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Expression of the Sodium Ion Pump Methylmalonyl-Coenzyme A-Decarboxylase from *Veillonella parvula* and of Mutated Enzyme Specimens in *Escherichia coli*

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The structural genes of the sodium ion pump methylmalonyl-coenzyme A (CoA)-decarboxylase from *Veillonella parvula* have recently been cloned on three overlapping plasmids (pJH1, pJH20, and pJH40) and sequenced. To synthesize the complete decarboxylase in *Escherichia coli*, the genes were fused in the correct order (*mmdADECB*) on a single plasmid (pJH70). A DNA region upstream of *mmdA* apparently served as promoter in *E. coli* because expression of the *mmd* genes was not dependent on the correct orientation of the *lac* promoter present on the pBluescript KS+-derived expression plasmid. To allow controlled induction of the *mmd* genes, the upstream region was deleted and the *mmd* genes were cloned behind a T7 promoter. The derived plasmid, pT7mmd, was transformed into *E. coli* BL21(DE3) expressing T7 RNA polymerase under the control of the *lac* promoter. The synthesized proteins showed the typical properties of methylmalonyl-CoA-decarboxylase, i.e., the same migration behavior during sodium dodecyl sulfate-polyacrylamide gel electrophoresis, stimulation of the decarboxylation activity by sodium ions, and inhibition with avidin. In methylmalonyl-CoA-decarboxylase expressed in *E. coli* from pT7mmd, the γ subunit was only partially biotinylated and the α subunit was present in substoichiometric amounts, resulting in a low catalytic activity. This activity could be considerably increased by coexpression of biotin ligase and by incubation with separately expressed α subunit. After these treatments methylmalonyl-CoA-decarboxylase with a specific activity of about 5 U/mg of protein was isolated by adsorption and elution from monomeric avidin-Sepharose. To analyze the function of the δ and ε subunits, the corresponding genes were deleted from plasmid pT7mmd. *E. coli* cells transformed with pJHΔ2, which lacks *mmdE* and the 3′-terminal part of *mmdD*, showed no methylmalonyl-CoA-decarboxylase activity. In addition, a core complex consisting of the remaining subunits (α, β, and γ) could not be isolated from this mutant. In contrast, catalytically active methylmalonyl-CoA-decarboxylase was expressed in *E. coli* from plasmid pJHΔ1, which contained a deletion of the *mmdE* gene only. The mutant enzyme could be isolated, reconstituted into proteoliposomes, and shown to function in the transport of Na^+ ions coupled to methylmalonyl-CoA decarboxylation. The small ε subunit therefore has no catalytic function within the methylmalonyl-CoA-decarboxylase complex but appears to increase the stability of this complex.

Methylmalonyl-coenzyme A (CoA)-decarboxylase (EC 4.1.1.41), oxaloacetate decarboxylase (EC 4.1.1.3), and glutaconyl-CoA-decarboxylase (EC 4.1.1.70) constitute a unique family of vectorial catalysts which use the free energy of the decarboxylation of these substrates as driving force for an active transport of Na^+ ions across the membrane (6, 7). Despite their different substrate specificities, these enzymes are remarkably similar in structure and function. All three decarboxylases are biotin-containing proteins and can be easily purified by affinity chromatography on monomeric avidin-Sepharose (1, 5, 8, 13). They all contain a water-soluble α subunit with an M_r of 60,000 to 65,000 that catalyzes a carboxyl transfer from each specific substrate to the prosthetic biotin group. In oxaloacetate decarboxylase, this biotin is bound to the C-terminal domain of the α subunit, whereas in methylmalonyl-CoA-decarboxylase and glutaconyl-CoA-decarboxylase, the prosthetic group is located on a separate subunit (γ). The biotin-carrying domains contain a proline/alanine-rich stretch which is probably responsible for the flexibility required for the movement of the prosthetic biotin group between different catalytic centers (19, 24).

All sodium ion-translocating decarboxylases have a highly hydrophobic β subunit with an M_r of about 38,000. An amino acid sequence alignment of the corresponding polypeptides from methylmalonyl-CoA-decarboxylase of *Veillonella parvula* (17) and oxaloacetate decarboxylase from *Klebsiella pneumoniae* (19) and *Salmonella typhimurium* (27) showed 60% identical amino acid residues. The homology was especially pronounced in those parts of the sequence where hydrophobic stretches that could traverse the membrane were evident (17, 27). The β subunit functions in the catalysis of the decarboxylation of the carboxylated biotin carrier protein and the coupled translocation of Na^+ ions across the membrane (16). A third subunit of oxaloacetate decarboxylase (γ; M_r of 8,900 [27]) and a fourth subunit of methylmalonyl-CoA-decarboxylase (δ; M_r of 11,900 [17]) and glutaconyl-CoA-decarboxylase have unknown functions. Because the amino acid sequences of the oxaloacetate decarboxylase γ subunit and the methylmalonyl-CoA-decarboxylase δ subunit contain a hydrophobic stretch near the N terminus, it has been proposed that these subunits are integral membrane proteins and that they could be involved in the Na^+ transport together with β (13). Recently, DNA sequence analysis of the genes encoding methylmalonyl-CoA-decarboxylase from *V. parvula* led to the discovery of a fifth subunit (17). This small ε subunit (M_r of 5,758) showed 47% sequence identity to the δ subunit of the same enzyme.

The aim of this work was to establish an expression system

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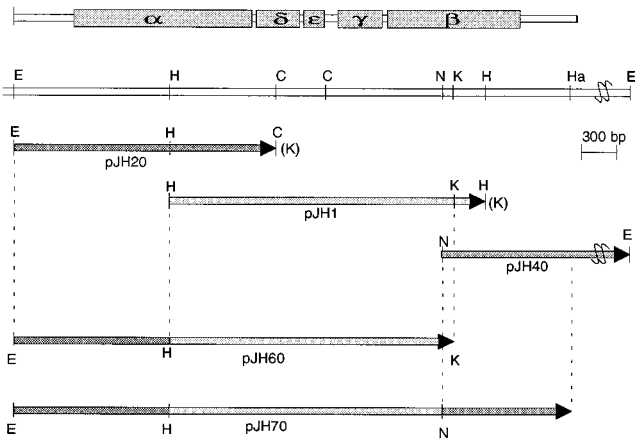

FIG. 1. Physical map of subclones pJH1, pJH20, and pJH40 and the derived constructs pJH60 and pJH70. The genes of the methylmalonyl-CoA-decarboxylase (grey boxes) and the sequenced part of the DNA are represented at the top. The individual cloning steps are described in Materials and Methods. The endonuclease restriction sites are abbreviated as follows: C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; Ha, *Hae*III. The restriction sites in parentheses were located in the polylinker.

for the *mmd* genes in *Escherichia coli* to analyze the role of the δ and ε subunits in the methylmalonyl-CoA-decarboxylase complex.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* DH5α (10) was generally used as the host for cloning and for expression of genes from the *lac* promoter on plasmids. *E. coli* BL21(DE3) (25) served as the host for expression of genes that had been cloned in pT7-7 (26) behind the T7 promoter; the strain is a λ lysogen carrying the gene for T7 RNA polymerase under the control of a *lacUV5* promoter. *E. coli* JM110 (28) was used in one case for plasmid isolation because of its *dam* genotype.

Recombinant DNA procedures were performed as described previously (22) or according to protocols provided by the manufacturers. Plasmids pJH1, pJH20, and pJH40 (17), which contain on pBluescript KS+ vectors (Stratagene) (pJH1 and pJH20) or pBR322 (pJH40) vectors different parts of the *mmd* operon from *V. parvula*, were used to construct a plasmid carrying the complete *mmd* operon (Fig. 1). For this purpose, a 2.4-kb *Hin*dIII-*Kpn*I fragment from pJH1 was cloned into pJH20 cut with *Hin*dIII and *Kpn*I. The resulting plasmid, pJH60, was digested with *Asp*718 (an isoschizomer of *Kpn*I), and the overhanging ends were filled with Klenow polymerase. Subsequently, the linearized pJH60 was cut with *Nhe*I, and the resulting 6.50-kb fragment was ligated with a 1.07-kb *Nhe*I-*Hae*III fragment from pJH40, resulting in plasmid pJH70, which carries the complete operon encoding methylmalonyl-CoA-decarboxylase.

To express the *mmd* genes in an inducible T7 expression system, the following plasmids were constructed (Fig. 2). After restriction of pJH20 with *Pst*I, a 1-kb *Pst*I-*Pst*I fragment was isolated and cloned into pBluescript KS+, resulting in pJH28. This plasmid served as template for the generation of a 730-bp PCR product (see below) containing the 5′-terminal part of *mmdA*, including an *Nco*I recognition site which overlaps the start codon of *mmdA*. For cloning the PCR product in the expression vector pT7-7 (26), it was cut with *Nco*I, treated with Klenow polymerase, and then cut with *Pst*I. This fragment was ligated with pT7-7 that had been digested with *Nde*I, treated with Klenow polymerase, and then cut with *Pst*I. The resulting plasmid was named pT7α′. Cloning of a 1.60-kb *Nru*I-*Cla*I fragment from pJH20 in pT7α′ resulted in plasmid pT7α, which served for expression of the *mmdA* gene by using the T7 promoter and the ribosome-binding site provided by the vector. To construct a plasmid for the expression of all five *mmd* genes under control of the T7 promoter, pT7α was digested with *Cla*I and the 5′ overhangs were refilled with Klenow polymerase. In a second step, the linearized pT7α was cut with *Bst*XI. The resulting 3.62-kb fragment was ligated with a 3.14-kb *Bst*XI-*Dra*I fragment from pJH70, resulting in plasmid pT7mmd. All pT7-7 derivatives were transformed in the expression host *E. coli* BL21(DE3) with or without pLysE (25).

For the construction of pJHΔ1 (see Fig. 6), pT7mmd was digested partially with *Afl*II and then completely with *Cla*I. After treatment with Klenow polymerase, the blunt-ended fragments were religated. To obtain a second *Cla*I site accessible for cleavage, pT7mmd was transformed into *E. coli* JM110 (*dam*) and then isolated. Subsequent deletion of the 0.44-kb *Cla*I-*Cla*I fragment from pT7mmd resulted in plasmid pJHΔ2 (see Fig. 6).

PCR. For the creation of an *Nco*I site at the start codon of the *mmdA* gene, PCR was performed with plasmid pJH28 as the template and one vector-specific primer (universal primer; 5′ TCAGTGCTGCAACAT 3′) and one *mmdA*-specific primer (5′ GCTACCATGGCAACAGTGCAGGA 3′) with the desired *Nco*I site. The reaction was performed in a Techne Thermocycler (model PHC-3), using Vent DNA polymerase (New England Biolabs). After 30 cycles with 1-min steps at 92, 50, and 72°C, the reaction mixture was analyzed by agarose gel electrophoresis. The 730-bp fragment obtained was used for the construction of plasmid pT7α′ (Fig. 2).

Media and growth conditions. All *E. coli* strains used were grown at 37°C in Luria-Bertani medium (22). The cultures expressing methylmalonyl-CoA-decarboxylase from plasmids were supplemented with 20 μM biotin and 200 μg of ampicillin per ml. The *birA* gene encoding biotin ligase was expressed from plasmid pCY216 (a gift from J. Cronan). Plasmid pCY216 contains the *birA* gene from *S. typhimurium* under the control of the arabinose promoter of *E. coli* and harbors the gene for chloramphenicol resistance. Cultures of pCY216-containing strains were therefore supplemented with 50 mg of chloramphenicol per liter, and the expression of the biotin ligase was induced with 0.1% L-arabinose when the cell density had reached an A_{560} of about 0.8. Synthesis of the chromosomally encoded T7 RNA polymerase under control of the *lacUV5* promoter in *E. coli*

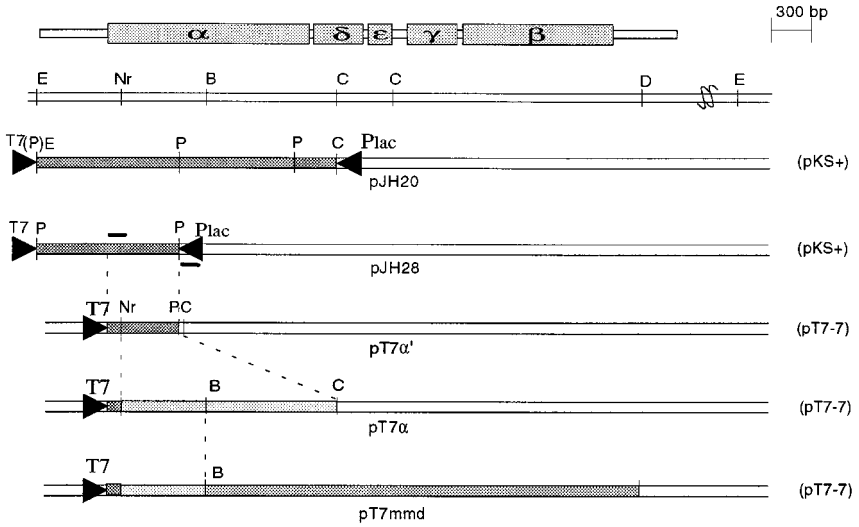

FIG. 2. Construction of the expression clones pT7α and pT7mmd. The origins of the vectors are shown in parentheses. Also indicated are the names of the subclones and the orientations of the promoters (▶, ◀). The oligonucleotides used for PCR are indicated by bars. Abbreviations for restriction enzymes used for cloning: B, *Bst*XI; C, *Cla*I; D, *Dra*I; Nr, *Nru*I; P, *Pst*I. The restriction site in parentheses was located in the polylinker.

BL21(DE3) was induced with 0.5 to 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an A_{560} of 1.0.

Biotinylation. For in vitro biotinylation, 1 ml of cell extract (total volume, 3.2 ml) prepared from cells of a 100-ml culture of *E. coli* DH5α/pCY216 in 50 mM potassium phosphate (pH 7.0) containing 5 mM $MgCl_2$ and 0.1 mM diisopropylfluorophosphate was added to 3 ml of cell extract from an *E. coli* strain synthesizing methylmalonyl-CoA-decarboxylase. Biotin, $MgCl_2$, and ATP were added to final concentrations of 0.1, 5, and 5 mM, respectively, and the mixture was incubated at 30°C for 1 h. For in vivo biotinylation, *E. coli* BL21(DE3) cells harboring one of the different pT7-7 derivatives described above were cotransformed with pCY216. The cells were grown in the presence of antibiotics and induced for expression of the desired genes as described above.

Reconstitution of the methylmalonyl-CoA-decarboxylase enzyme complex with cell extracts of separately expressed α subunit. As shown by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), cells expressing the *mmd* genes from plasmids pT7mmd, pJHΔ1, and pJHΔ2 formed substoichiometric amounts of the α subunit. Therefore, *mmdA* was expressed separately by using plasmid pT7α in *E. coli* BL21(DE3) with pLysE. Cells harvested from 100-ml cultures of the *E. coli* host harboring pT7α, pT7mmd, pJHΔ1, or pJHΔ2 were resuspended in 3.5 ml of 50 mM potassium phosphate buffer (pH 7.0) supplemented with 5 mM $MgCl_2$ and 0.1 mM diisopropylfluorophosphate and passed twice through a French pressure minicell at 123 MPa. Then 1.5 ml (one-half) of the cell extracts from the strains with pT7mmd, pJHΔ1, or pJHΔ2 was incubated with or without 1 ml (one-third) of the cell extract containing the separately synthesized α subunit for 20 min on ice and subsequently for 20 min at 25°C. After this incubation, the membranes were isolated as described previously (13) and methylmalonyl-CoA-decarboxylase was purified.

Purification of methylmalonyl-CoA-decarboxylase. Methylmalonyl-CoA-decarboxylase with or without reconstitution with the separately expressed α subunit (see above) was purified as described for the *V. parvula* enzyme (13) except that adsorption and elution from avidin-Sepharose were performed by a batch procedure. The membranes from cells of a 100- or 200-ml culture were homogenized in 2 ml of 2 mM potassium phosphate (pH 7.0) containing 1% Triton X-100, and KCl was added to a final concentration of 300 mM; 1 ml of monomeric avidin-Sepharose material (14) equilibrated with the same buffer and salt concentration was added and thoroughly mixed with the enzyme. After sedimentation of the Sepharose material by gravity, the supernatant was removed. Unbound proteins and Triton X-100 were removed by two washes with 5 ml of 10 mM potassium phosphate (pH 7.0) containing 300 mM KCl and 0.05% Brij 58. The decarboxylase was eluted from the affinity matrix by washing the avidin-Sepharose material twice with 2 ml of 10 mM potassium phosphate (pH 7.0) containing 150 mM KCl and 1.5 mM biotin. The resulting solution was concentrated to about 200 μl with a Centricon 30 microconcentrator (Amicon), washed with 10 mM potassium phosphate (pH 7.0), and concentrated again. All purification steps were carried out on ice and in the presence of 0.1 mM diisopropylfluorophosphate.

Decarboxylase activity. The activity of methylmalonyl-CoA-decarboxylase was assayed discontinuously by separating and quantitating methylmalonyl-CoA and propionyl-CoA via high-performance liquid chromatography (HPLC). If not indicated otherwise, the reaction was performed in 50 mM potassium phosphate (pH 7.0) containing 10 mM NaCl and 1 mM methylmalonyl-CoA and was initiated by the addition of enzyme. At the times indicated in the figures, samples of 50 μl were taken and the reaction was stopped by addition of 5 μl of 70% perchloric acid. The precipitate formed was removed by centrifugation at 14,000 × *g* for 3 min, and 45 μl of the supernatant was added to 3 μl of 10 M KOH. After a second centrifugation at 14,000 × *g* for 3 min, 40 μl of the resulting supernatant was added to 200 μl of HPLC buffer A (200 mM potassium phosphate [pH 6.0]). The analysis of the acyl-CoA compounds (12, 16) was modified as follows. A Hypersil ODS column (250 by 4 mm; particle size, 5 μm; Hewlett-Packard) was equilibrated with HPLC buffer A. The samples were eluted with a linear gradient (52 to 60% within 9 min) of HPLC buffer B (HPLC buffer A containing 20% [vol/vol] acetonitrile). The acyl-CoA compounds were detected at 254 nm. The retention time for methylmalonyl-CoA was 2.95 min; that for propionyl-CoA was 8.4 min. For quantification, peak areas were automatically integrated and compared with those of standards.

Sodium ion transport assay. To form liposomes, a suspension of 40 mg of phosphatidylcholine (soybean, type II-S; Sigma) in 1 ml of reconstitution buffer RB (30 mM potassium phosphate [pH 7.0], 1 mM disodium sulfate, 0.5 mM dithioerythritol) was strongly agitated with a vortex mixer for 3 min and afterwards sonicated twice for 30 s each time with a tip-type sonicator (40 W). The suspension was divided into two portions of 480 μl, and 8 or 12 μg of the isolated methylmalonyl-CoA decarboxylase was added. After an incubation for 15 min on ice, the samples were frozen in liquid nitrogen and thawed in an ice-water bath. The suspensions were sonicated again for 5 s at 20 W, and valinomycin was added to a final concentration of 10 μM. The proteoliposomes were collected by centrifugation (200,000 × *g*, 50 min) and resuspended in a final volume of 90 μl of buffer RB. Proteoliposomes (50 μl) were diluted into 460 μl of buffer RB containing $^{22}NaCl$. The final concentration of Na^+ was 2 mM with a specific activity of 1,300 cpm per nmol of Na^+. The reaction was started by the addition of the substrate methylmalonyl-CoA (final concentration, 100 μM). At the times indicated in Fig. 7, samples of 50 μl were taken and passed through a Dowex 50 K^+ column (0.4 by 1 cm), which was subsequently washed with 600 μl of Tris-phosphate buffer (50 mM [pH 7.5]). The radioactivity eluted from the column represents $^{22}Na^+$ entrapped inside the proteoliposomes. It was determined with a gamma counter.

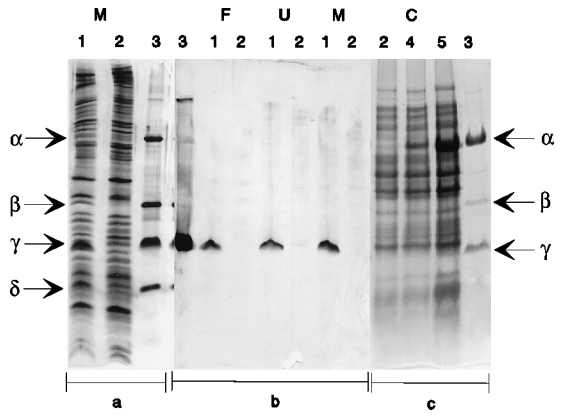

FIG. 3. Expression of the *mmd* genes in *E. coli*. Proteins separated by SDS-PAGE were either stained with silver (a) or subjected to Western blotting and stained for biotinylated proteins by using peroxidase conjugated to avidin (b) or stained with Coomassie brilliant blue (c). Lanes: 1, pT7mmd in *E. coli* BL21/pLysE; 2, vector control (pT7-7); 3, purified methylmalonyl-CoA-decarboxylase from *V. parvula*; 4, pT7α in *E. coli* BL21/pLysE; 5, pJH20 in *E. coli* BL21/pLysE. F, French press extract; M, membranes; U, supernatant (200,000 × *g*); C, cell lysate. The visible subunits of the decarboxylase are α, β, γ, and δ.

SDS-PAGE and Western blotting (immunoblotting). Proteins were separated by SDS-PAGE as described previously (23). A stacking gel of 4% and a separating gel of 12% were used. The polypeptide bands were visualized by staining with silver or Coomassie brilliant blue G250. For the detection of biotinylated proteins with avidin-peroxidase conjugate, the proteins were electroblotted onto a nitrocellulose membrane and treated as described previously (11).

Protein determination. Protein was determined by the Bradford method (3), using the Bio-Rad protein assay reagent mixture. If the samples contained unknown concentrations of detergents, the bicinchoninic acid protein assay (Pierce) was used. For both assays, bovine serum albumin served as the standard.

RESULTS

Expression of the *V. parvula mmd* genes in *E. coli*. Methylmalonyl-CoA-decarboxylase couples the exergonic decarboxylation of methylmalonyl-CoA to propionyl-CoA with the endergonic transport of sodium ions across the membrane to the outside of the cell. The protein is a complex consisting of five different subunits. Analysis of the purified enzyme from *V. parvula* allowed us to ascribe specific functions to three of these subunits. The α, β, and γ subunits were characterized as carboxyltransferase, carboxybiotin carrier protein decarboxylase, and biotin carrier protein, respectively (15). The functions of the δ subunit and of the recently detected ε subunit are not known. For that reason, we decided to establish an expression system in *E. coli* which should allow the analysis of mutant enzymes lacking one or both of these subunits.

In a first attempt, the expression of only the *mmdA* gene (encoding the α subunit) was investigated with different constructs. Plasmid pJH20, a pBluescript KS+ derivative, contains the complete *mmdA* gene and 400 bp of upstream DNA. In pT7α, the 400 bp of upstream DNA was deleted, and expression of the α subunit was under control of the T7 promoter, using the ribosome-binding site of the vector pT7-7 (Fig. 1). Cells from *E. coli* BL21(DE3) transformed with these plasmids synthesized a protein with the same migration behavior during SDS-PAGE as the purified α subunit, showing that *mmdA* was expressed (Fig. 3c). Since the *lac* promoter present on the vector pJH20 is in inverse orientation to *mmdA*, the 400-bp region upstream of the *mmdA* start codon must contain DNA sequences which act as promoter in *E. coli*. We previously have

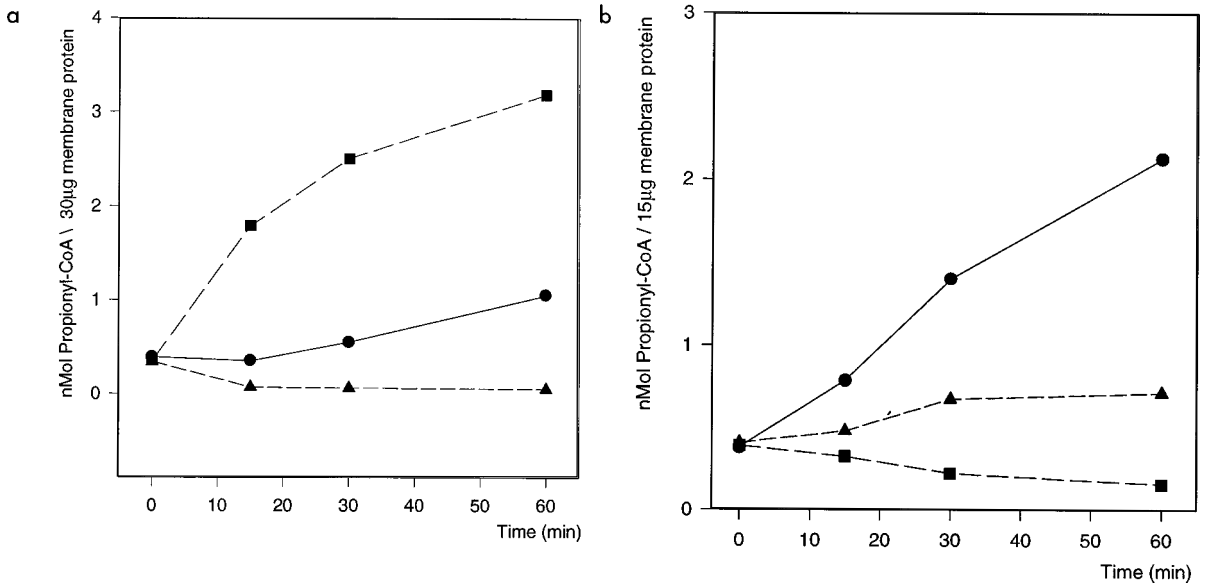

FIG. 4. (a) Kinetics of methylmalonyl-CoA decarboxylation by enzyme expressed in *E. coli*. Membranes isolated from *E. coli* DH5α transformed with pJH70 (●) and *E. coli* BL21/pLysE transformed with pT7mmd (■) and pT7-7 as a vector control (▲). The decarboxylation of methylmalonyl-CoA was determined by measuring the produced propionyl-CoA at various times. (b) Stimulation of methylmalonyl-CoA-decarboxylase by Na^+ and inhibition by avidin. Methylmalonyl-CoA-decarboxylase activity was determined with membrane fractions of *E. coli* BL21/pLysE/pT7mmd (internal Na^+ concentration of 0.8 mM) (▲) in the presence of 10 mM NaCl (●) and in the presence of 10 µg of avidin plus 10 mM NaCl (■).

indicated the presence of several putative *E. coli* σ^{70} promoter consensus sequences in this region (17).

We recently speculated that expression of the complete *mmd* operon in *E. coli* might be lethal to the cells (17). However, *E. coli* DH5α transformed with pJH70 was able to grow, albeit at a reduced growth rate compared with that of the vector control. Although this system in principle could be used to study the function of the δ and ε subunits, we decided to establish one which allowed a controlled induction of the genes. For this purpose, we constructed pT7mmd (Fig. 2). On this plasmid, the five *mmd* genes are exclusively expressed from the T7 promoter of the vector, since the 400-bp *mmdA* upstream region was deleted. Synthesis of the decarboxylase subunits was first analyzed by SDS-PAGE of membranes isolated from *E. coli* BL21(DE3) carrying pT7mmd or pT7-7 (vector control) followed by silver staining of the proteins. As shown in Fig. 3a, additional protein bands compared with the vector control, which corresponded to the β, γ, and δ subunits of purified methylmalonyl-CoA-decarboxylase, were visible. The α subunit is hardly visible because of its low level of expression from plasmid pT7mmd, and the ε subunit could not be detected presumably because of its small size (calculated M_r of 5,758). The biotinylation of the γ subunit was analyzed by immunoblotting with an avidin-peroxidase conjugate (Fig. 3b). Since the same amounts of protein were used for the silver-stained part and the blotted part of the gel, it was evident from Fig. 3a and b that the γ subunit formed in *E. coli* was biotinylated to a significantly lower extent than the γ subunit purified from *V. parvula.*

Methylmalonyl-CoA-decarboxylase activity expressed in *E. coli*. The activity of the decarboxylase formed in *E. coli* was analyzed by a discontinuous assay which involves the separation and quantification of methylmalonyl-CoA and propionyl-CoA via HPLC. As shown in Fig. 4a, membranes from *E. coli* BL21(DE3) with pT7mmd and from *E. coli* DH5α with pJH70 catalyzed the formation of propionyl-CoA from methylmalonyl-CoA, whereas membranes from *E. coli* with pT7-7 did not. The specific methylmalonyl-CoA-decarboxylase activity calculated for *E. coli* with pT7mmd was only 3.3 nmol/min/mg of membrane protein and thus about a factor of 100 lower than the decarboxylase activity in *V. parvula* membranes. It will be shown below that the reasons for this low activity are mainly the low level of expression of the α subunit (Fig. 3a) and a low extent of biotinylation of the γ subunit. It was also found that measurement of methylmalonyl-CoA-decarboxylase with crude *E. coli* membranes resulted in low values partially due to the action of phosphotransacetylase (EC 2.3.1.8), which converts propionyl-CoA to propionyl phosphate and CoASH. Addition of membranes from *E. coli* BL21 with pT7-7 (40 µg of protein) to a reaction mixture containing 5 µg of purified methylmalonyl-CoA-decarboxylase from *V. parvula* resulted in an apparent 10-fold decrease of the rate of propionyl-CoA formation from methylmalonyl-CoA. For a quantitative estimation of methylmalonyl-CoA-decarboxylase activity expressed in *E. coli*, it was therefore essential to purify the enzyme prior to activity determinations. Nevertheless, the measured activity was specific for methylmalonyl-CoA-decarboxylase since it was dependent on sodium ions and completely inhibited by avidin (Fig. 4b). Both of these properties are characteristic for this biotin-containing sodium ion-pumping enzyme (13).

Methylmalonyl-CoA-decarboxylase could be purified only in low yield from the *E. coli* clones by using monomeric avidin-Sepharose chromatography, probably because of the low degree of biotinylation of the γ subunit. Attempts to improve this reaction in vitro were not successful. Therefore, an enhanced in vivo biotinylation was tested by using pCY216. This plasmid expresses the *birA* gene from *S. typhimurium* encoding biotin ligase. Membranes from *E. coli* BL21(DE3) transformed with pT7mmd plus pCY216 exhibited a significantly higher methylmalonyl-CoA-decarboxylase activity (19 nmol/min/mg of protein) than membranes from the strain without pCY216, and it was subsequently possible to obtain reasonable amounts of the partially purified decarboxylase. Analysis of this enzyme by

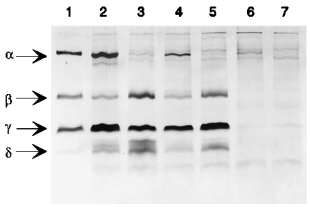

FIG. 5. Silver-stained SDS-polyacrylamide gel of methylmalonyl-CoA-decarboxylase partially purified by adsorption and elution from monomeric avidin-Sepharose. Lane 1, purified decarboxylase isolated from *V. parvula*. The following lanes show the expression from plasmids pT7mmd (lane 2 and 3), pJHΔ1 (lane 4 and 5), and pJHΔ2 (lane 6 and 7). The samples of lane 2, 4, and 6 were incubated with an extract of separately expressed α subunit before the methylmalonyl-CoA-decarboxylase was purified. In all expression clones, the *birA* gene encoding biotin ligase was coexpressed from plasmid pCY216.

SDS-PAGE revealed the presence of methylmalonyl-CoA-decarboxylase with a substoichiometric amount of the α subunit (Fig. 5, lane 3). This result may be due to the fact that the vector-derived ribosome-binding site of *mmdA* in pT7mmd is less efficient in translation initiation than the ribosome-binding sites of *mmdD*, *mmdE*, *mmdC*, and *mmdB*.

If extract from *E. coli*/pT7mmd was incubated with excess α subunit expressed by the pT7 α-containing strain (Fig. 2 and 3c), decarboxylase with higher amounts of α subunit was isolated by the affinity chromatography (Fig. 5, lane 2). The specific activity of the partially pure enzyme was 4.7 U/mg of protein and thus only about seven times lower than that of pure methylmalonyl-CoA-decarboxylase isolated from *V. parvula*. These results indicate that the low decarboxylase activity of membranes from *E. coli* carrying pT7mmd can be explained by interfering activities in the crude membranes, a low degree of biotinylation of the γ subunit, and low expression of the α subunit. These disadvantages are largely overcome by purifying the enzyme after enhanced in vivo biotinylation and preincubation with the separately expressed α subunit.

Characterization of the deletion mutants pJHΔ1 and pJHΔ2. To analyze the roles of the δ and ε subunits, we constructed derivatives of pT7mmd which lacked either the *mmdE* gene (pJHΔ1) or the *mmdE* gene plus the 3′-terminal half of the *mmdD* gene (pJHΔ2) (Fig. 6). After transformation into *E. coli*, the strains were tested for methylmalonyl-CoA-decarboxylase activity. Whereas membranes from the strain harboring pJHΔ1 exhibited specific activities equal to those from *E. coli* with pT7mmd, those from the strain harboring pJHΔ2 did not form detectable amounts of propionyl-CoA from methylmalonyl-CoA. These results indicate that the ε subunit but not the δ subunit is dispensable for methylmalonyl-CoA decarboxylation. To further analyze these mutant enzymes, their purification by avidin-Sepharose was attempted from strains cotransformed with pCY216 to ensure a high biotinylation of the γ subunit. Part of the samples was preincubated with the α subunit prior to the purification to compensate for the low level of expression of this subunit in our *E. coli* clones. Figure 5 shows a silver-stained SDS-polyacrylamide gel of the purified methylmalonyl-CoA-decarboxylase from *V. parvula* and of the *E. coli* expression clones. It is obvious from these results that methylmalonyl-CoA-decarboxylase complexes were partially purified from the *E. coli* clones carrying pT7mmd or pJHΔ1 but not from those carrying pJHΔ2. A decarboxylase complex must therefore exist in the absence of the ε subunit, but no complex formation apparently occurs in the additional absence of part of the δ subunit. In the latter case, neither the β nor γ subunit is present in the isolates. In addition, the amount of the biotin-containing γ subunit, which should be retained by the affinity matrix regardless of whether the other subunits are present, was very low in comparison with the isolates from the other constructs that had been treated in the same way. This subunit may therefore be degraded if no complex is formed in vivo. The only other subunit that can be seen in the sample derived from pJHΔ2 is the α subunit, and this is present only if the extract was incubated with the α subunit prior to the isolation procedure. These results indicate binding of the α to the γ subunit.

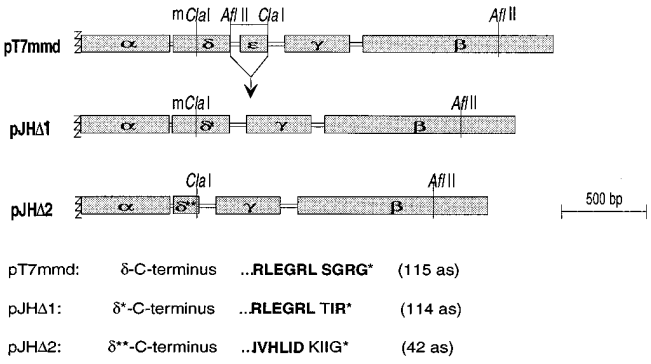

FIG. 6. Construction of the deletion mutants pJHΔ1 and pJHΔ2. Construction of the deletion mutants for *mmdE* and *mmdD* is shown at the top. Details of the cloning procedure are described in Materials and Methods. m*Cla*I symbolizes a methylated *Cla*I site in the *dam*$^+$ host strain (*E. coli* DH5α). Below are shown the original and modified C termini of the δ-subunit derivatives and the number of amino acids (as) present in the truncated δ subunits. The amino acids in boldface are identical to those of the wild-type form of the δ subunit.

A similar subunit pattern was seen in the isolates from the strains carrying pT7mmd and pJHΔ1 if no preincubation with the α subunit was performed. A comparison with the wild-type enzyme clearly shows that substoichiometric amounts of α are present under these conditions. In the enzymes isolated after incubation with α, the amount of this subunit increased, but only in the pT7mmd-derived isolate was this increase sufficient to yield a proportion between α and the other decarboxylase subunits similar to that in the *V. parvula* enzyme. We conclude from these results that in the absence of the ε subunit, an enzyme complex in which the α subunit is bound only loosely is formed. The presence of the ε subunit apparently improves this binding and therefore plays an important role in stabilizing the methylmalonyl-CoA-decarboxylase enzyme complex.

Na^+ transport and decarboxylation activity of the ε-free methylmalonyl-CoA-decarboxylase. The specific methylmalonyl-CoA-decarboxylase activity obtained after partial purification from the clone lacking the ε subunit was 3 U/mg of protein and thus lower than that obtained from the pT7mmd-containing strain (4.7 U/mg of protein). As the activity of the former enzyme increased upon incubation with the α subunit, the difference can be explained by the different amounts of α subunit in the two forms of the enzyme. To investigate whether the decarboxylase activity of the ε-free enzyme was still coupled to Na^+ transport, the wild-type and mutant forms of methylmalonyl-CoA-decarboxylase were reconstituted into liposomes. These proteoliposomes then served to measure sodium ion transport and decarboxylation activity in parallel (Fig. 7). Although the same amounts of units were used for the preparation of the liposomes, the activity resulting from enzyme without the ε subunit was lower than that of the proteoliposomes containing the wild-type enzyme. This result is further evidence for a stabilizing function of the ε subunit. The results of Fig. 7 show methylmalonyl-CoA-decarboxylase-coupled $^{22}Na^+$ uptake into proteoliposomes not only for the wild-

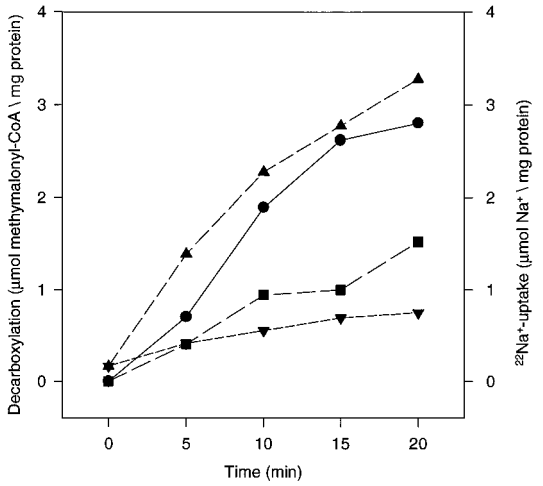

FIG. 7. Kinetics of decarboxylation of methylmalonyl-CoA and Na^+ transport into proteoliposomes. The *E. coli* clones used for expression of methylmalonyl-CoA-decarboxylase contained in *E. coli* BL21(DE3) plasmid pT7mmd (●, ▲) or pJHΔ1 (■, ▼). These clones also contained plasmid pCY216 from which the *birA* gene encoding biotin ligase was coexpressed. Cell extracts were incubated with a cell extract of *E. coli* BL21(DE3) with pLysE containing pT7α to compensate for the low amount of α subunit in the extracts from the pT7mmd- or pJHΔ1-containing clones. The decarboxylase reconstituted with the separately expressed α subunit was then isolated by the batch affinity purification method with monomeric avidin-Sepharose. The purified enzymes were reconstituted into proteoliposomes, which were used to measure $^{22}Na^+$ uptake (▲, ▼). From the data obtained in the presence of methylmalonyl-CoA, $^{22}Na^+$ uptake in the absence of the substrate was subtracted. The activity of the methylmalonyl-CoA-decarboxylase was determined from propionyl-CoA formation by HPLC under the same reaction conditions used for $^{22}Na^+$ uptake experiments (pT7mmd [●] and pJHΔ1 [■]). For details, see Materials and Methods.

type enzyme but also for the form lacking the ε subunit. Therefore, the ε subunit seems not important for the catalysis of either decarboxylation or Na^+ transport.

DISCUSSION

We describe here the expression of methylmalonyl-CoA-decarboxylase from *V. parvula* in *E. coli*. The apparent specific activity of the decarboxylase synthesized in the *E. coli* expression clone pT7mmd was low as a result of several factors, including incomplete biotinylation of the γ subunit, the presence of substoichiometric amounts of the α subunit, and disturbances of the activity measurements in the crude membrane system by interfering enzymes, e.g., phosphotransacetylase. After coexpression of biotin ligase and supplementation of the extracts from pT7mmd with separately expressed α subunit, a methylmalonyl-CoA-decarboxylase complex derived which after purification by affinity chromatography showed the typical subunit pattern on SDS-PAGE of the wild-type enzyme besides a number of impurities. In addition, the specific activity of the partially pure enzyme isolated from the *E. coli* clone pT7mmd after reconstitution with the α subunit (4.7 U/mg of protein) was only about seven times less than that of pure methylmalonyl-CoA-decarboxylase isolated from *V. parvula*. The decarboxylase expressed in *E. coli* exhibited the typical characteristics of the *Veillonella* enzyme, i.e., activation by Na^+, inhibition by avidin, and after reconstitution into proteoliposomes, Na^+ uptake upon methylmalonyl-CoA decarboxylation.

It was not the aim of these studies to develop a readily available source for methylmalonyl-CoA-decarboxylase by expression in *E. coli*. As moderate expression of catalytically active enzyme resulted in growth inhibition, it appears unlikely that *E. coli* will tolerate high-level expression of active methylmalonyl-CoA-decarboxylase. The reason for this growth inhibition is probably an interference with fatty acid biosynthesis by the catalysis of malonyl-CoA decarboxylation. It was possible, however, to overexpress the α subunit and the remaining subunits in *E. coli* from plasmids pT7α and pT7mmd, respectively, and to reconstitute a more active methylmalonyl-CoA-decarboxylase in vitro by combining the extracts of these clones.

Interestingly, deletion of the small ε subunit in the mutant pJHΔ1 did not abolish methylmalonyl-CoA-decarboxylase or Na^+ transport activity. A functional role of the ε subunit in the catalysis can therefore be excluded. The lack of the ε subunit, however, affected the stability of the complex, especially the binding of the α subunit. In all purified enzyme specimens lacking the ε subunit, the amount of α subunit was considerably less than in the specimens handled similarly but containing the ε subunit. Substoichiometric amounts of the α subunit in the complex were reflected by a low methylmalonyl-CoA-decarboxylase activity which could be reactivated upon incubation with separately expressed α subunit. Similar specific activities were obtained after reconstitution with the α subunit of the complexes with or without ε subunit, in agreement with the notion that this subunit does not function in catalysis. A stabilizing effect of the ε subunit was also evident from faster inactivation of purified enzyme lacking this subunit after storage at −20°C compared with the wild-type enzyme and from reconstitution into proteoliposomes: starting from the same amount of activity, the proteoliposomes formed with the ε-free enzyme contained only about 30% of the decarboxylase or Na^+ transport activity of proteoliposomes of the ε-containing enzyme complex. The proposed role of the ε subunit to stabilize the enzyme complex is in accord with recent results from our laboratory on the methylmalonyl-CoA-decarboxylase from *Propionigenium modestum* (2a). DNA sequence analyses from this organism revealed a gene cluster for methylmalonyl-CoA-decarboxylase similar to that found in *V. parvula*. A striking difference, however, was the lack of the gene for the ε subunit. Attempts to isolate the decarboxylase from *P. modestum* by the protocol used for the *V. parvula* enzyme were not successful because of the instability of the complex. The majority of the methylmalonyl-CoA-decarboxylase was lost if the membranes were separated from the cytoplasm by ultracentrifugation. The enzyme eluted with biotin from the subsequent monomeric avidin-Sepharose column was inactive and completely devoid of the α subunit.

The mutant pJHΔ2 contained, in addition to the deletion of the ε subunit, a deletion within the C-terminal region of the δ subunit. While methylmalonyl-CoA-decarboxylase activity was maintained by deletion of the ε subunit, it was completely abolished by the additional deletion of part of the δ subunit. In addition, a core complex consisting of the remaining subunits α, γ, and β could not be isolated from this mutant strain. Only small amounts of the biotin-containing γ subunit were present after the attempt to purify the enzyme by avidin-Sepharose affinity chromatography. Small amounts of α subunit were also seen after SDS-PAGE if extracts of the pJHΔ2-containing strains were incubated with an extract of the α subunit from strain pT7α prior to the purification, but no traces of the β subunit were detectable under both conditions (Fig. 5). These results may indicate an interaction between the α and γ subunits but no direct interaction between the α or γ subunit with the β subunit. Binding of the β subunit to γ (and α) apparently requires an interaction with the δ subunit. The very hydrophobic β subunit could therefore bind to the δ subunit, most

probably to its N-terminal hydrophobic anchor, while the hydrophilic C-terminal part of this protein could be responsible for the binding of the γ subunit. The binding of α to the γ subunit could then form a loosely bound four-subunit complex, the stability of which would be considerably improved by an interaction with the fifth subunit, ε. The binding between α and γ subunits is supported by studies of the biotin enzyme transcarboxylase from *Propionibacterium shermanii*, in which the binding of the 1.3S biotin subunit to the 12S carboxyltransferase subunit could be attributed to the first 14 amino acid residues of the small polypeptide (18). A similar sequence motif has been identified in the related γ subunit of methylmalonyl-CoA-decarboxylase. The α subunit of this enzyme is highly homologous to the 12S subunit of transcarboxylase. Binding between the decarboxylase α and γ subunits therefore seems likely. This binding was further supported by the presence of residual amounts of the γ subunit within the α-subunit band after SDS-PAGE, as revealed from N-terminal protein sequencing (unpublished results).

An inspection of the related Na^+ pump oxaloacetate decarboxylase reveals similar features of subunit association. Here, the α subunit and the biotin domain are connected even by a covalent linkage. The γ subunit of oxaloacetate decarboxylase, which is related to the δ subunit of methylmalonyl-CoA-decarboxylase, is absolutely essential for catalytic functioning and for a proper assembly of the complex. After deletion of the gene for the oxaloacetate decarboxylase γ subunit, no activity and no association of the α subunit to the membrane containing the β subunit were observed. In contrast, expression of only the β and γ subunits led to a membrane-bound complex that became fully functional after reconstitution with the α subunit (5a). Oxaloacetate decarboxylase forms a stable complex like methylmalonyl-CoA-decarboxylase from *V. parvula* but lacks a small subunit related to the ε subunit of the latter complex. The β subunit of oxaloacetate decarboxylase contains an extended cytoplasmic loop that is 57 amino acid residues longer than the corresponding region in the methylmalonyl-CoA-decarboxylase β subunit. Possibly, this loop region in oxaloacetate decarboxylase has a function similar to that of the ε subunit in methylmalonyl-CoA-decarboxylase, i.e., to increase the stability of the complex.

The small ε subunit (55 amino acid residues) is 48% identical to the C-terminal part of the δ subunit, indicating that *mmdE* may have evolved from *mmdD* (17). It may have been retained and modified during evolution to improve its function to stabilize the methylmalonyl-CoA-decarboxylase enzyme complex. In the 2-oxoacid dehydrogenase multienzyme complex, the dihydrolipoyl dehydrogenase (E3) subunit and the dihydrolipoyl acyltransferase (E2) hold together by a noncovalent interaction with the small domain BD (21). This 33-amino-acid-residue-long BD domain shows a folding motif similar to that of a substructure of E3 (4). It has been speculated that the BD domain that is responsible for attaching E3 to E2 may have formed during evolution by splicing of fragments of the E3 gene. Similarly, the small ε subunit of methylmalonyl-CoA-decarboxylase, which probably evolved by duplication of part of the δ-subunit gene, may have been maintained and optimized during evolution for tight binding of individual subunits within the decarboxylase enzyme complex.

In conclusion, the ε subunit of methylmalonyl-CoA-decarboxylase has no catalytic function but stabilizes the complex by strengthening the binding of the α subunit, while the δ subunit is required for catalytic activity as well as for the proper assembly of the individual subunits to an enzyme complex.

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