linear gradient of 0.1 % trifluoroacetic acid in water and 100 % acetonitrile at a flow rate of 1 ml/min. The elution profile was monitored at 214 nm wavelength and recorded on a Merck D6000 HPLC Manager data system. The tryptic peptides were collected, dried in a speedvac and analysed on an Applied Biosystems 477A pulsed liquid-phase sequencer.

Cloning, sequencing and heterologous expression of the cDNA for human mitochondrial NADP$^+$-ME

Degenerate oligonucleotides corresponding to peptides 1 and 3 (Table 1) were synthesized. To facilitate cloning of amplified fragments, an *Eco*RI restriction site was added at the 5′ end of the two oligonucleotides. A PCR reaction at low stringency (40 s 94 °C, 45 s 45 °C, 3 min 72 °C, 30 cycles) with 1 μg of bovine heart cDNA (Clontech BL 1017b) amplified a 850 bp DNA fragment which was subcloned into the *Eco*RI site of a pUC18 vector (Yanisch-Perron et al., 1985). DNA sequencing showed some similarity to published sequences of other human ME isoforms (Loeber et al., 1991, 1994). This fragment was radioactively labelled with ^{32}P-dCTP using random primers (Feinberg and Vogelstein, 1983) to a specific activity of 10^9 c.p.m./μg DNA and used to screen for human ME sequences in a human hippocampus cDNA library (Stratagene 936205) in a standard radioactive plaque hybridization (Benton and Davis, 1977) with reduced hybridization-stringency at 40 °C in 50 % formamide and 3 × SSC (SCC = 150 mM NaCl/15 mM sodium citrate). Positive signals were plaque-purified twice and recloned into the *Eco*RI site of a Bluescript (SK +) vector (Stratagene, La Jolla) using standard procedures (Sambrook et al., 1989). The nucleic acid sequence was determined from the double-stranded plasmid on a ABI 373A sequencer with 30 sequentially made primers specific for the human mitochondrial NADP$^+$-ME on both strands of the DNA.

To express the coding region minus the putative mitochondrial leader sequence of mitochondrial NADP$^+$-ME (amino acids 46 to the stop codon) in *E. coli*, 2 oligonucleotides (5′ GATTA TGCCT CGAGA GGTTG AGGTG ATTTT ATGCT GAAGA AGCGC GGATA CG 3′) and 5′GGAAG CTTGC CTCAG ACCGT CTGAA CATTC ATGGC 3′) were synthesized. PCR amplification of the full-length cDNA with these primers results in a linear DNA segment containing an *Xho*I restriction site, a bacterial ribosomal binding-site, an ATG start codon, DNA coding for amino acids 46–605 of the ME cDNA, a stop codon plus a *Hind*III restriction site. This expression cassette was ligated into an *Xho*I–*Hind*III-digested pRH281T expression vector (Loeber et al., 1991, 1994) and cloned into *E. coli* XL1 blue cells (Stratagene, La Jolla). Expression from this plasmid in *E. coli* was induced with 50 μg/ml indoleacrylic acid. After induction (3 h to overnight), bacteria were collected, lysed in 50 ml lysis buffer (as above) and the recombinant human NADP$^+$-ME was purified using the same procedure as described for the bovine protein.

Enzyme assays

ME activities were assayed in a Beckman DU640i spectrophotometer at 340 nm as described by Mandella and Sauer (1975). The standard reaction mixture to assay the ME decarboxylation reaction contained 50 mM Tris (pH 7.4), 3 mM $MnCl_2$, 5 mM malate and 0.12 mM NADP$^+$. ME carboxylation reactions ('reverse reaction') were measured in 50 mM Tris/HCl, 50 mM pyruvate, 75 mM $KHCO_3$, 1 mM $MnCl_2$, 0.2 mM NADP, pH 7.4 (Zelewski and Swierczynski, 1991). K_m analyses for pyruvate were carried out in carboxylation buffer at ten different pyruvate concentrations between 10 mM and 35 mM. Kinetic data were processed using a Hanes–Woolf plot.

RNA analysis

For Northern blots, multiple tissue Northern blots from Clontech (7759-1 and 7760-1) were used. Insert fragments were labelled with α-^{32}P-dCTP by random priming (Feinberg and Vogelstein, 1983) to a specific activity of 10^9 c.p.m./μg DNA and hybridizations were carried out in 0.5 M Na-phosphate, 7 % SDS, pH 7.2, for 18 h as described by Church and Gilbert (1984). The blots were washed repeatedly in 40 mM Na-phosphate, 0.5 % SDS, pH 7.2, at 65 °C. Membranes were exposed to Kodak XAR-5 X-ray films with intensifier screen at −70 °C for 16–72 h.

RESULTS

Purification, peptide sequencing and isolation of a specific cDNA fragment of bovine NADP$^+$-ME

Mitochondrial NADP$^+$-ME is expressed predominantly in non-dividing tissues. We were unable to detect significant activities of

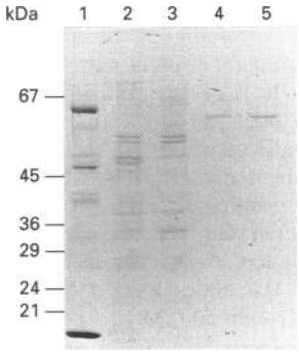

Figure 1 Purification of bovine mitochondrial NADP+-dependent malic enzyme

Coomassie-Blue stained SDS/polyacrylamide gel of: lane 1, bovine heart lysate (20 μg protein); lane 2, bovine heart mitochondrial extract (20 μg protein); lane 3, protein pool after ion-exchange chromatography (20 μg protein); lane 4, protein pool after NADP+-affinity purification (5 μg protein); lane 5, purified NADP+-ME after final MonoQ ion-exchange chromatography (5 μg protein). Positions of molecular mass markers are shown on the left.

this isoform in any human cell culture line (Loeber et al., 1994 and unpublished work). Therefore, we have purified NADP+-ME from bovine heart mitochondria to homogeneity using ion-exchange chromatography, affinity purification on an NADP+-affinity column and f.p.l.c. anion-exchange chromatography. This procedure resulted in a homogenous protein with an apparent molecular mass of 63 kDa as determined by SDS/PAGE (Figure 1) which had a specific activity of 11.1 units/mg protein (1 unit of enzyme catalyses the formation of 1 μmol substrate per min). The purified protein was cleaved with trypsin and the tryptic digest was separated on a reverse-phase h.p.l.c. column. Amino acid sequences were determined for six different peptides (Table 1). The sequence similarity of the peptides to the related human cytoplasmic NADP+-ME was between 33% and 84%. Peptides 1 and 3 were selected as a template for the synthesis of degenerate oligonucleotides (256- and 64-fold degenerate) specific for the bovine mitochondrial NADP+-ME. To reduce the degeneracy for peptide 1 to 128-fold, 2 oligonucleotides based on peptide 1 were synthesized, differing only in their codon for the C-terminal aspartic acid. A PCR with 30 cycles at a low stringency yielded for one of the oligonucleotide pairs a DNA fragment with a length of approx. 850 bp, which was the expected size for an ME-specific fragment, based on the position of the homologous peptides in the related cytoplasmic ME. DNA sequencing of this fragment showed significant similarity to known human ME sequences (60% identity with human mitochondrial NAD+-ME cDNA, 69% identity with human cytoplasmic NADP+-ME cDNA). Cytoplasmic ME is almost identical in different mammalian species, the human and the rat or mouse enzymes have 90% identity at the amino acid level (Loeber et al., 1994). Therefore, it was concluded that this clone represented a distinct isoform of ME.

Isolation of a full-length cDNA clone of human mitochondrial NADP+-ME

The partial bovine mitochondrial NADP+-ME cDNA clone was used to screen a human hippocampus cDNA library for a full-length cDNA clone. In total, 10^6 phage clones were screened. Six plaques gave positive signals after repeated plaque purification, one of them contained an insert with a length of approx. 2000 bp.

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   1 CGGAAGGAGAGGACCGAGGTCTGCCAAGGACCATGGGTGCCGCGCTGGGGACAGGCACGC
                                 M  G  A  A  L  G  T  G  T  R
  61 GGCTGGCTCCCTGGCCGGGCCGGGCCTGCGGCGCCCTCCCGCGCTGGACACCCACCGCGC
     L  A  P  W  P  G  R  A  C  G  A  L  P  R  W  T  P  T  A  P
 121 CCGCCCAAGGCTGCCACTCCAAGCCTGGCCCGGCGCGCCCTGTGCCCCTGAAGAAGCGCG
     A  Q  G  C  H  S  K  P  G  P  A  R  P  V  P  L  K  K  R  G
 181 GATACGATGTCACCAGGAACCCTCATCTCAACAAGGGGATGGCCTTTACCCTTGAAGAAA
     Y  D  V  T  R  N  P  H  L  N  K  G  M  A  F  T  L  E  E  R
 241 GGCTGCAGCTTGGAATCCACGGCCTAATCCCGCCCTGCTTTCTGAGCCAGGACGTCCAGC
     L  Q  L  G  I  H  G  L  I  P  P  C  F  L  S  Q  D  V  Q  L
 301 TCCTCCGAATCATGAGATATTACGAGCGGCAGCAGAGTGACCTGGACAAGTACATCATTC
     L  R  I  M  R  Y  Y  E  R  Q  Q  S  D  L  D  K  Y  I  I  L
 361 TCATGACACTCCAAGACCGGAACGAGAAGCTCTTCTACCGAGTGCTGACTTCGGATGTGG
     M  T  L  Q  D  R  N  E  K  L  F  Y  R  V  L  T  S  D  V  E
 421 AGAAGTTCATGCCAATCGTGTACACGCCTACCGTGGGGCTGGCCTGTCAGCACTATGGCC
     K  F  M  P  I  V  Y  T  P  T  V  G  L  A  C  Q  H  Y  G  L
 481 TGACTTTCCGCAGGCCCCGTGGACTGTTCATCACCATTCATGACAAAGGTCATCTTGCAA
     T  F  R  R  P  R  G  L  F  I  T  I  H  D  K  G  H  L  A  T
 541 CAATGCTGAATTCTTGGCCAGAAGACAATATTAAGGCCGTGGTGGTGACTGATGGGGAGC
     M  L  N  S  W  P  E  D  N  I  K  A  V  V  V  T  D  G  E  R
 601 GCATCCTGGGCCTGGGAGACCTGGGCTGCTACGGCATGGGCATCCCTGTGGGCAAGCTGG
     I  L  G  L  G  D  L  G  C  Y  G  M  G  I  P  V  G  K  L  A
 661 CCCTGTACACGGCATGCGGAGGGGTGAACCCGCAGCAGTGCCTCCCTGTGCTGCTGGACG
     L  Y  T  A  C  G  G  V  N  P  Q  Q  C  L  P  V  L  L  D  V
 721 TCGGCACCAACAATGAGGAGCTGCTCAGAGACCCTCTGTACATCGGCCTGAAACACCAGC
     G  T  N  N  E  E  L  L  R  D  F  L  Y  I  G  L  K  H  Q  R
 781 GCGTGCACGGGAAGGCATACGATGACTTGCTGGATGAGTTCATGCAGGCTGTGACAGACA
     V  H  G  K  A  Y  D  D  L  L  D  E  F  M  Q  A  V  T  D  K
 841 AGTTTGGAATAAATTGCCTCATCCAATTTGAAGACTTCGCCAATGCCAATGCCTTCCGCC
     F  G  I  N  C  L  I  Q  F  E  D  F  A  N  A  N  A  F  R  L
 901 TGCTCAACAAATACCGTAACAAGTACTGCATGTTCAATGATGACATCCAAGGCACAGCCT
     L  N  K  Y  R  N  K  Y  C  M  F  N  D  D  I  Q  G  T  A  S
 961 CCGTTGCTGTGGCAGGGATCTTGGCTGCTCTGCGAATCACCAACAACAAGCTTTCCAATC
     V  A  V  A  G  I  L  A  A  L  R  I  T  N  N  K  L  S  N  H
1021 ACGTGTTTGTTTTCCAAGGTGCAGGCGAGGCAGCTATGGGCATTGCCCACCTCCTTGTCA
     V  F  V  F  Q  G  A  G  E  A  A  M  G  I  A  H  L  L  V  M
1081 TGGCCCTAGAGAAAGAAGGTGTACCGAAGGCAGAGGCCACAAGAAAGATCTGGATGGTGG
     A  L  E  K  E  G  V  P  K  A  E  A  T  R  K  I  W  M  V  D
1141 ACTCTAAAGGGCTCATTGTCAAGGGGAGGAGCCACCTGAACCATGAAAAGGAGATGTTTG
     S  K  G  L  I  V  K  G  R  S  H  L  N  H  E  K  E  M  F  A
1201 CCCAAGACCATCCTGAAGTCAACTCCCTGGAGGAGGTGGTGAGGCTGGTGAAGCCCACAG
     Q  D  H  P  E  V  N  S  L  E  E  V  V  R  L  V  K  P  T  A
1261 CCATCATAGGTGTTGCTGCCATCGCAGGAGCCTTCACGGAGCAGATTCTGAGGGACATGG
     I  I  G  V  A  A  I  A  G  A  F  T  E  Q  I  L  R  D  M  A
1321 CCTCCTTCCACGAGCGCCCTATCATCTTTGCCCTGAGCAACCCCACCAGCAAGGCCGAGT
     S  F  H  E  R  P  I  I  F  A  L  S  N  P  T  S  K  A  E  C
1381 GCACGGCTGAGAAGTGCTACCGGGTCACCGAGGGCCGAGGGATTTTTGCCAGTGGAAGTC
     T  A  E  K  C  Y  R  V  T  E  G  R  G  I  F  A  S  G  S  P
1441 CTTTTAAGAGTGTGACTCTGGAAGATGGCAAGACCTTCATTCCTGGGCAGGGAAACAATG
     F  K  S  V  T  L  E  D  G  K  T  F  I  P  G  Q  G  N  N  A
1501 CTTACGTGTTCCCCGGGGTGGCACTGGGAGTCATCGCCGGCGGGATCCGGCACATCCCAG
     Y  V  F  P  G  V  A  L  G  V  I  A  G  G  I  R  H  I  P  D
1561 ATGAGATCTTCCTCCTGACAGCAGAGCAAATTGCCCAGGAAGTCTCTGAGCAGCATCTGT
     E  I  F  L  L  T  A  E  Q  I  A  Q  E  V  S  E  Q  H  L  S
1621 CCCAGGGGAGACTCTATCCACCACTCAGCACCATCCGAGACGTGTCTTTGAGAATTGCCA
     Q  G  R  L  Y  P  P  L  S  T  I  R  D  V  S  L  R  I  A  I
1681 TCAAAGTTCTCGACTACGCGTACAAACACAACCTGGCTTCCTACTACCCAGAGCCTAAGG
     K  V  L  D  Y  A  Y  K  H  N  L  A  S  Y  Y  P  E  P  K  D
1741 ACAAGGAGGCTTTTGTAAGATCCCTGGTCTACACTCCAGACTATGACTCCTTTACACTGG
     K  E  A  F  V  R  S  L  V  Y  T  P  D  Y  D  S  F  T  L  D
1801 ACAGCTACACTTGGCCCAAGGAAGCCATGAATGTTCAGACGGTCTGAGGCAGTCTCAGAG
     S  Y  T  W  P  K  E  A  M  N  V  Q  T  V  *
1861 GCTAGTATGGGGCTAGATGAAGCCCAGAGTAACACCCACAATATAAATGGGTTCCAAAAT
1921 GGCCCAAGTG  1930
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Figure 2 cDNA and deduced amino acid sequence of the human mitochondrial NADP+-dependent ME

The deduced amino acid sequence is shown below the corresponding nucleotide sequence. Positions of the oligonucleotide primers used for DNA sequence and analysis are underlined. The DNA sequence has been submitted to the Genbank/EMBL database and has the accession number X 79440.

The insert of this clone was subcloned and the cDNA was sequenced directly from the double-stranded plasmid. The DNA sequences from both ends of the ME-specific insert were obtained using the M13 universal and reverse-sequencing primers. Sequence information obtained from this set of experiments allowed the custom synthesis of subsequent ME-specific primers, which permitted determination of the complete nucleotide sequence of the 1930 bp fragment for both strands of the DNA (Figure 2). The insert contained an open reading frame coding for a protein of 604 amino acids with a predicted molecular mass of 67 kDa and a predicted isoelectric point of 7.8. The predicted protein has significant homology to other known sequences of ME (Figure

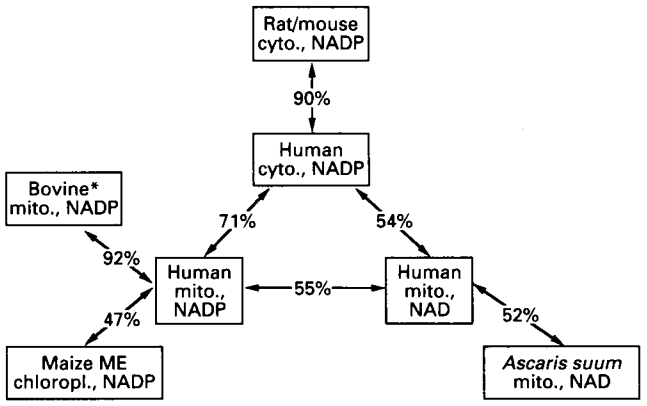

Figure 3 Comparison of malic enzyme proteins from different sources

The degree of amino acid identity (%) is shown in the Figure. References for the published sequences are in the main text. *Bovine mitochondrial NADP$^+$-ME: amino acid identity given for amino acids 74–368 as determined for the cDNA sequence from a PCR-amplified fragment (see text).

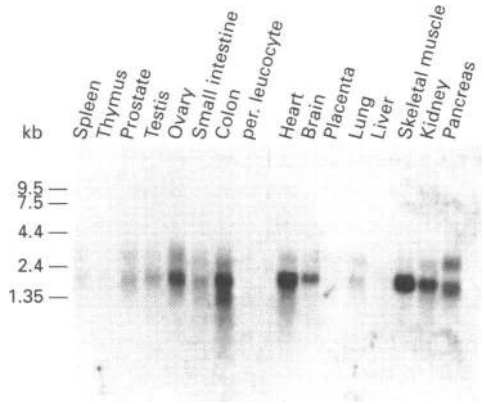

Figure 4 Tissue-specific transcription of the mitochondrial NADP$^+$-ME gene

Northern-blot analysis of a ^{32}P-labelled specific fragment of the mitochondrial NADP$^+$-ME cDNA (bp 1460–1820) to 2 μg of Poly(A)$^+$-selected mRNA from 16 different adult human tissues (multiple-tissue Northern Blot; Clontech, Palo Alto, CA, U.S.A.). The autoradiograph was exposed for 48 h at −70 °C.

3). The 30 N-terminal amino acids have a high probability of forming an amphipathic α-helix and contain several arginine, leucine and threonine residues, whereas acidic amino acids, valines and isoleucines are not present in this stretch. Thus, this segment has the characteristics of a mitochondrial leader peptide as characterized by von Heijne (1986).

To express functional mitochondrial NADP$^+$-ME in *E. coli* we have fused an ATG start codon in frame in front of the leucine codon at position 46. We chose this position for the introduction of a start codon for two reasons. (1) The length of mitochondrial targetting sequences is variable among different proteins, and in the case of the human mitochondrial NAD$^+$-ME the presence of the leader peptide, which is not removed in *E. coli*, interferes with the activity of the recombinant protein (Loeber et al., 1991). By choosing a position so far downstream of the endogenous ATG we assured that no interference with the residual leader peptide took place. (2) Significant similarity to the other 2 human ME isoforms starts at amino acid 46, whereas no significant similarity was present in the first 45 amino acids, and an ATG codon introduced at the corresponding position of the NAD$^+$-ME resulted in a recombinant protein with the same properties as ME from human cells (Loeber et al., 1991). We have expressed this newly created open reading frame spanning amino acids 46–604 in *E. coli* under the control of the inducible *trp*-promoter. Induction of exponentially growing bacteria with the inducer indoleacrylic acid resulted in expression of the human ME, which was easily separated from the endogenous bacterial ME on an anion-exchange column. The recombinant human mitochondrial NADP$^+$-ME elutes from a MonoQ column at salt concentrations of around 50 mM (Loeber et al., 1994), whereas the bacterial ME elutes at a KCl concentration of 180 mM (Loeber et al., 1991). The yield was between 0.5 to 1 mg of purified recombinant human ME per litre of fermentation broth.

Properties of mitochondrial NADP$^+$-ME

The NADP$^+$-ME from bovine heart and the human recombinant protein from *E. coli* were purified to homogeneity and their enzymic properties were characterized in detail. The specific activities for both proteins were in the same range, namely 12 units/mg protein for the human recombinant and 11.1 units/mg for the bovine protein. Human cytoplasmic NADP$^+$-ME can carboxylate pyruvate at the same rate as the forward reaction (malate + NADP$^+$ → pyruvate + NADPH + CO_2; Zelewski and Swierczynski, 1991). The recombinant human and the bovine mitochondrial NADP$^+$-ME can also reverse the decarboxylation reaction, but only with significantly lower efficiency than the forward reaction, and the K_m for pyruvate is very high (between 25 and 30 mM).

Tissue-specific transcription of the mitochondrial NADP$^+$-ME gene

To address the question of whether the known tissue-specific expression is regulated on the transcriptional level, we performed a Northern-blot analysis. To minimize cross-hybridization with the other human ME isoforms, a fragment corresponding to bp 1460–1820 was PCR amplified and radioactively labelled. This fragment has less than 60% homology to the other two human ME isoforms and contiguous stretches of homology are missing. Hybridization of this fragment to a Northern blot resulted in single band of approx. 2.2 kbp (Figure 4). As expected from the literature, a strong expression of the mRNA could be detected in heart, skeletal muscle and brain tissue; however, colon, kidney, pancreas and ovary tissue also showed strong signals. Small intestine, testes and lung were also positive, although to a lesser extent. A prolonged exposure of the blot showed minimal signals in the lanes of lung, thymus and spleen tissue. No significant transcription, even after prolonged exposure, could be found in liver, placenta and in peripheral leucocytes.

DISCUSSION

In this paper, we describe the characterization, cDNA cloning, heterologous expression in *E. coli* and the mRNA tissue-distribution of human mitochondrial NADP$^+$-dependent ME. This ME isoform is not expressed in any established human cell line tested so far. Since primary human material was not available to the extent necessary for the purification of sufficient protein for amino acid analysis, we chose to approach the purification indirectly by purifying the mitochondrial NADP$^+$-ME from bovine heart, obtaining sequence information of tryptic peptide fragments of the purified ME and then PCR amplifying a cDNA fragment of mitochondrial NADP$^+$-ME from a bovine heart cDNA library. This fragment was used to isolate the cor-

Table 2 Conserved regions among the human malic enzymes

Comparison of highly conserved regions among the three human MEs. Amino acids are given in the single letter code. | = identical amino acid, : = highly similar amino acid, . = low degree of similarity, no symbol = unrelated amino acid. (a) Putative malate binding domain. The cysteine which is labelled by the substrate analogue 3-bromo-1-pyruvate is emphasized by an arrow. (b) Possible domain involved in dinucleotide binding. Consensus sequence for ADP binding $\beta\alpha\beta$-fold as identified by Wierenga et al. (1985). h = Hydrophilic amino acid, G = glycine, n = neutral amino acid, (.) = any amino acid.

(a)

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                                        ↓
  81  RNEKLFYRVLTSDIEKFMPIVYTPTVGLACQQYS    cyto., NADP+
      |||||||||||||:|||||||||||||||||:|:
 116  RNEKLFYRVLTSDVEKFMPIVYTPTVGLACQHYG    mito., NADP+
      |||||||||:| .|:|.:|||||||||||||| :||
  91  RNEKLFYRILQDDIESLMPIVYTPTVGLACSQYG    mito., NAD+
```

(b)

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                hn.G.G..G...n..n........n.n  consensus
 146  KAIVTDGERILGLGDLGCNGMGIPVGKLALYTACGG  cyto., NADP+
      ||:|||||||||||||||| ||||||||||||||||
 181  DAVVVTDGERILGLGDLGCYGMGIPVGKLALYTACGG mito., NADP+
      ||||||||||||||||||.||||||||||.|||||:|
 156  KAVVVTDGERILGLGDLGVYGMGIPVGKLCLYTACAG mito., NAD+
```

responding cDNA from a human heart cDNA library. The cDNA sequence and deduced amino acid composition differs significantly from the other two human MEs described (Loeber et al., 1991, 1994), with the cytoplasmic NADP+-ME cDNA (71% identity) showing a closer relationship than the mitochondrial NAD+-ME (54% identity).

The relationship of ME isoforms of different species [NADP+-ME proteins from rat (Magnusson et al., 1986), mouse (Bagchi et al., 1987), human (Loeber et al., 1994) and maize chloroplasts (Rothermel and Nelson, 1989); NAD+-ME proteins from human (Loeber et al., 1991) and *Ascaris suum* (Kulkarni et al., 1993)] is shown in Figure 3. It is evident from the comparison that the same isoform of ME is highly conserved among different mammalian species. Between human and mouse/rat cytoplasmic NADP+-ME amino acid identity is 90%, and human and bovine mitochondrial NADP+-ME (amino acid sequence of the bovine enzyme was translated from the PCR-amplified piece of cDNA) have an identity of 92% over a stretch of 270 amino acids. In both cases, amino acid exchanges are mostly conservative, replacing an amino acid with a highly similar one. There are, however, significant differences between the three different human isoforms. The two human mitochondrial ME isoforms share only 54% of their amino acids, which is the same range of homology when comparing human MEs with MEs from *Ascaris suum* mitochondria or maize chloroplasts (Figure 3).

Table 2 presents two regions which are highly conserved among the human MEs. Region 1 spans amino acids 115–149 in the mitochondrial NADP+-ME. The segment contains a peptide with a cysteine residue which is covalently labelled by the substrate analogue 3-bromo-1-pyruvate (Satterlee and Hsu, 1991). Between the two human NADP+-MEs, 31 out of 34 amino acids are identical. This region is probably involved in malate binding. The second region with high homology is located between amino acids 181 and 218. Here, 35 and 34 amino acid residues out of 37 are conserved when comparing the mitochondrial NADP+-ME with the cytoplasmic NADP+- or the mitochondrial NAD+-ME respectively. This region contains an element with a consensus sequence of an 'ADP-binding $\beta\alpha\beta$-fold' as identified by Wierenga et al. (1985). Probably, this domain is involved in the cofactor binding.

The recombinant human protein made in *E. coli* has the same properties as the native protein from bovine mitochondria. The specific activity of the mitochondrial NADP+-ME is 12 units/mg, thus it is lower than in the other two human isoforms. The specific activity of the mitochondrial NAD+-ME is approx. 35 units/mg protein and the activity of the cytoplasmic NADP+-ME is between 40 and 55 units/mg (Zelewski and Swiercynski, 1991; Loeber et al., 1994). Human cytoplasmic NADP+-ME carboxylates pyruvate to malate at almost the same rate as the forward (decarboxylating) reaction (Zelewski and Swiercynski, 1991). Biegniewska and Skorkowski (1987) reported that in cod heart mitochondria, NADP+-ME carboxylates pyruvate at a rate 5-fold higher than the decarboxylation reaction, indicating an important anaplerotic function of ME in fish. In mammalian mitochondria, an anaplerotic role is unlikely because of the low rate of the pyruvate to malate reaction and because of the high K_m of human ME to pyruvate (25–30 mM). Simpson and Estabrook (1969) found high activity of mitochondrial NADP+-ME in adrenal cortex and correlated the expression of this isoform with the β-hydroxylation reactions occurring during steroid hormone synthesis, which required NADPH. Our data of the expression of mitochondrial NADP+-ME in various tissues support this, since we find significant expression of this isoform in ovary and testes, two other tissues with steroid hormone production. The highest levels of expression of this isoform however are found in heart, skeletal muscle, kidney and colon, tissues with no steroid hormone synthesis. In colon, the mitochondrial NADP+-ME is possibly expressed in the submucosal smooth muscle layers, since we were unable to find activity of this isoform in epithelial colon cells scraped off the luminal side of the gut wall (results not shown). The latter tissues and brain, which also expresses relatively high rates of mitochondrial NADP+-ME, show a high rate of aerobic metabolism. One possible role for mitochondrial NADP+-ME in those tissues is an involvement in the detoxification of reactive oxygen radicals generated in the mitochondria during respiration. Glutathione reductase activity, which is in part mitochondrial (Halliwell and Gutteridge, 1989), requires NADPH to recycle oxidized glutathione for the glutathione peroxidase system, and mitochondrial NADP+-ME might contribute to the generation of reducing equivalents. Interestingly, the list of tissues expressing high levels of this isoform of ME overlaps to a large extent the list of rodent tissues suffering extensive mitochondrial damage after artificially induced glutathione deficiency (Meister, 1994).

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REFERENCES

Bagchi, S., Wise, L. S., Brown, M. L., Bregman, D., Sul, H. S. and Rubin, C. S. (1987) J. Biol. Chem. **262**, 1558–1565

Benton, W. D. and Davis, R. W. (1977) Science **196**, 180–185

Biegniewska, A. and Skorkowski, E. F. (1987) Comp. Biochem. Physiol. B. Comp. Biochem. **86B**, 731–735

Blaire, P. V. (1967) Methods Enzymol. **10**, 78–80

Chang, G., Wang, J., Huang, T., Lee, H., Chou, W. and Meng, C. (1991) Eur. J. Biochem. **202**, 681–688

Church, G. M. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 1991–1995

Dozin, B., Magnuson, M. A. and Nikodem, V. M. (1985) Biochemistry **24**, 5581–5586

Feinberg, A. P. and Vogelstein, B. (1983) Anal. Biochem. **132**, 6–13

Frenkel, R. (1975) Curr. Top. Cell. Regulation **9**, 157–181

Halliwell, B. and Gutteridge, J. M. C. (1989) in Free Radicals in Biology and Medicine, 2nd Edn. Oxford University Press, Oxford

Kulkarni, G., Cook, P. F. and Harris, B. G. (1993) Arch. Biochem. Biophys. **300**, 231–237

Lin, R. C. and Davis, E. J. (1974) J. Biol. Chem. **249**, 3867–3875
Loeber, G., Infante, A. A., Maurer-Fogy, I., Krystek, E. and Dworkin, M. B. (1991) J. Biol. Chem. **266**, 3016–3021
Loeber, G., Dworkin, M. B., Infante, A. A. and Ahorn, H. (1994) FEBS Lett. **344**, 181–186
Magnusson, M. A., Morioka, H., Tecce, M. F. and Nikodem, V. (1986) J. Biol. Chem. **261**, 1183–1186
Mandella, R. D. and Sauer, L. A. (1975) J. Biol. Chem. **250**, 5877–5884
McKeehan, W. L. (1982) Cell. Biol. Int. Rep. **6**, 635–647
Meister, A. (1994) Cancer Res. (suppl.) **54**, 1969–1975
Nagel, W. O., Dauchy, R. T. and Sauer, L. (1980) J. Biol. Chem. **255**, 3849–3854
Petty, K. J., Desvergne, B., Mitsuhashi, T. and Nikodem, V. M. (1990) J. Biol. Chem. **265**, 7395–7400
Rothermel, B. A. and Nelson, T. (1989) J. Biol. Chem. **264**, 19587–19592
Saito, T. and Tomita, K. (1972) J. Biochem. (Tokyo) **72**, 807–815
Satterlee, J. and Hsu, R. Y. (1991) Biochim. Biophys. Acta **1079**, 247–252
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Press, New York
Sauer, L. A. (1973) Biochem. Biophys. Res. Commun. **50**, 524–531
Sauer, L. A. and Dauchy, R. T. (1978) Cancer Res. **38**, 1751–1756
Simpson, E. R. and Estabrook, R. W. (1969) Arch. Biochem. Biophys. **129**, 384–395
Taroni, F., Gellera, C. and DiDonato, S. (1988) Biochim. Biophys. Acta **916**, 446–454
von Heijne, G. (1986) EMBO J. **5**, 1335–1342
Wierenga, R. K., De Maeyer, M. C. H. and Hol, W. G. L. (1985) Biochemistry **24**, 1346–1357
Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene **33**, 103–119
Zelewski, M. and Swiercynski, J. (1991) Eur. J. Biochem. **201**, 339–345
Zolnierowicz, S., Swiercynski, L. and Zelewski, L. (1988) Biochem. Med. Metab. Biol. **39**, 208–216

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