Mar. Biotechnol. 6, 527–540, 2004
DOI: 10.1007/s10126-004-4203-x

MARINE BIOTECHNOLOGY

Isolation and Identification of 3-Methylcrotonyl Coenzyme A Carboxylase cDNAs and Pyruvate Carboxylase, and Their Expression in Red Seabream (*Pagrus major*) Organs

Shunnosuke Abe, Chhoun Chamnan, Kenichi Miyamoto, Yasutaka Minamino, and Makoto Nouda

Laboratory of Molecular Cell Biology, Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama City, 790-8566, Japan

Abstract: We determined complementary DNA sequences of biotin-containing (*MCCC1*) and non-biotin-containing (*MCCC2*) subunits of 3-methylcrotonyl coenzyme A carboxylase (MCCase) and pyruvate carboxylase (*PC*ase) using reverse transcriptase polymerase chain reaction of RNA extracted from seabream skeletal muscle and liver. We determined the complete coding sequences of *MCCC1* and *PC* and a partial coding sequence of the major part of *MCCC2*. Molecular sizes of *MCCC1, MCCC2*, and *PC* were 4300, 2400, and 6500 nucleotides, respectively, according to Northern blot analysis. The length of *MCCC1* from cDNA sequencing was 4249 nucleotides, indicating the full-length messenger RNA sequence was obtained. Northern blot analyses showed that *PC* was expressed in muscle, heart, liver, and ovary, but not in spleen. *MCCC1* and *MCCC2* were expressed at high levels in muscle and ovary, but only trace levels in heart, spleen, and liver. MCCase appears to be particularly important in muscle and ovary, which are active in protein metabolism, while PCase is important in organs active in glycolysis, such as liver.

Key words: red seabream, MCCase, PCCase, PCase, biotin, mRNA.

INTRODUCTION

Pyruvate carboxylase (PCase, EC 6.4.1.1), propionyl-coenzyme A (CoA) carboxylase (PCCase, EC 6.4.1.3), and 3-methylcrotonyl-CoA carboxylase (MCCase, EC 6.4.14) are known as mitochondrial biotin-dependent carboxylases that play a pivotal role in organic acid metabolic pathways. PCase has been found widely in diverse organisms, and its structure and functions are well characterized. PCase catalyzes the ATP-dependent carboxylation of pyruvate to form oxaloacetate in gluconeogenesis, lipogenesis, synthesis of certain amino acids, and formation of neurotransmitters (Jitrapakdee et al., 1996; Wallace et al., 1998).

PCase was first discovered in chicken (Utter and Keech, 1963), and many studies on PCase have been performed in vertebrates, invertebrates (Crabtree et al., 1972), and bacteria (Goss et al., 1981). The gene or complementary DNA encoding PCase has also been reported in many organisms including zebrafish (Yoder and Litman, 2000) but not yet in any commercial fishery species. MCCase has been implicated as a component enzyme of the ''mevalonate shunt''

Received March 13, 2003; accepted July 3, 2003; online publication February 2, 2005.

Corresponding author: Shunnosuke Abe; e-mail: abe@mcb.agr.ehime-u.ac.jp

(Popjak, 1971), it catalyzes the carboxylation of 3-methylcrotonyl-CoA to form 3-methylglutaconyl-CoA, and it is involved in the catabolism of leucine and isovalerate (Moss and Lane, 1971). MCCase has also been isolated from soybean (Song et al., 1994), tomato (Wang et al., 1994), *Arabidopsis thaliana* (Weaver et al., 1995), and mammals (Lau et al., 1980).

The full-length cDNAs encoding MCCase have been obtained from soybean and tomato for the biotin, containing subunit (Song et al., 1994, Wang et al., 1994), and from *Arabidopsis* for the non-biotin-containing subunit (McKean et al., 2000). Both the cDNA and the genomic sequences encoding MCCase in human have been demonstrated for the biotin-containing subunit (Obata et al., 2001) and for the non-biotin-containing subunit (Holzinger et al., 2001), but comparable sequences have not been fully reported in fish.

These biotin-dependent enzymes are also important in nutritional and physiologic aspects, and numerous studies have been carried out on biotin-dependent carboxylase deficiency in humans and other animals. Biotin deficiency in humans is characterized by decreased ability of the carboxylation reactions that affect the metabolism of carbohydrates, fats, and proteins, resulting in retarded growth, as well as other developmental and morphologic abnormalities (Wolf et al., 1981; Mock et al., 1985). In other animals biotin deficiency can cause poor growth, dermal lesions such as parakeratosis, alopecia, and achromotrichia, and fetal and other abnormalities, including biological changes such as depression of the activities of biotin-dependent enzymes (Whitehead, 1985). Biotin deficiency is also teratogenic in some species of mammals such as in mice (Watanabe, 1983) and human (Zempleni and Mock, 2000).

In the commercial fishery industry, both biotin dietary supplementation and its relation to biotin-dependent enzyme levels have been studied. The symptoms of biotin deficiency are as poor growth, low food consumption, increased mortality, degeneration of the gill lamellae, and skin disease (BASF, 1998) Biotin requirements for optimal growth of fish have been studied for rainbow trout (Woodward and Frigg, 1989), for mirror carp (Gunther and Meer-Burgdorff, 1990), and also for trout and salmon (National Research Council, 1973). However, the molecular basis of physiology and nutrition related to the biotin-dependent enzymes has not been established yet in fish.

We report here cloning and analysis of complete cDNA sequences for the biotin-containing subunits of MCCase (MCCC1) and PCase (PC), and identification of PCase, MCCase, and PCase proteins in a member of the *Sparidae*, red seabream (*Pagrus major*), which is an important commercial fish. We also identified the cDNA encoding the non-biotin-containing subunit of MCCase (MCCC2) and studied the coexpression of genes encoding PCase and subunits of MCCase in various organs from red seabream.

Materials and Methods

Protein Extraction and Isolation of Biotinylated Proteins

Proteins were extracted from skeletal muscle of 2 to 3-year-old red seabream (*Pagrus major*). Muscle tissue was ground in 5 volumes of CDB containing 200 mM Tris-HCl (pH 8.5), 450 mM KOAc, 25 mM $Mg(OAc)_2$,and 2% PTE (Abe and Davies, 1995) with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) using a mortar and pestle at ice-cold temperature, then transferred to a Teflon homogenizer to continue homogenization at 5 strokes per second for 15 to 20 minutes. The homogenate was filtered through Miracloth, then centrifuged for 15 minutes at 27,000*g* (Kubota model 6800-rotor, type RA300). The supernatant was filtered through a membrane filter (ADVANTEC) using a vacuum pump, then applied to an avidin affinity column prepared by conjugating egg white avidin to an NHS-activated Sepharose column (Hitrap-NHS activated) from Amersham Biotech according to the manufacturer instructions, using a peristatic pump at 0.5 ml/min flow rate. The effluent was applied again to the same column at least 3 times. After the application the column was washed with 5 volumes of CDB, 5 volumes of a washing buffer containing 5 mM Hepes-KOH (pH 7.5), 10 mM $Mg(OAc)_2$, 2 mM EGTA, and 0.1 mM PMSF, and 5 volumes of 1 mM biotin solution in the washing buffer. After the column was washed proteins were eluted with 5 ml of 6 M guanidine thiocyanate into 10 fractions. Each fraction was precipitated with 4 volumes of acetone at −20°C overnight and analyzed for proteins using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and Detection of Biotin-Containing Proteins

The protein samples obtained from the avidin affinity column chromatography were dissolved in a sample buffer

containing 2% LDS and 0.01 M Tris-HCl (pH 6.8), 20% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, and heated for 5 minutes at 95°C. These samples were electrophoresed in an 8.5% acrylamide gel, and transferred onto a PVDF membrane (Immobilon-P, Millipore) at 2 mA cm^{-2} for 1 hour using an electroblotting apparatus (BE300, Biocraft) for detection of biotin-containing proteins. To detect biotin-containing proteins, the blotted membrane was blocked in 5% dry milk in TBS for 1 hour, washed with TBS (10 mM Tris HCl [pH 7.6], 0.8% NaCl) containing 0.5% Tween 20) 5 times, then hybridized with a streptavidin-alkaline phosphatase conjugate for 30 minutes. After washing the membrane 5 times with TBS, the binding of alkaline phosphatase conjugate was detected in 100 mM diethanolamine (pH 9.5) with BCIP and NBT as substrates.

Determination of Partial Amino Acid Sequences of Avidin Binding Proteins Digested by V8 Protease

The avidin-affinity column fractions containing avidin-binding proteins were pooled by separated by (SDS-PAGE). Bands of avidin-binding proteins stained with Brilliant Blue R-250 were cut out and loaded on a stacking gel, and the protein was digested with *Staphylococcus aureus* V8 protease (EC 3.4.21.19) by the method described by Cleveland et al. (1977). The digested polypeptides were separated on 15% acrylamide gels using SDS-PAGE and electroblotted onto a PVDF membrane (Immobilon-P, Millipore). The transferred peptides were detected by staining with Brilliant Blue R-250, and peptide bands were excised and sequenced using a protein sequencer (model 476A, PerkinElmer, Applied Biosystems Division). These streptavidin-binding proteins are referred to as SBPs hereafter.

Cloning and cDNA Sequence Analysis

Partial nucleotide sequences were obtained by RT-PCR using primers derived from the homologous nucleotide sequences corresponding to the genes coding for MCCase and PCase of human (BAA99407), mouse (NP_076133), and *Arabidopsis* (Q42523). The PCR products obtained were subcloned into pMOSblue vector, and the positive colonies were isolated and purified using a FlexiPrep kit (Amersham Pharmacia Biotech). DNA sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 377 DNA Sequencer (PE Applied Biosystems). The 3'- and 5'-cDNA extremities were determined by the rapid amplification of cDNA ends (RACE) method.

The poly (A+) RNA was purified from total RNA (100 μg) using the mRNA Purification kit (Amersham Pharmacia Biotech). The blunt-ended double-stranded cDNA was synthesized by incubating 1 μg of poly(A+) RNA with 10 pmol gene-specific primers under a ReverTra *Ace* reverse transcriptase (TOYOBO Co., Ltd.) for 1 hour at 42°C. This was followed by the simultaneous inclusion of DNA polymerase I (9 $U \cdot \mu g^{-1}$), *Escherichia coli* DNA ligase (6 $U \cdot \mu g^{-1}$, Takara), and T4 DNA polymerase (9 $U \cdot \mu g^{-1}$) in the presence of RNase H (1 $U \cdot \mu l^{-1}$) in a reaction buffer composed of 33.4 mM Tris-HCl (pH 8.5), 4.6 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 6.6 mM 2-mercaptoethanol, 0.06 mM EDTA, 0.005% bovine serum albumin (BSA) 100 mM KCl, and 0.15 mM β-NAD, and incubated at 16°C for 90 minutes. The double-stranded cDNA obtained was ligated with 12.5 pmol of an adapter consisting of complementary oligo DNA (5'-gtaatacgactcactatagggcacgcgtggtcgacggcccgggctg gt-3') and (3'-gggcccgacca-5'), using 4 $U \cdot \mu g^{-1}$ T4 DNA ligase at 16°C overnight. (T4 DNA ligase, DNA polymerase I, and RNase H were obtained from TOYOBO).

RACE-PCR was performed in 25-μl-volume reactions amplified by the adapter primer (5'-gtaatacgactcactatagggc-3') with gene-specific primers using a XL-PCR kit (PE Applied Biosystem). The target fragments from the RACE-PCR were subcloned and sequenced as above. Nucleotide sequences were analyzed using the basic local alignment search tool (BLAST) algorithm program of the National Center for Biotechnology Information, and deduced amino acid sequences were analyzed with the CLUSTAL W multiple alignment algorithm (DNA Data Bank of Japan) and the GENETYX-WIN (Version 6.1.0) tree-generation program.

Primers for Cloning

Gene-specific primers for cloning cDNA fragments, along with the adapter primer, are summarized in Table 1. A forward primer, MCCC1-Fa, and a reverse primer, MCCC1-Ra, were derived from conserved *MCCC1* sequences in human (AB029826) and fugu genome databases (CAAB01000411). A forward primer, MCCCl-Fb, and a reverse primer, MCCC1 Rb, were designed from the partial *MCCC1* sequence obtained in the present study (AB081831), for amplifying the 3'- and 5'-cDNA ends, respectively.

To obtain a cDNA fragment for *MCCC2*, a forward primer, MCCC2-Fa, and a reverse primer, MCCC2-Ra, were designed using conserved *MCCC2* sequences for human (AB029826) and *Arabidopsis* (AF059510) *MCCC2*

Table 1. Gene Specific Primers for Cloning cDNA Fragments for *MCCC1*, *MCCC2*, and *PC*

Name of primer	Sequence 5' – 3'	Source
MCCC1-Fa	tgcaggtggaacatcctgt	AB029826, CAAB01000411
MCCC1-Ra	gccactttgtctccagcttt	AB029826, CAAB01000411
MCCC1-Fb	cgtgtacaacaaagacggaag	AB081831
MCCC1-Rb	cgagcttggcgatcattggg	AB081831
MCCC2-Fa	cgcaagcagggaaccat	AB029826, AF059510
MCCC2-Ra	cacatgccgtagtttcc	AB029826, AF059510
MCCC2-Rb	gctctccatttagcaagaaaggcg	AB082535
PC-Fa	caacttcagcaacctcttcag	AF295372
PC-Fb	gaggttgtacgcaagatggg	AF295372
PC-Ra	agtgtaggttggtgtactgg	AF295372
PC-Fc	tgccctggagaaggtgtttg	AB081832
PC-Rb	tcctggagtctcttccatgg	AB081832
PC-Rc	agagatggctatggagcgag	AB081832
Tag primer	ctgaaggttccagaatcgat	
Adapter primer	gtaatacgactcactatagggc	

cDNAs. For amplifying the 5'-cDNA end, a reverse primer, MCCC2-Rb, was designed using the partial sequence for *MCCC2* obtained in the present study (AB082535).

To obtain a cDNA fragment for *PC*a, forward primers PC-Fa and PC-Fb and a reverse primer, PC-Ra, were designed using the zebrafish sequence for *PCase* (AF295372). A forward primer, PC-Fc, for the 3'-cDNA end and reverse primers, PC-Rb and PC-Rc, for the 5'-cDNA end were designed from the partial *PC* sequence obtained in the present study (AB081832). The adapter and tag primers for amplifying the 5'- and 3'-cDNA ends, respectively, are also shown in Table 1.

Northern Blotting Analyses

Total RNA was isolated from heart, spleen, brain, muscle, liver, and ovary tissues from the same fish, using a RNA Extraction Kit (Amersham Pharmacia Biotech). Each total RNA (20-μg) fraction was denatured at 65C for 5 minutes in 1× MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0), 2.2 M formaldehyde, and 50% (v/v) formamide. The denatured RNA was separated on a 1.0% agarose gel containing 0.66 M formaldehyde, then transferred to a Hybond XL nylon membrane (Amersham Pharmacia Biotech), and the membrane was baked at 80°C for 2 hours. The membranes with immobilized RNA were pre hybridized for 30 minutes at 42°C in a plastic bag in hybridization buffer containing 5× SSC (750 mM NaCl, 75 mM Na_3-citrate), 5× Denhardt's reagent (0.1 % [w/v] BSA, 0.1% [w/v] Ficoll-400, 0.1% [w/v] polyvinylpyrrolidone), 5% dextran sulfate, 0.5% SDS, 100 $\mu g \cdot ml^{-1}$ salmon sperm DNA, and 50% (v/v) formamide. Then the randomly primed ^{32}P-labeled probe (specific activity, 1.0×10^6 $cpm \cdot \mu g^{-1}$) was added to the bag, and hybridization was carried out at 42°C overnight with constant agitation.

The membranes were washed at 60°C twice with 2 × SSC and 0.1% SDS for 5 minutes, followed by 1 × SSC and 0.1% SDS at 60°C for 15 minutes, and finally washed at 60°C twice with 0.1 × SSC and 0.1% SDS for 10 minutes. The membrane was exposed to Imaging Plate BAS III (Fuji Fihn, Co. Ltd.) for 12 hours and analyzed using Bio Image Analyzer (BAS-2000, Fuji Film, Co. Ltd.). The membrane was hybridized with α-^{32}P-labeled cDNA probes of *MCCC1, MCCC2*, and *PC* obtained in the present study. The same membrane was consecutively reprobed with α-^{32}P-labeled cDNA probes for *MCCC1, MCCC2*, and *PC*. The hybridized probe was stripped after each probing. For preparing probes a Rediprime II random prime labeling system was used (Amersham Pharmacia Biotech). The same specific activity (1.0×10^6 $cpm \cdot \mu g^{-1}$) of randomly primed α-^{32}P-labeled probe was hybridized with each probe at 42°C overnight in 5 × SSC (750 mM NaCl, 75 mM Na_3-citrate) containing hybridization buffer.

Radioactivity on the probed membrane was visualized and quantified by BAS-2000. The amounts of total RNA

loaded were equalized according to densitometric estimation of relative amounts in agarose gels.

Results

Isolation of Biotin-Containing Proteins and Their Identification as PCase, MCCase, and PCCase

Figure 1 shows Brilliant Blue-stained proteins obtained by avidin-affinity column chromatograpy in an SDS-PAGE gel and detection of SBP using streptavidin-conjugated alkaline phosphatase with BCIP and NBT as substrates as described in "Materials and Methods." Six major proteins, of 209 121, 76, 72, 56, and 43 kDa, were visible in the stained gel (lane 2). Among them, 3 protein bands, of 121, 76, and 72 kDa, reacted with streptavidin-conjugated alkaline phosphatase; therefore, they are annotated as 121-kDa SBP, 76-kDa SBP, and 72-kDa SBP (lane 3).

Table 2 shows the results of analyses of partial amino acid sequencing and the homology search in databases for the peptide fragments obtained by V8 proteinase digestion. A fragment of 121-kDa SBP was 100% identical to zebrafish, human, and mouse PCases; a fragment of 76-kDa SBP was 100% identical to MCCase from human and mouse; and a fragment of 72-kDa SBP was 90% identical to human and mouse PCCase. According to the molecular mass, the 56-kDa protein band was considered to be a mixture of non-biotin-containing subunits of MCCase and PCCase (Oei and Robinson, 1985). The 209-kDa and 43-kDa proteins were not identified in this study.

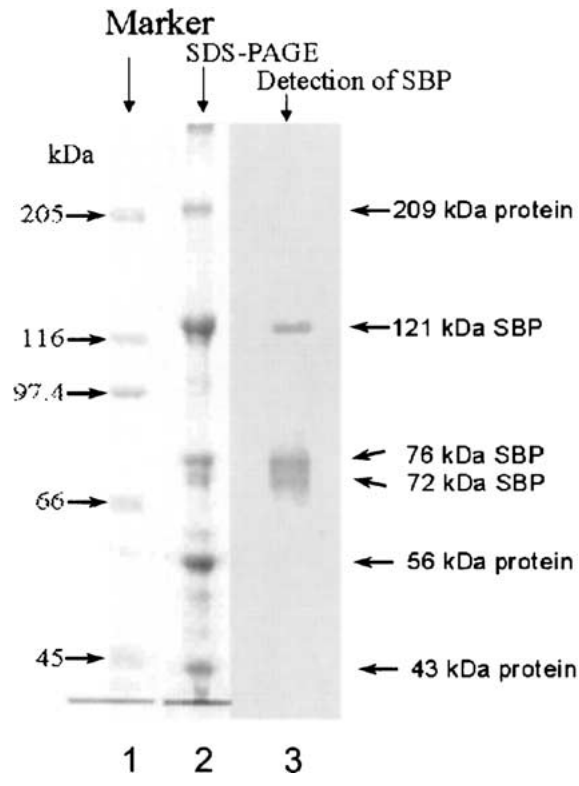

Figure 1. Isolation and detection of biotin-containing proteins from fish skeletal muscle tissue. Avidin-binding proteins obtained from skeletal muscle tissue of red seabream were separated by SDS-PAGE, electroblotted onto PVDF membrane, and either stained with Brilliant Blue R-250 (lane 2) or processed for detection of biotin-containing proteins using streptavidin-conjugated alkaline phosphatase with NBT and BCIP as substrates (lane 3). Brilliant Blue-stained molecular mass markers are shown in lane 1. Major protein bands in lane 2 are annotated at 43, 56, 72, 78, 121, and 209 kDa. The SBP bands in lane 3 are protein bands that reacted with the streptavidin conjugate.

Cloning of cDNAs Encoding the Biotin-Containing Subunit of MCCase (*MCCC1*)

A partial nucleotide sequence of 949 nucleotides of red seabream cDNA encoding 316 amino acids (AB081831) was obtained by RT-PCR with a forward primer, MCCC1-Fa, and a reverse primer, MCCC1-Ra. 5' RACE was performed to obtain the 5' end of cDNA sequence using a gene-specific reverse primer, MCCC1-Rb, and the adapter primer. Sequences assembled from these RT-PCR products revealed a cDNA of 2140 nucleotides. 3' RACE was then performed using the tag primer and a gene-specific primer, MCCC1-Fb, and furnished a 2109 nucleotide-long 3' sequence containing the stop codon and the canonical poly (A) signal (5'-aataaa-3'), which was also polyadenylated at the 3' end. The compiled sequence for red seabream *MCCC1* was 4249 nucleotides long, and it was deposited in the database (AB100429). This full-length cDNA sequence contained a canonical translation initiation motif, [a/g]nnatgg (Kozak, 1999), at 185 to 191, with the initiating methionine beginning at 188 and a stop codon occurring at 2339. This defined an open reading frame of 2151 nucleotides that putatively encoded a polypeptide of 717 amino acid residues. An in-frame stop codon at base 12 occurs upstream of the putative initiating methionine, consistent with the methionine codon at 188 being identified as the true initiating methionine and the recovered 5' sequences as the 5'-untranslated region (UTR). The partial amino acid sequences of V8 fragments (Table 2: E-XQVF-GDMHG) of the 76-kDa biotin-containing protein, along with the cleavage site E for V8 proteinase, were also located in the encoded amino acid sequence at 925 to 952.

Table 2. Identification of SBP by Partial Protein Sequencing[a]

SBP (kDA)	V8 fragment (kDa)	Sequence	Identification	Identity (%)	Protein ID	Species
121	15	NEIPGGQYTN	PCase	100	AF295372	Zebrafish
					L09192	Mouse
					BC011617	Human
76	25	XQVFGDMHG	MCCase	100	BAA99407	Human
72	27	AAPIGYPVMI	PCCase	90	LXM_049016	Human

[a]Molecular masses of SBP were calculated from the results shown in Figure 1.

Figure 2 shows a comparison of the amino acid sequences between the encoded polypeptide and MCCases from different organisms. All of the hallmarks for MCCase were present: CPSase L-chain domain (37-149), CPSase L-chain D2 domain (152-267), biotin carboxylase c domain (372-479), biotin lipoyl biotin requiring enzyme domain (640-692), biotin-binding motif (AMKME), and the binding site (K); D-Ala D-Ala ligase domain (135-327), which is a component of D-Ala-D-Ala ligase enzymes (EC:6.3.2.4).

The overall homologies of the red seabream protein to the biotin-containing subunits of MCCases were 73% to mouse (*Mus musculus*), 71% to human (*Homo sapiens*), 57% to mosquito (*Anopheles gambiae* str.), 56% to fruit fly (*Drosophila melanogaster*), and 47% to *Arabidopsis* (*Arabidopsis thaliana*), while low overall homologies (40%) to the biotin containing subunit of PCCases from human (XM_049016) and mouse (NM_144844) were obtained. There was also 49% overall homology between the red seabream protein and a biotin-containing carbamoyl-phosphate synthase L chain from a nematode (*Caenorhabditis elegans* (NP_501777).

Cloning of cDNA Encoding the Non-Biotin-Containing Subunit of MCCase (*MCCC2*)

We also cloned a cDNA fragment encoding most of the coding sequence of the non-biotin-containing subunit of MCCase, *MCCC2* (*MCCB*), by RT-PCR. A partial nucleotide sequence of 595 nucleotides of red seabream cDNA encoding 198 amino acids was obtained by RT-PCR with forward primer MCCC2-Fa and a reverse primer MCCC2-Ra, which were derived from conserved *MCCC2* sequences in human (AB029826) and *Arabidopsis* (AF059510). 5' RACE PCR using a gene-specific reverse primer, MCCC2-Rb, and the adapter primer was also performed to recover a complete 5' sequence including the start codon, and the compiled sequence of 1361 bp was obtained and deposited in the GenBank database (AB099519). The initiating methionine codon was found at nucleotides 48 to 50, and the mRNA encoded 438 amino acid residues. The encoded amino acid sequence is highly homologous to the non-biotin-containing subunit of MCCases from human, mouse, insects, bacteria, and plant, and these are compared in Figure 3. This red seabream protein contained a complete domain characteristic of *MCCC2*, AccD for acetyl-CoA carboxylase β subunit (COG0777) at 81 to 255. In addition, other essential domains for MCCC2 were found: the α and β subunits of carboxyltransferase domain (COG4799, 40-438); and the carboxyltransferase domain (pfam01039, 61-437). When the red seabream protein was compared with the non-biotin-containing subunits of PCCases from human and mouse, there were conserved amino acid residues but less homology to those for MCCC2. Overall homologies to human, fly, mosquito, *Agrobacterium*, and *Arabidopsis* were 80%, 69%, 68%, 72%,

Figure 2. Comparison of amino acid sequences of *MCCC1* between red seabream and other organisms. The deduced amino acid sequence derived from the coding sequence of the red seabream cDNA (AB097824) was compared with the *MCCC1* amino acid sequence of mouse (NP_076133), human (BAA99407), mosquito (EAA14349), fruit fly (NP_651896), nematode (NP_501777), and *Arabidopsis* (Q42523). The motif representing biotin binding is boxed. Genus and species abbreviations: red seabream (Pgm), human (Hsap), mouse (Mmus), mosquito (Agam), fruit fly (Dmel), nematode (Cele), and *Arabidopsis* (Athal). Dashes represent gaps inserted by the program to optimize the alignment. Domains identified are also shown as shaded areas: D-Ala D-Ala ligase biotin-requiring enzymes, carbamoyl-phosphate synthase (CPSase), phosphoribosylglycinamide synthetase (GARS). Asterisks and dots below the peptide sequence rows indicate completely matched and similar amino acid residues, respectively.

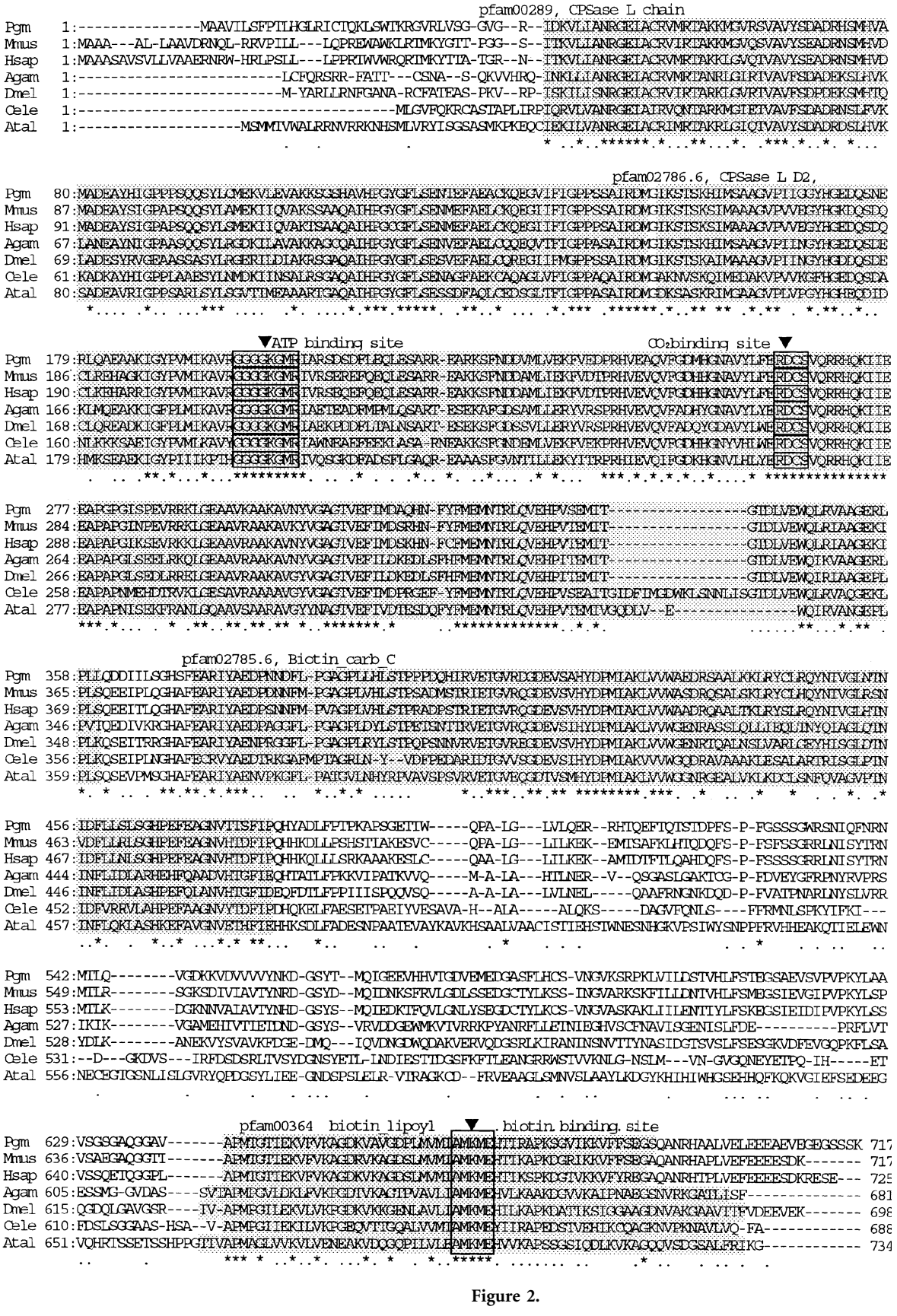
pfam00289, CPSase L chain
pfam02786.6, CPSase L D2,
ATP binding site
CO2binding site
pfam02785.6, Biotin_carb_C
pfam00364 biotin_lipoyl
biotin_binding_site
Pgm
Mmus
Hsap
Agam
Dmel
Cele
Atal

Figure 2.

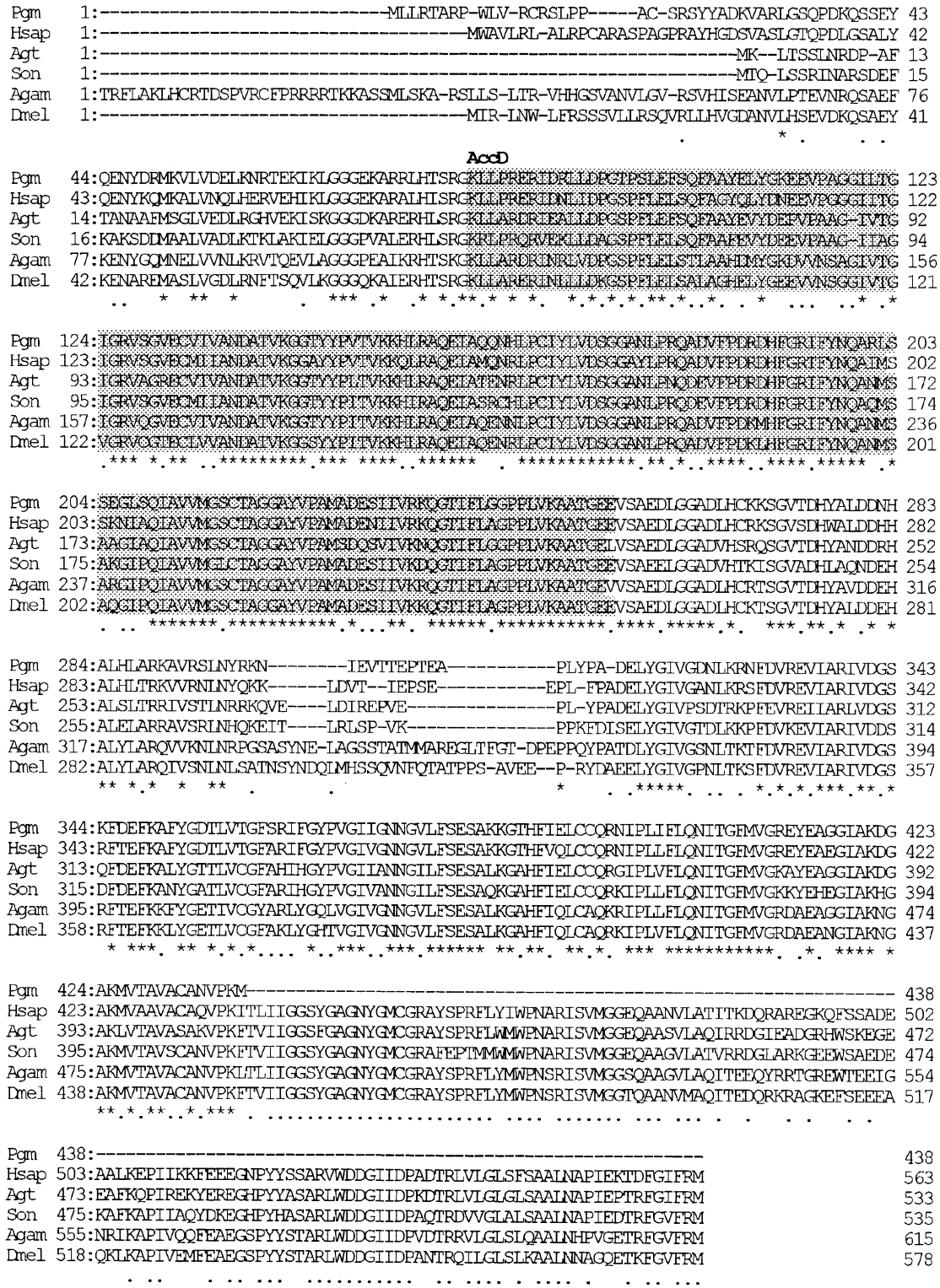

Figure 3. Alignment of MCCC2 from red seabream and from other organisms. The deduced amino acid sequence derived from the partial coding sequence of the red seabream protein obtained in this study (AB099519) compared with the *MCCC2* amino acid sequence of human (NP_071415), fruit fly (NP_652724), mosquito (EAA07889), and *Arabidopsis* (AF059511). *MCCC2* from 2 bacteria, *Shewanella oneidensis* MR-1 (NP_717504) and *Agrobacterium tumefaciens str* (NP_533975), were also included for this comparison. AccD, the characteristic domain for acetyl-CoA carboxylase β subunit (COG0777), is shaded. Asterisks and dots below the peptide sequence rows indicate completely matched and similar amino acid residues, respectively. Genus and species abbreviations are as in Figure 2, except Agt is *Agrobacterium*; Son, *Shewanella*.

and 61%, respectively, yet overall homology was distinctly low (40%) to the non-biotin-containing subunit of PCCase in human (NM_000532) and mouse (NM_025835), which is a biotin-requiring enzyme family closely related to MCCases.

Cloning of cDNA Encoding PCase

A cDNA fragment was obtained by RT-PCR using forward primer PC-Fa and reverse primer PC-Ra, which was based on the zebrafish sequence for *PCase* (AF295372). It was subcloned, sequenced, and shown to be 880 nucleotides long. The 3' region was obtained by RT-PCR using a gene-specific primer based on this sequence, PC-Fc, and the tag primer. RT-RCR was also performed using a forward primer based on the zebrafish *PC*ase sequence (AF295372), PC-Fb, and a reverse primer designed from the seabream cDNA obtained above, PC-Rb. We thus identified a 3170-nucleotides sequence encoding a polypeptide of 1029 amino acid residues highly homologous to zebrafish PCase, and the compiled sequence was deposited in the Genbank database (AB081832).

To obtain the complete 5' sequence for red seabream *PC*, 5' RACE was performed using a gene-specific primer based on the cDNA sequence, PC-Rc, and the adapter primer. We thus obtained the complete coding sequence of red seabream PCase and deposited it in the GenBank database (AB086371). This sequence was 4010 bp long with an open reading frame from 389 to 3928 encoding a polypeptide of 1179 amino acid residues. This encoded polypeptide was highly homologous to PCases from zebrafish (AAG37836), human (JC2460), mouse (A47255), chicken (AAM92771), and a nematode (NP_505977), as shown in Figure 4. All of the important conserved domains for PCases were also found in the red seabream protein: CPSase L chain (36–149), CPSase-L 02 (151–364), biotin carboxylase c domain (375–482), HMGL-like domain (569–733), pyravate carboxylase conserved domain (861–1069), and biotin-binding domain (1109–1177). The biotin-binding motif (AMKME) from 1142 to 1146 with the biotin-binding site (lysine) at 1144, and D-Ala-D-Ala ligase (EC:6.3.2.4) domain were also found (151–326), as in *MCCC1*. The partial N-terminal sequence of a V8 proteinase fragment of the 121-kDa biotin-containing protein (Table 2) was found from 864 to 873 including the cleaving site E at 863 (including white letters in black box: ENE-IPGGQYTN, with the first E being the cleavage site for V8 proteinase).

Phylogenetic Trees for MCCase Subunits and PCase in Various Organisms

Figure 5 shows phylogenetic trees for proteins MCCC1, MCCC2, and PC. There were 4 distinct classes of MCCC1 and MCCC2 consistent with evolution of these organisms: i.e., insects, vertebrates, nematode, and plant. The red seabream proteins were close to those in mammals, and fit in well with vertebrate MCCC1 and MCCC2. As expected, the plant orthologs were the most distant from any of the others presented here.

Red seabream PCase was most homologous to zebrafish PCase (89%), highly homologous to mammalian (82%) and avian (77%) PCases, and more distant from a nematode PCase (67%). There was a deviation between fish and bird PCases from their typical phylogeny, suggesting a novel functional evolution in birds.

Expression Analysis of Genes Encoding PCase and Biotin-Containing and Non-Biotin-Containing MCCase Subunits

Figure 6 shows transcript abundance of the different genes using total RNA isolated from various organs of red seabream blotted onto a nylon membrane and probed successively with *MCCC1, MCCC2*, and *PC*. In all panel lanes represent total RNA from heart, liver, spleen, muscle, and ovary. Molecular markers and the total RNA from heart electrophoresed under the same conditions are also shown.

Discussion

In the present study we isolated cDNAs encoding 2 important biotin-containing mitochondrial enzymes, MCCase and PCase, in a commercially important fish, red seabream (*Pagrus major*). For MCCase we determined the complete cDNA sequence for the biotin-containing subunit (*MCCC1 or MCCA*), which is essential for the catalytic role. The overall homologies of the biotin-containing subunits of MCCases were high to mammals (71%–73%) and other lower animals (56%–57%), but only 47% to plant, as expected. In addition, all of the hallmarks for MCCase including the biotin-binding site (K) and motif (AMKME) were present. However, the homology of red seabream *MCCC1* was only 47% to mammalian *PCC1*, which is a paralogous gene to *MCCC1* with a very similar domain structure. These results justify our assignment of the red

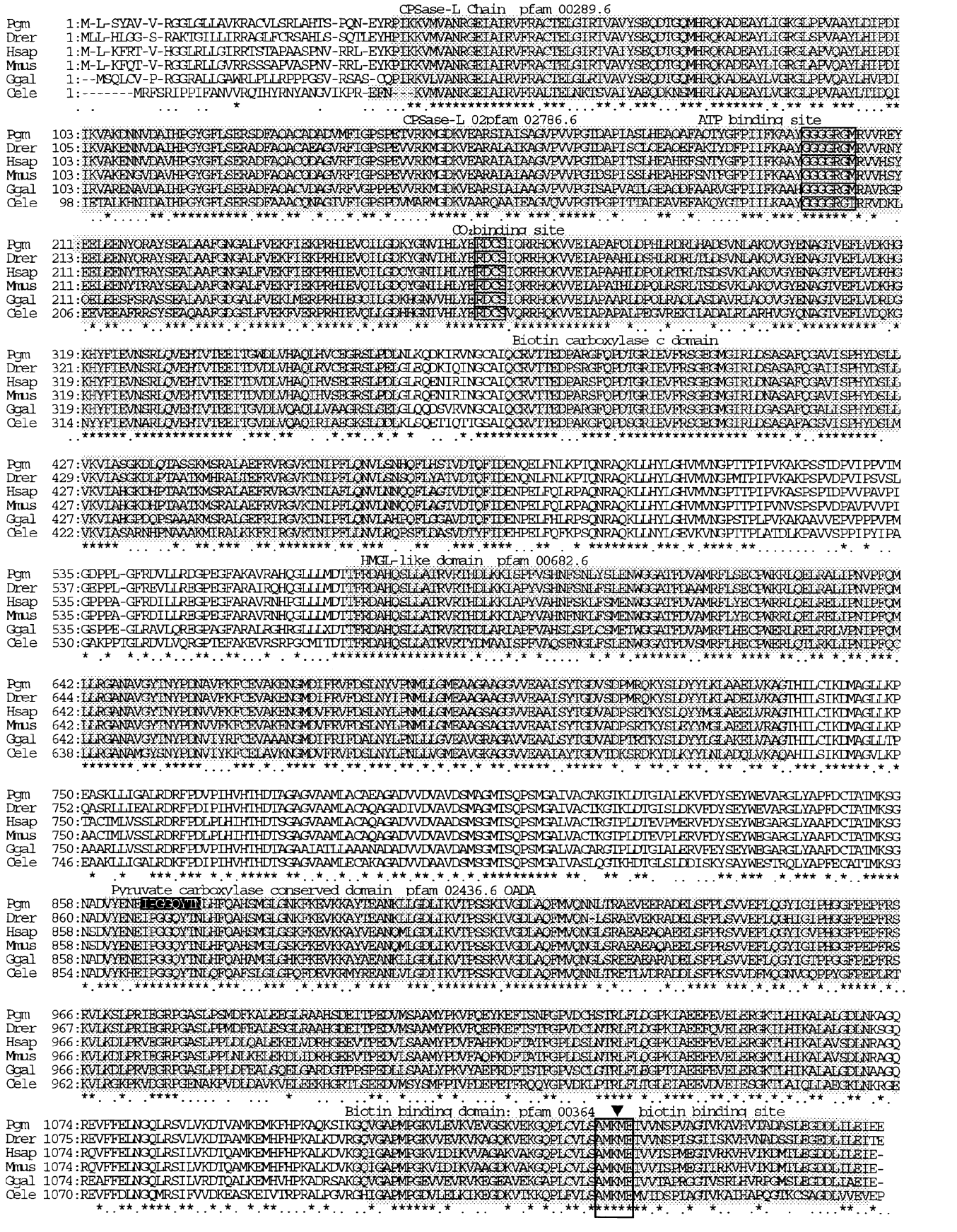
CPSase-L Chain pfam 00289.6
CPSase-L 02pfam 02786.6
ATP binding site
CO2binding site
Biotin carboxylase c domain
HMGL-like domain pfam 00682.6
Pyruvate carboxylase conserved domain pfam 02436.6 OADA
Biotin binding domain: pfam 00364
biotin binding site
Pgm
Drer
Hsap
Mmus
Ggal
Cele

Figure 4.

Figure 4. Comparison of amino acid sequences of PCases between red seabream and other organisms. The deduced amino acid sequence derived from the full coding sequence of the red seabream cDNA (AB097824) was compared with the PCases from zebrafish (AAG37836), human (JC2460), mouse (A47255), chicken (AAM92771), and a nematode (NP_505977). The motif representing biotin binding is boxed. Asterisks and dots below the peptide sequence rows indicate completely matched and similar amino acid residues, respectively. Genus and species abbreviations are as in Figure 2, except Ggal is chicken.

seabream cDNA obtained in the present study (AB100429) as *MCCC1*. The fish *MCCC1* shown here was the most similar to mouse *MCCC1* in terms of both homology (73%) and length of encoded polypeptide (717 amino acid residues). Because the mRNA was 4300 nucleotides long according to the Northern blot (Figure 6), and which is very close to that predicted from the nucleotide sequence obtained in the present study (AB100429), we presume that the sequence for red seabream *MCCC1* obtained in the present study is full length. This is about 200 nucleotides longer than that in human (Obata et al., 2001).

Such a long 3' UTR can often be found in fish gene transcripts, and it is sometimes difficult to obtain a full-length sequence of the 3' UTR from fish (Gray et al., 2001). We also obtained most of the coding sequence of *MCCC2* containing the start codon. Although 438 amino acid residues were encoded by the cDNA fragment here, the encoded polypeptide was highly homologous to MCCC2 in other organisms and especially to human (84% over 429 amino acids overlap out of 438 amino acids) and 67% to 71% homologous in other organisms, including the most distant species, *Arabidopsis*. Human *MCCC2* encodes 563 amino acid residues (Obata et al., 2001). However, when compared with the paralogous *PCC2*, even from mammals, the homology was only 35% to 37% in a 127 amino acid overlap with red seabream *MCCC2*. In addition, the mRNA hybridized with this red seabream cDNA was about 2400 nucleotides long (Figure 6, B), which is close to the reported size of the full-length *MCCC2* cDNA in human of 2016 nucleotides (AB050049). The expression pattern was also exactly matched with that *MCCC1* (Figure 6, A). These results strongly imply that the partial cDNA sequence obtained here corresponds to red seabream *MCCC2*.

PCase is composed of only one type of subunit, and the cDNA for the complete coding sequence of PCase in red seabream was obtained in the present study. In this

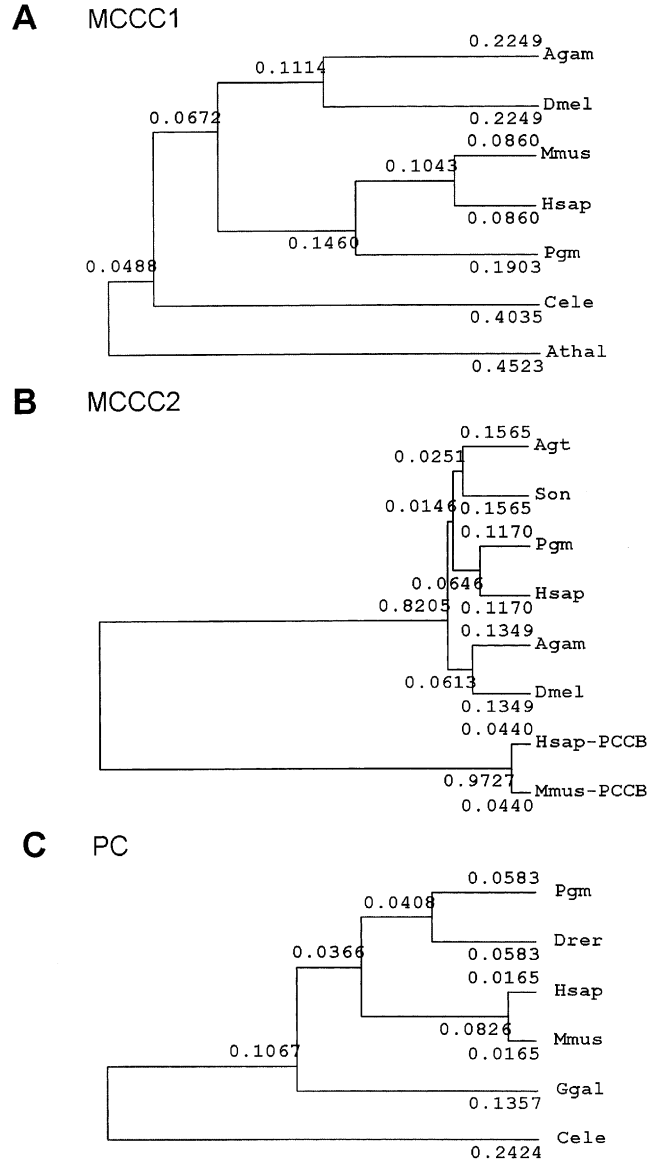

Figure 5. Phylogenetic analysis of MCCC1, MCCC2, and PC in divergent species of organisms. A phylogenetic tree was constructed using UPGMA method in the alignment tool (GENETYX-WIN, Version 6.1.0). Genus and species abbreviations are as in Figures 2–4. Divergence distances calculated by GENETYX-WIN are shown on individual branches. **A**: The tree derived from amino acid sequence alignments of 2 vertebrate MCCC1 sequences from human (BAA99407) and mouse (NP_076133); 2 insects, fruit fly and mosquito; one lower animal, nematode; and one plant, *Arabidopsis*, and the red seabream MCCC1 obtained in this study (Pgm) is shown. **B**: The tree derived from partial amino acid sequence alignments of the red seabream MCCC2 (Pgm) is shown. Phylogenetic relations to a paralogous gene, *PCCB*, are also shown. Hsap-PCCB and Mmus-PCCB represent *PCCB* from human and mouse, respectively. **C**: The tree derived from amino acid sequence alignments in Figure 4 including the red seabream protein (Pgm) is shown.

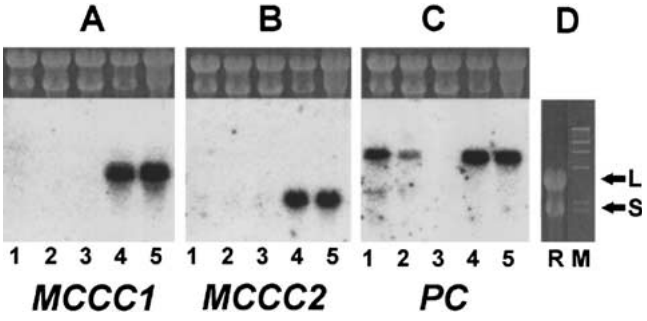

Figure 6. Northern blotting analysis of *MCCC1*, *MCCC2*, and *PC* in red seabream organs. Total RNA isolated from various organs of red seabream was blotted and successively probed with ^{32}P-labeled cDNAs for *MCCC1* (**A**), *MCCC2* (**B**), and *PC* (**C**) as isolated and identified in this study. In all panels, the ethidium-bromide-stained gel showing total RNA from heart (lane 1), liver (lane 2), spleen (lane 3), muscle (lane 4), and ovary (lane 5) is given. **D**: M indicates molecular markers (*Hin*d III digest of λ DNA) electrophoresed under the same conditions; R, ribosomal RNA from red seabream. Randomly primed ^{32}P-labeled probe (specific activity, 1.0×10^6 cpm·μg^{-1}) was hybridized for each probe. The mRNAs for *MCCC1*, *MCCC2*, and *PC* in the blots are 4300, 2400, and 6500 nucleotides long, respectively.

encoded polypeptide, all of the hallmarks for PCase were present. PCase mRNA in red seabream was a single transcript of 6500 nucleotides according to the Northern blot (Figure 6, C). The cDNAs encoding *PC* were 3543 to 4000 nucleotides long in zebrafish (Yoder and Litman, 2000) and virtually identical length in red seabream. In addition, both zebrafish and red seabream sequences lack the canonical poly(A) signal (aataaa). In humans a very similar length (4111-nucleotide) *PC* was also reported (NM_000920), and this human sequence contains the canonical poly(A) signal and is thought to be full length. However, considering that the mRNA was significantly longer (6500 nucleotides) in the Northern blot (Figure 6, C) than that predicted from the cDNA sequence (4010 nucleotides) in red seabream, we anticipate that the compiled cDNA sequence for *PC* may not be complete at its 3' UTR.

Transcripts for both the biotin and non-biotin-containing subunits of MCCase were abundant in skeletal muscle and ovary, but only trace amounts were present in other organs (Figure 6A and B). In contrast, the more essential pyruvate carboxylase was expressed significantly in all organs except spleen. We do not know why the transcript levels for these nuclear-encoded, mitochondrial enzymes were so low in the spleen. PCase is one of the key enzymes to incorporate pyruvate into mitochondria, and its expression is more ubiquitous (being significantly expressed in liver and highly in heart, muscle, and ovary) than that of MCCase subunits. In particular, *PC* transcripts were more abundant than MCCase subunit transcripts in heart, and may play an essential role in metabolizing pyruvate in heart. Extremely strong expression of MCCase in muscle and ovary indicates very active metabolism of amino acids in these organs, including active protein synthesis and degradation (Obata et al., 2001). This might be related to a physiologic factor such as laying eggs, which was occuring in the red seabream we used Northern blot analysis (Jitrapakdee et al., 1996) has shown that in mouse, PC mRNA is not expressed in spleen or skeletal muscle, while it is higher in liver, and except for spleen, this expression pattern in mouse organs is different from that in red seabream. Indeed, little or no expression of these biotin-dependent enzyme transcripts takes place, suggesting that protein turnover in heart, liver, and spleen might not be important in red seabream. However, expression and levels of these biotin-dependent enzymes may be affected by environmental and physiologic factors such as nutrition in mouse (Goldberg and Chang, 1978) and human (Murayama et al., 1997), and such factors should be considered in future studies.

We have previously reported protein bands reacting with a streptavidin-conjugated alkaline phosphatase in different classes of organisms including plants, animals, and several fish species (Abe et al., 1992, 1996; Abe and Davies 1995), and we identified some of them as the biotin-containing subunit of MCCase in a plant, *Pisum sativum* (AB075695, AB075695), and in human (Obata et al., 2001). We confirmed that the 76-kDa and 121-kDa biotin-containing proteins are a subunit of MCCase and a biotin-containing enzyme, PCase (Table 2), while homology searches for the partial amino acid sequence of the 72-kDa biotin-containing protein suggested that the protein is likely to be PCCase in red seabream (Figure 1, Table 2). Therefore, the protein bands at 56 kDa, which are found in abundant amounts but do not react with streptavidin, are likely to be the non-biotin-containing subunits of MCCase and PCCase (Oei and Robinson, 1985). However, the guanidine-HCl methods we used are not suitable for isolating these enzymes in active form, which would require a biotin gradient. This makes it very simple to isolate biotin-containing enzymes, for analyses such as protein sequencing. The detection method for these biotin-containing enzymes (using a streptavidin conjugate on transferred proteins) is also simple and sensitive, and may be useful for analyzing the amounts of these biotin-containing enzymes under different physiologic conditions in fish and other species.

Acknowledgments

We thank Dr. Koichi Akiyama of Centre for Gene Research, Ehime University, for sequence analyses and advice on Northern blot analysis. We also thank Dr. Eric Davies for proof reading this manuscript.

References

Abe, S., and Davies, E. (1995). Methods for isolation an analysis of the cytoskeleton. *Methods Cell Biol B* 50:223–236.

Abe, S., Ito, Y., and Davies, E. (1992). Cosedimentation of actin, tubulin and membranes in the cytoskeleton fractions from peas and mouse 3T3 cells. *J Exp Botany* 43:941–949.

Abe, S., Ito, Y., Doi, H., Shibata, K., Azama, K., and Davies, E. (1996). Distribution of actin and tubulin in the cytoskeletal fraction from a variety of plant and animal tissues. Memoir of College of Agriculture, Ehime University. 41:1–10.

BASF (1998). Vitamin nutrition in cold-water fish, II. Animal Nutrition News NN9093, Bulletins. Mount Olive, N.J.: BASF Cooperation.

Cleveland, D.W., Fisher, S.G., Kirschner, M.W., and Laemmli, U.K. (1977) "Peptide mapping by limited proteolysis in sodium dodecylsulfate and analysis by gel electrophoresis" J Biol Chem 252: 1102–1106.

Crabtree, B., Higgins, S.J., and Newsholme, E.A. (1972). The activities of pyruvate carboxylase, phosphoenolpyruvate carboxylase and fructose diphosphatase in muscles from vertebrates and invertebrates. *Biochem J* 130:391–396.

Goldberg, A.L., and Chang, T.W. (1978). Regulation and significance of amino acid metabolism in skeletal muscle. *Fed Proc* 37:2301–2307.

Goss, J.A., Cohen, N.D., and Utter, M.F. (1981). Characterization of the subunit structure of pyruvate carboxylase from *Pseudomonas citronellolis*. *J Biol Chem* 256:11819–11825.

Gray, T.A., Azama, K., Whitmore, K., Min, A., Abe, S., and Nicholls, R.D. (2001). Phylogenetic conservation of the makorin-2 gene, encoding multiple zinc-finger protein, antisense to the RAF1 proto-oncogene. *Genomics* 77:119–126.

Gunther, K.D., and Meyer-Burgdorff, K.H. (1990). Studies on biotin supply to mirror carp (*Cyprinus carpio L.*). *Aquaculture* 84:49–60.

Holzinger, A., Roschinger, W., Lagler, F., Mayerhofer, P.U., Lichtner, P., Kattenfeld, T., Thuy, L.P., Nyhan, W.L., Koch, H.G., Muntau, A.C., and Roscher, A.A. (2001). Cloning of the human MCCA and MCCB genes and mutations therein reveal the molecular cause of 3-methylcrotonyl-CoA: carboxylase deficiency. *Hum Mol Genet* 10:1299–1306.

Jitrapakdee, S., Booker, G.W., Cassady, A.I., and Wallace, J.C. (1996). Cloning, sequencing, and expression of rat liver pyruvate carboxylase. *Biochem J* 316:631–637.

Kozak, M. (1999). Initiation of translation in prokaryotes and eukaryotes. *Gene* 234:187–208.

Lau, D.P., Cochran, B.C., and Fall, R.R. (1980). Isolation of 3-methylcrotonyl-CoA carboxylase from bovine kidney. *Arch Biochem Biophys* 205:352–359.

McKean, A.L., Ke, J., Song, J., Che, P., Achenbach, S., Nikolau, B.J., and Wurtele, E.S. (2000). Molecular characterization of the non-biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase. *J Biol Chem* 275:5582–5590.

Mock, M.D., Baswell, D.L., Holman, R.T., and Sweetman, L. (1985). Biotin deficiency complicating parental alimentation: diagnosis, metabolic repercussions, and treatment. *J Pediatr* 106:762–769.

Moss, J., and Lane, M.D. (1971). The biotin-dependent enzymes. *Adv Enzymol* 35:321–442.

Murayama, K., Kimura, M., Ymaguchi, S., Shinka, T., and Kodama, K. (1997). Isolated 3-methylcrotonyl-CoA carboxylase deficiency in a 15-year-old girl. *Brain Dev* 19:303–305.

National Research Council. (1973). Nutrient Requirements of Trout, Salmon and Catfish. Washington, D.C.: National Research Council.

Obata, K., Fukuda, T., Morishita, R, Abe, S., Asakawa, S., Yamaguchi, S., Yoshino, M., Ihara, K., Murayama, Keiko, Shigemoto, K., Shimizu, N., and Kondo, I. (2001). Human biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase gene (MCCA): cDNA sequence, genomic organization, localization to chromosomal band 3q27, and expression. *Genomics* 72:145–152.

Oei, J., and Robinson, B.H. (1985). Simultaneous preparation of the three biotin-containing mitochondrial carboxylases from rat liver. *Biochim Biophys Acta* 840:1–5.

Popjak, G. (1971). Specificity of enzymes of sterol biosynthesis. *Harvey Lect* 65:127–156.

Song, I, Wurtele, E.S., and Nikolau, B.J. (1994). Molecular cloning and characterization of the cDNA coding for the biotin-containing subunit of 3-methylcrotonyl-CoA carboxylase: identification of the biotin carboxylase and biotin carrier domains. *Proc Natl Acad Sci U S A* 91:5779–5783.

Utter, M.F., and Keech, D.B. (1963). Pyruvate carboxylase, I: nature of the reaction. *J Biol Chem* 23:2603–2608.

Wallace, J.C., Jitrapakdee, S., and Chapman-Smith, A. (1998). Pyruvate carboxylase. *Int J BiochemCell B* 30:1–5.

Wang, X., Wurtele, E.S., Keller, G., McKean, A.L., and Nikolau, B.J. (1994). Molecular cloning of cDNA and genes coding for B-methylcrotonyl-CoA carboxylase of tomato. *J Biol Chem* 269:11760–11768.

Watanabe, T. (1983). Teratogenic effects of biotin deficiency in mice. *J Nutr* 113:574–581.

Weaver, L.M., Lebrun, L., Franklin, A., Huang, L., Hoffman, N., Wurtele, E.S., and Nikolau, B.J. (1995). Molecular cloning of the biotinylated subunit of 3-methylcrotonyl-coezyme A carboxylase of *Arabidopsis thaliana*. *Plant Physiol* 107:1013–1014.

Whitehead, C.C. (1985). Assessment of biotin deficiency in animals. *Ann N Y Acad Sci* 447:86–96.

Wolf, B., Hsia, Y.E., Sweetman, L., Feldman, G., Boychuk, R.B., Bart, R.D., Crowell, D.H, Di Mauro, R.M., and Nyhan, W.L. (1981). Multiple carboxylase deficiency: clinical and biochemical improvement following neonatal biotin treatment. *Pediatrics* 68:113–118.

Woodward, B., and Frigg, M. (1989). Dietary biotin requirements of young rainbow trout determined by weight gain, hepatic biotin concentration and maximal biotin-dependent enzyme activities in liver and white muscle. *J Nutr* 119:54–60.

Yoder, J.A., and Litman, G.W. (2000). The zebrafish *fth1, slc3a2, men1, pc, fgf3 and cycd1* genes define two regions of conserved synteny between linkage group 7 and human chromosome 11q13. *Gene 261:235–242.*

Zempleni, J., and Mock, D.M. (2000). Marginal biotin deficiency is teratogenic. *Proc Soc Exp Biol Med* 223:14–21.